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C1GALT1C1-Associated Mosaic Disorder of Glycosylation in a Female

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ABSTRACT

Cosmc, encoded by the X-linked *C1GALT1C1*, is a molecular chaperone in the endoplasmic reticulum and a master regulator of O-glycosylation of mammalian glycoproteins. Recently, we described a germline mutation in *C1GALT1C1* in two male patients, giving rise to a congenital disorder of glycosylation—*COSMC*-CDG. Here, we have identified a female patient with a *de novo* mosaic variant in *C1GALT1C1* (c.202C>T, p.Arg68*), which results in a truncated and nonfunctional form of Cosmc (Cosmc-R68). The patient is mosaic, as ~27% of her buccal cells carry the mutation. The patient is now a 5-year old who presented with nonimmune hydrops fetalis. As Cosmc is essential for the generation of normal O-glycans through regulating T-synthase activity, thereby enabling the formation of the universal Core 1 O-glycan Galβ1-3GalNAcα1-Ser/Thr (T-antigen), the loss of Cosmc leads to the expression of the unusual precursor O-glycan termed Tn-antigen (CD175) (GalNAcα1-Ser/Thr). Owing to the mutational mosaicism, only a significant minority of cells would exhibit abnormal O-glycosylation. Analysis of red blood cells (RBCs), leukocytes, and serum from this patient indicated reduced expression of Cosmc and T-synthase proteins and lower T-synthase activity. Consistent with these findings, we observed reduced normal O-glycans in serum glycoproteins and RBCs from the patient, along with elevated expression of the Tn-antigen in serum glycoproteins compared to controls. This case represents the first description of a true mosaic loss-of-function variant in *C1GALT1C1*, that is, one that occurred postzygotically during embryogenesis, and raises interesting questions about the role of O-glycosylation during fetal development and its consequences on the clinical presentation.

1 | Introduction

Glycosylation of proteins is an essential process for normal development. Alterations in pathways that produce glycoproteins often result in altered protein glycosylation, which typically leads to multiple clinical pathologies. This is most evident in the congenital disorders of glycosylation (CDG), one of the fastest-growing groups of inborn errors of metabolism [1–3]. One of the major and most common pathways of protein glycosylation is the addition of O-glycans by modification of Ser/Thr residues

with the monosaccharide *N*-acetylgalactosamine (GalNAc) to generate the Tn-antigen GalNAcα1-Ser/Thr (Tn-antigen/CD175) [4, 5]. This is a precursor in all cells to the universal Core 1 O-glycan Galβ1-3GalNAcα1-Ser/Thr (T-antigen), which is generated by modification of the Tn-antigen by T-synthase [6]. The Tn-antigen is not found in normal cells, as it is modified efficiently by T-synthase, and the subsequent Core 1 O-glycans are typically further modified and extended by additional sugars [5, 7]. The generation of the Core 1 O-glycan requires the expression of two genes—one encodes the enzyme T-synthase (*C1GALT1*), which adds galactose to the Tn-antigen, and the other encodes the molecular chaperone Cosmc (*C1GALT1C1*, an X-linked gene on Xq24) [8], which functions as a private chaperone within the endoplasmic reticulum (ER) to regulate folding of T-synthase and acquisition of activity (Figure 1) [13, 14]. Engineered loss of Cosmc or T-synthase in mice results in embryonic lethality and uniform expression of the immature Tn-antigen in all cells [15, 16].

We recently identified a germline variant in *C1GALT1C1* that leads to a significant reduction in Cosmc protein, decreased expression of normal Core 1 O-glycans, along with elevated expression of the Tn-antigen, leading to a congenital disorder of glycosylation known as C1GALT1C1-CDG (also termed *COSMC*-CDG) [9]. Owing to the X-linked nature of the pathology, the variant was first identified in two hemizygous sons of a mildly affected heterozygous mother. There are significant developmental abnormalities in *COSMC*-CDG as a consequence, including developmental delay, short stature, immunodeficiency, and acute kidney injury (AKI) resembling atypical hemolytic uremic syndrome (aHUS) [9]. Interestingly, in earlier studies, we identified somatic, postnatally acquired variants in *C1GALT1C1* within hematopoietic precursors in patients with Tn syndrome [10], a rare autoimmune disorder in which a large population of blood cells of all lineages express the Tn-antigen [17]. A number of patients with Tn syndrome have been identified, and all carry an acquired somatic variant in *C1GALT1C1* [18, 19], but it is limited to hematopoietic lineages. In contrast to typical CDGs, this represents a type of acquired disorder of glycosylation (ADG).

Here, we report a female patient carrying a mosaic variant in *C1GALT1C1* (c.202C>T, p.Arg68*), here termed Cosmc-R68. The variant was originally detected in blood by trio genome sequencing and subsequently detected as mosaic in a buccal sample (allelic fraction 27%). This is associated with a loss of normal O-glycans in serum and RBCs, as well as elevated expression of the Tn-antigen in serum glycoproteins. The systemic pathology associated with mosaic expression of the Tn-antigen raises many interesting questions about the nature of the COSMC mutation and X-inactivation pathways.

2 | Results

2.1 | Clinical Presentation

A 5-year-old female was born prematurely at 33 weeks gestation after a pregnancy complicated by nonimmune hydrops fetalis. Extensive prenatal and postnatal evaluation did not yield an underlying etiology for the hydrops. The neonatal period was complicated by thrombocytopenia (lowest $65\times10^3/\mu L$), coagulopathy, pulmonary hypertension, elevated liver transaminases, and hyperferritinemia, all of which resolved by hospital discharge on Day 43 of life.

Growth was slow during infancy, but her height and weight are now both approximately 5th percentile for her age. She had global developmental delay with persistent speech delay. At age 18 months, she screened positive for autism but has not completed a formal neuropsychological evaluation. A diagnosis of focal epilepsy was made after seizure onset at 12 months. Brain MRI is normal. She has persistent proteinuria but normal renal function, urine protein/creatinine ratio, renal ultrasound, and serum complement factors 3 and 4 at age 4 years. Her complete blood count remains normal. For additional clinical details, see the Supporting Data S1.

2.2 | Genetic Testing

Conventional karyotype (46,XX) and chromosomal microarray were reported as normal for a female. The patient underwent clinical molecular testing utilizing rapid next-generation whole-genome sequencing (WGS) performed as a trio with her parents. A heterozygous, apparently *de novo* variant denoted c.202C>T (p.Arg68*) in *C1GALT1C1* was detected in the whole blood sample and classified as pathogenic (Figure 1A,B). The variant was confirmed by Sanger sequencing on whole blood. WGS coverage data at ChrX: 119760820 is 25× with 19 reads for the reference base and 6 reads for the variant. Based on a follow-up study utilizing a buccal swab sample for targeted variant analysis to evaluate for mosaicism, the NM_152692.4:c.202C>T (p.Arg68*) variant was detected at a 27% allelic fraction with 201× coverage (Figure 2A,B).

Because of her evolving phenotype, reanalysis of the original WGS data was carried out and detected an additional likely pathogenic variant in *IRF2BPL* denoted c.1293C>G (p.Tyr431*), paternally inherited.

2.2.1 | Leukocytes From Cosmc-R68 Patient Exhibit Overall Decreased Levels of Endogenous Cosmc and T-Synthase Proteins, Along With Reduced T-Synthase Activity

Cosmc is a specific molecular chaperone important for T-synthase function; in the absence of Cosmc, T-synthase is inactive [13] (Figure 1A). The Cosmc-R68 patient has a heterozygous, mosaic expression of Cosmc, with 54% of the cells containing the mutation (Figure 2B,C). The mutation in Cosmc (p.Arg68*) is expected to produce a truncated protein consisting of 1–68 amino acids compared to the WT, which consists of 1–318 amino acids (Figures 1B and 3E). To study the steady-state level of Cosmc-R68 expression, we first prepared a leukocyte lysate from patient Cosmc-R68 and analyzed the expression of Cosmc and T-synthase proteins using SDS-PAGE and Western blot. We observed a significant reduction of Cosmc in patient cells compared to healthy controls (Figure 3A,B). We also observed a significant reduction of T-synthase protein expression (Figure 3A,C), which is consistent with our prior work on Cosmc deletion in cell culture, mice models, and human patients, where

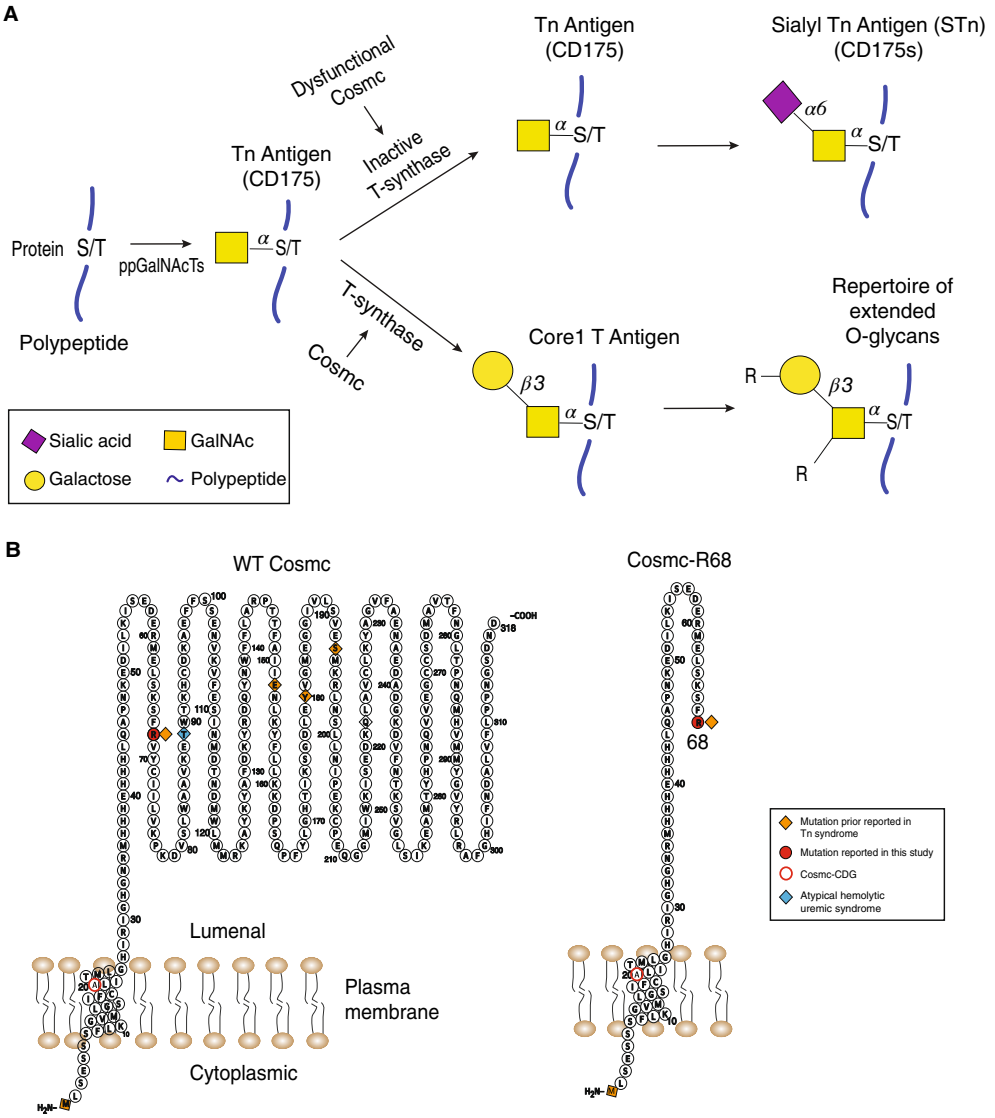

FIGURE 1 | (A) Mucin-type O-glycosylation pathway. Cosmc is a Type II membrane protein that serves as a specific chaperone for the proper folding and functioning of T-synthase. Functional Cosmc generates active T-synthase, which is critical for the production of extended O-glycans in many glycoproteins (bottom branch). However, in the absence of Cosmc function, T-synthase is not active, which results in aberrant O-glycosylation (top branch). (B) Visualization of the Cosmc protein in the context of the ER membrane. On the left, the full-length WT Cosmc showing disease-causing mutations. The red circle with the alanine residue within the transmembrane domain is reported in a *COSMC*-CDG patient [9]. The yellow square indicates previously reported mutations in Tn-syndrome patients [5]. The red-filled circle with arginine shows the mutation within the reported patient (heterozygous, non-mosaic *de novo C1GALT1C1* gene variant c.202C>T, p.Arg68*), as seen previously in a Tn-syndrome patient [10]. The light blue square represents a mutation previously observed in atypical hemolytic uremic syndrome [11]. On the right, visualization of the truncated Cosmc protein as a result of the mutation, p.Arg68*. Protein visualization modified from Protter output and *COSMC*-CDG [9, 12]. The red circle is seen in the patient previously reported in the Tn-syndrome patient [10].

loss of Cosmc generates an inactive T-synthase that is typically degraded in the ER/proteasomal pathway [9, 16, 20]. In parallel, we performed a T-synthase activity assay from the leukocyte lysates and found that T-synthase activity was also significantly reduced in leukocytes from Cosmc-R68 compared to controls (Figure 3D).

To further investigate Cosmc-R68 expression and function, we generated a recombinant Cosmc containing an N-terminus tagged version of both patient Cosmc-R68 and the full-length form of Cosmc (HA-Cosmc-R68 and HA-tagged wild-type Cosmc [HA-Cosmc]) using a mammalian expression vector. These were expressed by transient transfection in an established CosmcKO HEK cell line that lacks endogenous Cosmc [20]. We observed expression of Cosmc-R68 with the expected truncated molecular weight in SDS-PAGE-WB analysis (Figure 3F, HA blot, lower arrow; Cosmc blot for Cosmc expression). Next, we asked whether Cosmc-R68 could rescue T-synthase protein; the results demonstrate that Cosmc-R68 cannot rescue T-synthase protein (Figure 3F, T-syn blot). In a parallel experiment using freshly prepared lysates, we also investigated whether Cosmc-R68 could rescue some level of T-synthase activity. The results demonstrated that Cosmc-R68 did not rescue the activity of T-synthase (Figure 3G). Thus, despite the expression of

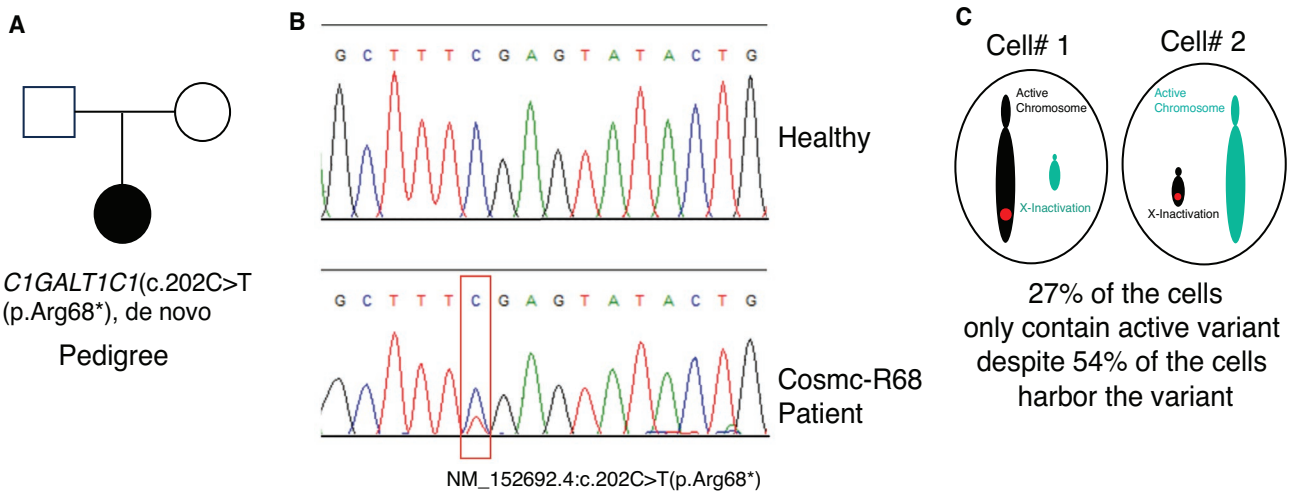

FIGURE 2 | Pedigree and Sanger sequencing. (A) Pedigree. The black-filled circle shows the affected *C1GALT1C1* c.202C>T *de novo* mosaic female patient (as shown in the mutation in B), showing two generations. (B) Sanger sequencing data demonstrating the mutation in the Cosmc-R68 patient (c.202C>T), bottom, red box. (C) Model showing that 50% of cells express the variant due to X-inactivation. The red circle indicates the mutation.

a truncated version of Cosmc-R68, its ability to chaperone T-synthase appears to be lost both in vitro and in vivo.

2.2.2 | Cosmc-R68 Patient Shows Reduced Expression of Normal O-Glycan Structures and Consequently Increased Expression of Aberrant O-Glycans (Tn-Antigen)

Cosmc regulates normal Core 1 O-glycans that may be further modified. In the absence of Cosmc function, the O-glycome collapses to the Tn-antigen, and in some cases, a sialylated derivative, Sialyl-Tn, may also be formed (Figure 1A). Due to the mosaic nature (54% of cells) of the *C1GALT1C1* mutation in the patient, with assumed random X-inactivation, we expected that the majority of glycoproteins would exhibit normal O-glycan expression, but a significant minority might contain the Tn-antigen.

To test the expression level of O-glycans, we treated the samples obtained from healthy control and patient with glycosidases, resolved them on SDS-PAGE, prepared lectin blots, and probed the blots with lectins specific for various glycan structures (Figure 4A). We first analyzed samples for binding by peanut agglutinin (PNA), a lectin that binds strongly to the non-sialylated Core 1 O-glycan Galβ1-3GalNAcα1-Ser/Thr [21]. We aimed to determine if there is a slight decrease in staining with PNA in the patients compared to healthy controls and a slight increase in *Vicia villosa* agglutinin (VVA), a plant lectin that binds to the Tn-antigen [22] (Figure 4A). The results demonstrated that serum glycoproteins from the patient express normal O-glycans as seen in the sample treated with neuraminidase (NeuA) to expose the Core 1 O-glycan bound by PNA (Figure 4B, Lanes 2 and 6). For the specificity of the PNA staining, the samples were treated with NeuA + O-glycosidase, which specifically removes the Core 1 O-glycan disaccharide [23] (Figure 4A); as a result the binding of PNA is largely lost (NeuA + O-glycosidase) (Figure 4B, Lanes 3 and 7). Next, we compared the slight degree of change of PNA staining between healthy and patient serum, and found significantly reduced PNA staining in patient material (Figure 4B,C, Lanes 2 and 6 quantified), which is consistent with the reduced Cosmc expression.

Similarly, we analyzed a serum sample from patient Cosmc-R68 for Tn-antigen expression using VVA lectin, which is commonly used to test Tn-antigen expression [24]. Glycoproteins in the patient's serum showed a significantly higher abundance of the Tn-antigen in high molecular weight glycoproteins (Figure 4D, Lanes 3 and 7 and Figure 4E). It is important to note that treatment of serum samples with NeuA + O-glycosidase removes normal O-glycans and can enhance identification of the Tn-antigen in samples. The expression of the Tn-antigen does not disappear (Figure 4D, Lane 8) when we remove N-glycans by treatment with PNGase-F [25], indicating that binding is specifically to O-glycans. This result is consistent with our previous studies, in which dysfunctional Cosmc results in the expression of the Tn-antigen [8, 10].

Our prior work indicated that mutations in *C1GALT1C1* do not alter the expression of N-glycans [16]. To determine the N-glycosylation status in the glycoproteins in Cosmc-R68 patient serum, we processed the sample similarly to Figure 4B,D and probed the lectin blots with the plant lectin concanavalin A (ConA), which binds to many types of N-glycans [26] (Figure 4A). Our analysis indicates that serum glycoproteins from the patient have normal N-glycans (Figure 4F, Lanes 1–4 and 5–8). PNGase-F treatment removes N-glycans; as a result, we saw reduced staining by ConA upon treatment, which serves as a specificity control for ConA staining (Figure 4F, Lanes 4 and 8), and secondary reagents alone also showed no signal, as expected (Figure 4G). Our data suggest that the patient's serum has a low level of normal O-glycans and a higher level of Tn-antigen compared to controls, whereas N-glycan expression is not appreciably altered.

We next analyzed the expression level of extended O-glycans on Cosmc-R68 patient's RBCs. For this, we prepared the whole-cell lysates and processed the samples with glycosidases, similar to the serum protein analysis described above. The processed samples were analyzed on SDS-PAGE-LB and probed for PNA

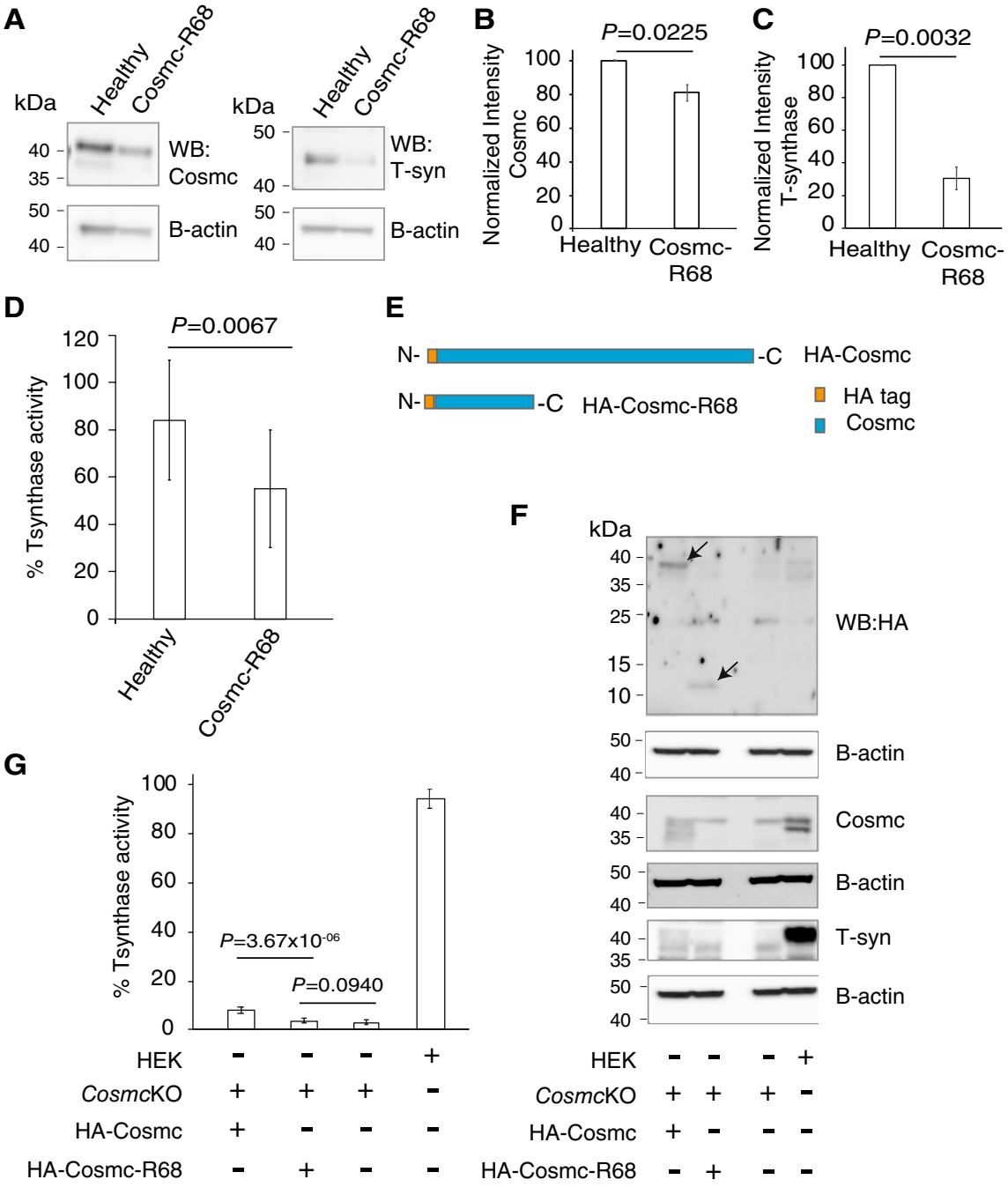

FIGURE 3 | Cosmc-R68 patient leukocytes exhibit reduced Cosmc and T-synthase expression and function. (A) Whole-cell lysates prepared from healthy and patient leukocytes were analyzed on SDS-PAGE western blot and probed for endogenous Cosmc and T-synthase as indicated on the right. Beta-actin serves as the loading control for the respective blots. All images are representative examples of $n = 3$. (B) ImageJ quantification of the Cosmc blot in Panel A, normalized with respective beta-actin, $n = 3$, an average of 3, ±1 SD. (C) ImageJ quantification of the T-synthase blot in Panel A, normalized to the respective beta-actin, $n = 3$, an average of 3, ±1 SD. (D) Similar to (A), whole-cell lysates from healthy and Cosmc-R68 leukocytes were analyzed to measure the T-synthase activity as indicated at the bottom. $n = 7$, two independent experiments of $n = 3$ and 4, an average of 7, ±1 SD. (E) Schematics of WT Cosmc and Cosmc-R68 with an N-terminus HA tag. (F) HA-Cosmc-R68 shows truncated expression of the Cosmc protein. Whole-cell lysates prepared from transiently transfected HA-Cosmc and HA-Cosmc-R68 in the CosmcKO cell line or mock were analyzed by SDS-PAGE-WB and probed as indicated on the right. HEK cells were used as a WT control. The arrow shows the HA-tagged expressed Cosmc proteins. $n = 3$, a representative example of three independent experiments. Beta-actin serves as a loading control (all images are representative examples of $n = 3$). (G) Parallel to (F), freshly prepared lysates were measured for T-synthase activity. $n = 12$, three independent experiments of $n = 4$, an average of 12, ±1 SD.

binding. We observed glycoproteins from Cosmc-R68 patient's RBCs expressing normal extended O-glycans (Figure 4H, NeuA lanes). To test the specificity of PNA binding, we treated the sample with NeuA and NeuA + O-glycosidase, as described above in the serum analysis, and demonstrated that PNA staining is specific and lost after O-glycosidase treatment (Figure 4H,

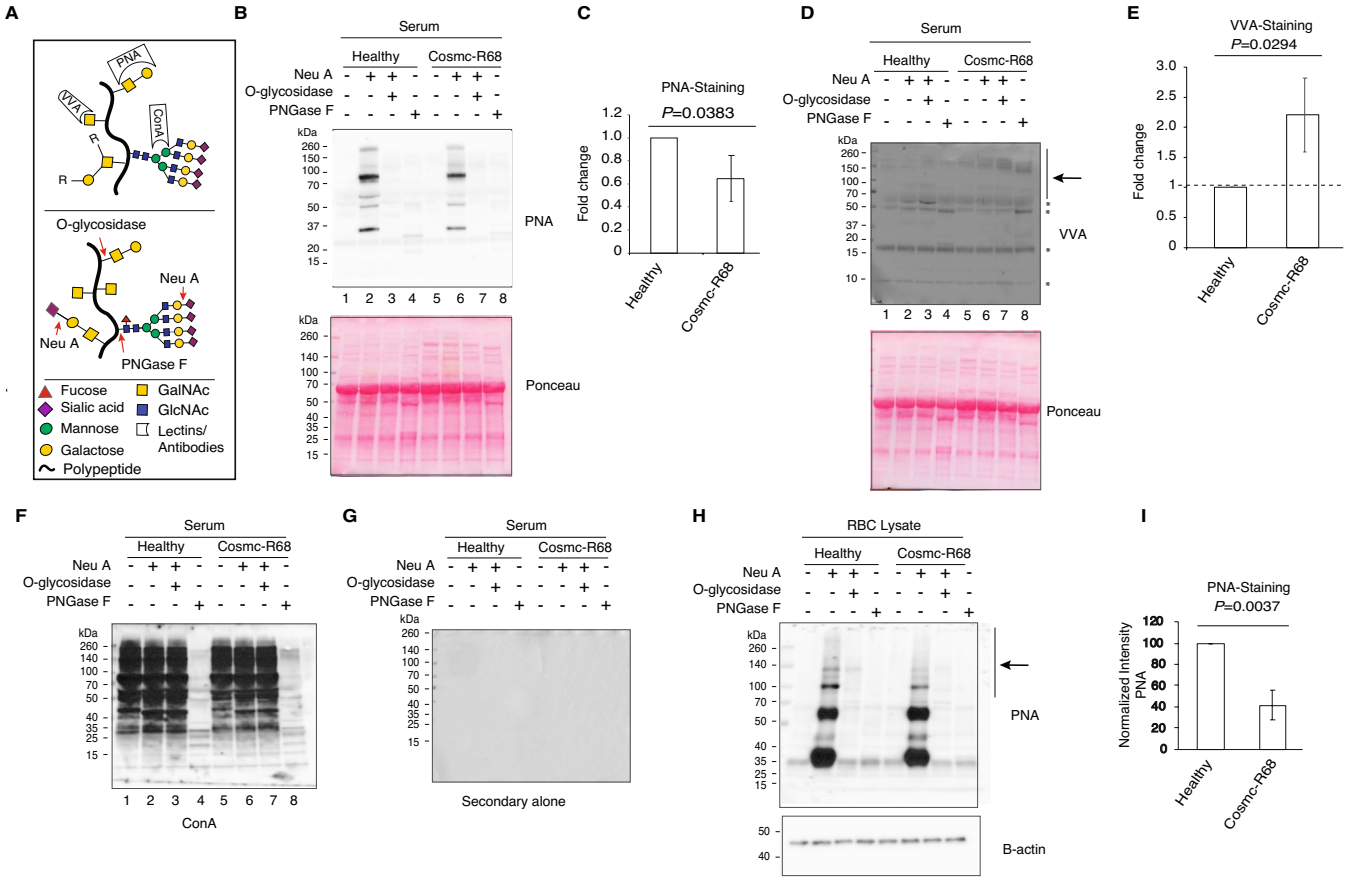

FIGURE 4 | Characterization of glycoproteins from Cosmc-R68 patient serum and RBCs. (A) Glycan structures and their respective interacting lectins and glycosidases are shown. The red arrow indicates the digestion sites within the glycan structure where the glycosidases release the glycans. Glycan symbols are shown at the bottom. (B) Analysis of serum glycoproteins. Diluted serum samples were treated with neuraminidase (Neu A, removes sialic acid), Neu A + O-glycosidase (removes non-sialylated T-antigen), PNGase-F (removes all N-glycans), or mock-treated. Samples were analyzed on SDS-PAGE-lectin blot (LB) probed with PNA (binds to normal non sialylated O-glycan, T-antigen). Ponceau staining serves as a loading control. LB images are representative examples of four independent experiments. (C) ImageJ quantification of LB from (B) (only Lanes 2 and 6 were quantified, and whole lanes were used in quantification). $n = 4$, average of 4, ±1 SD. Fold change in total signal intensity is reported. (D) Similar to (B), samples were processed and analyzed on SDS-PAGE-LB and probed with VVA (binds to truncated O-glycans, Tn-antigen). Ponceau staining serves as a loading control. An asterisk (*) on the LB indicates nonspecific binding. LB images are representative examples of four independent experiments. (E) ImageJ quantification of LB from (D) (only Lanes 3 and 7 included; the arrow shows the area used in quantification). $n = 4$, average of 4, ±1 SD. Fold change in total signal intensity is reported. The dashed line below indicates the background signal of VVA. (F, G) Similar to (B) and (D), samples were processed and analyzed on SDS-PAGE-LB probed with ConA lectin (binds to mannosylated N-glycans) as shown in (F) and secondary antibody alone in (G). (H) Analysis of whole RBC lysate for extended O-glycans. RBC lysates were processed with glycosidases as defined in (B) and analyzed using SDS-PAGE-LB and probed with PNA. LB image is a representative example of four independent experiments. (I) ImageJ quantification of LB from (H) (only lanes with neuraminidase-treated samples included; the arrow shows the area covered in quantification). $n = 4$, average of 4, ±1 SD. Normalized intensity of PNA with respective beta-Actin intensity is reported.

NeuA and NeuA + O-glycosidase lanes). Next, we further analyzed any possible changes in PNA staining. Our results demonstrated a significant reduction in PNA staining in Cosmc-R68 patient RBCs compared to the healthy control (Figure 4H,I, Lanes 2 and 6, quantified). Together, the results suggest that the Cosmc-R68 patient has low levels of extended O-glycans in their RBCs.

3 | Discussion

The studies presented here describe the first mosaic mutation in X-linked *C1GALT1C1* (Xq24) that is present at significant levels in the analyzed cells. Cosmc, encoded by *C1GALT1C1*, is a unique molecular chaperone required within the ER of all vertebrate cells for the functional folding of its single client enzyme, the T-synthase (*C1GALT1*) [10, 16]. The T-synthase activity generates the universal disaccharide T-antigen Galβ1-3GalNAcα1-Ser/Thr on vertebrate glycoproteins. Consequently, cells lacking Cosmc accumulate the precursor Tn-antigen GalNAcα1-Ser/Thr (CD175), whose expression does not typically occur, which can result in significant organismal and cellular pathologies [5]. Our results demonstrate that Cosmc-R68 leads to the expression of the Tn-antigen in glycoproteins expressed by leukocytes and in serum glycoproteins.

The *de novo* mutation of *C1GALT1C1* (c.202C>T, p.Arg68*) identified here, which results in a truncated and nonfunctional

form of Cosmc (Cosmc-R68), presents as an unusual mosaicism, in which ~54% of the cells carry the mutation heterozygously, with the remaining cells having a normal genotype, suggesting the acquisition of the mutation very early in development. Interestingly, this same acquired *de novo* mutation was observed by us in a patient with Tn syndrome [10], an acquired polyagglutination syndrome resulting from mutations in *C1GALT1C1* in hematopoietic cell precursors. This leads to the expression of the Tn-antigen on RBCs [19]. However, in that patient, the mutation arose only in hematopoietic precursors and was not multisystemic.

Because of X-inactivation, in which one X chromosome in each female cell is randomly silenced early in development to become a Barr body [27], assuming completely random inactivation, only about 27% of the patient's cells should be Cosmc-deficient. However, there is little information available about the frequency and phenotypic characteristics of X chromosomal mosaicism [28]. Such mosaicism is unusual, but likely underlies some of the unusual clinical symptoms of the patient with the Cosmc-R68 mutation, which include nonimmune hydrops fetalis and relatively slow growth. These conditions are often observed in patients with various congenital disorders of glycosylation, but they are not unique to such patients [29]. However, in the case of patient Cosmc-R68, the majority of cells likely do express functional Cosmc, which likely reduces the potentially serious consequences of *C1GALT1C1* mutation, as actual loss of Cosmc systemically, as we observed in mice, results in embryonic lethality [16]. We recently identified patients with a germline mutation in *C1GALT1C1* (*COSMC*-CDG) in which the mutation is hypomorphic. While Cosmc protein is vastly decreased, the low residual amount is sufficient to rescue a significant amount of T-synthase enzyme activity, leading to normal O-glycosylation alongside some expression of the Tn-antigen [9]. The male patients exhibited developmental delay, short stature, immunodeficiency, thrombocytopenia, elevated liver enzymes, isolated seizures, and AKI. The mother and maternal grandmother of these boys have mild and somewhat nonspecific findings of borderline short stature and learning differences with transient or mild cytopenia. Although there is some phenotypic overlap in the described patient's borderline short stature, epilepsy, proteinuria, transient elevation of liver enzymes, and developmental delay, the clinical presentation differs prenatally. We hypothesize that this is explained by the different phenotypic effects of decreased but nonzero T-synthase activity in all cells in patients with *COSMC*-CDG versus a stochastically distributed complete loss of T-synthase activity in a (significant) subset of cells in patient Cosmc-R68. To date, after the neonatal period, our patient has a normal complete blood count and no evidence of aHUS. Our patient's height is within the normal range at 5% without skeletal disproportion.

Unrelated to the original hydrops fetalis phenotype, an additional genetic variant that may be contributing to her neurodevelopmental phenotype was also detected upon reanalysis of genomic data. The *IRF2BPL* variant is classified by the clinical laboratory as likely pathogenic based on ACMG guidelines [30], although the clinical significance is not entirely clear. The variant remains novel, but it is a truncating variant and is expected to result in loss of protein function. Other nonsense variants are reported to be pathogenic based on functional data [31]. *IRF2BPL*-related phenotypes are variable, ranging from *de novo* childhood-onset epileptic encephalopathy, progressive myoclonic encephalopathy to late-onset familial movement disorder [20, 21, 32]. Although most individuals harbor apparently *de novo* variants, there are several familial cases now reported [32]. Given that the symptoms can be variable, progressive, and later onset, we cannot be certain if the proband's focal epilepsy and developmental delay or the father's learning difficulties might be related, or if one or both may experience additional symptoms in the future.

In relation to our studies, there are additional phenotypes observed in individuals with defects in galactose pathways affecting protein galactosylation. As seen for individuals with mutations in the X-linked UDP-galactose transporter *SLC35A2*, and variants in *SLC39A8*, a transporter for manganese, which is a required cofactor for T-synthase and other galactosyltransferases, both are associated with delayed development and neurodisorders [33, 34].

In summary, our studies present novel insights into mosaic mutations in glycosylation pathways involving X-linked genes, and suggest the possibility that less mosaic mutations might exist, but may be missed due to less severe clinical consequences, as seen recently in patients with HUS arising from a de novo mutation in *C1GALT1C1* [11]. Related examples of de novo mutations in X-linked genes include the *PIG-A* gene, required for glycosylphosphatidylinositol (GPI)-anchor–based disorder paroxysmal nocturnal hemoglobinuria (PNH) [35], and congenital mutations in the O-GlcNAc transferase encoded by OGT in patients with OGT-CDG [36]. Although the overall frequency of (relevant) postzygotic mosaicism for *C1GALT1C1* mutations is unknown, we should consider that *C1GALT1C1* be included in the molecular genetic workup of developmental delays in female patients.

4 | Materials and Methods

4.1 | Ethics Approval

The Corewell Health Institutional Review Board has approved the study for which this patient is enrolled. All adult probands and the legal guardians of the minor have given verbal and written consent for study participation, publication of experimental and clinical data, and use of photographs. Blood samples from the patient and healthy control were collected under IRB–approved protocol (#2021-058).

4.2 | Rapid Next-Generation Genome Sequencing (NGS)

Rapid NGS was performed as a trio with her parents (Rady Children's Institute for Genomic Medicine, San Diego, CA) utilizing whole blood cells at Day 3 of life. Alignment and variant calling were performed using the Edico DRAGEN pipeline with Reference Build 37.1. Variants are curated and classified in accordance with the American College of Medical Genetics and Genomics Guidelines [30]. A buccal swab sample was collected on Day 20 of life for targeted variant analysis to evaluate for mosaicism (Fulgent Genetics, Temple City, CA). Reanalysis and confirmation of the original genome data were requested and carried out by Rady Children's Institute for Genomic Medicine. The *C1GALT1C1* variant was confirmed by Sanger sequencing. The following primers

were used: forward, 5′-CTCTGCTTTGTCACAGTGTTTG-3′ and reverse, 5′-TGCTAGGACACATTAGGATTGG-3′.

4.3 | Lysate Preparation and T-Synthase Activity Assay

HEK cells were trypsinized and collected (500*g*, 5 min) and washed with 1 mL ice-cold PBS. Using 200 μL of lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, one tablet protease inhibitor/10 mL) for 30 min (vertexing every ~2 min) on ice, followed by sonication (amplitude 39, 7× for ~2 s each). After centrifugation at 15000*g* for 15 min at 4°C, the supernatant was collected. For leukocyte lysate preparation, snap-frozen pellets in PBS (−80°C) were thawed on ice, and protease inhibitor and Triton X-100 (1% final concentration) were added. Cells were lysed, and lysate was prepared as defined for HEK cells, except the centrifugation step was not included. For T-synthase activity, thawed leukocyte lysates (−80°C) on ice, and freshly prepared CosmcKO or WT HEK cell lysates were used following an established protocol [37].

4.4 | Western Blot Analysis

Whole-cell lysates prepared as defined above were resolved on SDS-PAGE (4%–16%), and the resolved proteins were transferred to nitrocellulose membranes, which were blocked with 5% milk in 1× TBST for an hour at room temperature (RT). Membranes were incubated overnight with primary antibodies prepared in 1× TBST containing 5% milk, including anti-Cosmc (mouse, H-10, Santa Cruz #SC-271829, 1:2000), anti-T-synthase (mouse, F31, Santa Cruz #SC-100745, 1:2000), anti-β-actin (HRP-conjugated, Sigma-Aldrich A3854, 1:10000), and anti-HA (rabbit, Cell Signaling, HA-Tag (C29F4) mAb #3724, 1:1000 in 5% BSA in 1× TBST). For secondary antibodies, Peroxidase-AffiniPure Goat Anti-Mouse IgG (H + L) (Jackson ImmunoResearch, 115-035-062, 1:5000), ECL Anti-rabbit IgG HRP–linked F(ab′)2 fragment (from donkey) (GE Healthcare, # NA9340V, 1:5000), prepared as the primary antibody, were incubated at RT for an hour, followed by washing five times for 5 min each with 1× TBST. The signals were detected using SuperSignal West Pico PLUS Chemiluminescent Substrate or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) using an Amersham Imager 600 (GE Healthcare Life Sciences).

4.5 | Serum Collection

Blood samples from the patient and healthy control were collected based on the IRB–approved protocol. Collected blood samples were kept at RT for an hour and then centrifuged at 2000 rpm for 10 min at RT.The supernatant was carefully collected as serum and stored.

4.6 | Serum Processing and Lectin Blot Analysis

Serum from −80°C was thawed on ice and analyzed. For SDS-PAGE lectin blot analysis, ~150 μg of serum protein was boiled for 10 min in 1× glycoprotein denaturing buffer (NEB). The boiled samples were then treated with either mock treatment, 1 μL of α2-3,6,8,9 NeuA (Cat#P0722S), a combination of NeuA and O-glycosidase (Cat#P0733S), or PNGase-F (Cat#P0708S) for 2 h at 37°C, following the manufacturer's protocol from NEB. Approximately 5–10 μg of the processed samples were resolved on SDS-PAGE and transferred to prepared lectin blots on nitrocellulose membranes. Membranes were blocked with 5% BSA in 1× TBST (300 mM NaCl) for 1 h at RT under constant shaking (30 rpm/min) and probed with the following biotinylated lectins: *Vicia villosa* lectin (VVA, Cat#B-1235-2, diluted to 0.2 μg/mL), PNA (Cat#B-1075-5, diluted to 0.2 μg/mL), and ConA (Cat#B-1005-5, diluted to 0.1 μg/mL) in 1× TBST (300 mM NaCl) overnight at 4°C. Membranes were washed two times for 5 min with 1× TBST (300 mM NaCl), followed by incubation with HRP–labeled streptavidin (Cat#SA-5014-1, Vector Laboratories) at a 1:15000 dilution for 1 h at RT. Membranes were washed at least five times for 5 min each with 1× TBST (300 mM NaCl) and developed as defined above.

4.7 | Lymphocyte Collection

Blood was collected using an anticoagulant and then diluted 1:1 with PBS.The diluted blood was carefully layered onto Ficoll-Paque PLUS in clear 15 mL tubes (with an internal diameter of ~1.3 cm, maintaining the same height of Ficoll-Paque (~2.4 cm) to blood height (~3 cm)). The samples were centrifuged using a swing-out rotor at 400*g* for 30–40 min (18°C–20°C). After centrifugation, the upper plasma layer was removed carefully. Using a Pasteur pipette, the lymphocyte (buffy coat) was carefully aliquoted and kept in a clean centrifuge tube. At least three volumes of PBS were then added to the lymphocytes and gently mixed by pipetting up and down with a Pasteur pipette. The samples were then centrifuged at 60–100*g* for 10 min at 18°C–20°C. The supernatant was removed, and the process was repeated one more time. Immediately afterward, the cell pellet was resuspended in 1 mL of PBS and was snap-frozen in liquid nitrogen.

4.8 | RBC Collection and SDS-PAGE Lectin Blot Analysis

Collected frozen RBCs were thawed on ice and resuspended in 200 μL of lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and Protease Inhibitor Cocktail Tablet; Roche 11836170001). The cell suspension was vortexed every ~2 min on ice for 30 min before sonication (seven times, 2 s, amplitude = 39). The lysate was then centrifuged at 15000*g* for 15 min at 4°C, and the supernatant was collected for analysis. The concentration of the lysate was quantified using the BCA assay (Thermo Scientific #23225) with BSA standards (Thermo Scientific #23208).

4.9 | Quantification of SDS-PAGE Lectin Blot

Lectin blot images were analyzed using ImageJ software. For fold change calculation, intensities from the healthy control are normalized to 1, and the patient intensities are accordingly calculated and reported as fold change on the *Y*-axis.

4.10 | Generation of the HA-Cosmc and the Truncated HA-Cosmc68 (1–68) and Transient Transfections

N-terminally HA-tagged Cosmc constructs were generated using the mammalian vector pGen2.1 (GenScript). Cells at 80%–90% confluence were transiently transfected using the *X-tremeGENE* HP DNA Transfection Reagent (Sigma-Aldrich). Two micrograms of plasmid or empty vector, directly from the miniprep, was mixed with the transfection reagents as specified in the company's protocol and transfected into CosmcKO (SimpleCells) cultured in six-well plates in complete DMEM medium (penicillin/streptomycin, amphotericin B, and glutamine) at 37°C with 5% CO_2. After 72 h of transfection, cells were collected and immediately processed for T-synthase activity as described above.

Author Contributions

R.P.A., L.H.S., and R.D.C. designed the research and interpreted the data. L.H.S. managed patient care and patient data collection. R.P.A. and A.R. performed research, data collection, and data analysis. C.B. performed research and data collection. C.N. performed Sanger sequencing data collection and analysis. J.H.-M, S.F.C., F.E., and B.B.B. managed resources. F.E. and B.B.B. performed data analysis. R.P.A., L.H.S., and R.D.C. wrote the paper. All authors reviewed and approved the final paper.

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Ethics Statement

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Declaration of Helsinki of 1975, as revised in 2000 [5].

Consent

Informed consent was obtained from all patients for inclusion in the study, and the information is included in this article.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data are presented in the manuscript or are available upon request with no restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.