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GTDC2 modifies *O*-mannosylated α-dystroglycan in the endoplasmic reticulum to generate *N*-acetyl glucosamine epitopes reactive with CTD110.6 antibody

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ABSTRACT

Hypoglycosylation is a common characteristic of dystroglycanopathy, which is a group of congenital muscular dystrophies. More than ten genes have been implicated in α-dystroglycanopathies that are associated with the defect in the *O*-mannosylation pathway. One such gene is *GTDC2*, which was recently reported to encode *O*-mannose β-1,4-*N*-acetylglucosaminyltransferase. Here we show that GTDC2 generates CTD110.6 antibody-reactive *N*-acetylglucosamine (GlcNAc) epitopes on the *O*-mannosylated α-dystroglycan (α-DG). Using the antibody, we show that mutations of GTDC2 identified in Walker–Warburg syndrome and alanine-substitution of conserved residues between GTDC2 and EGF domain *O*-GlcNAc transferase resulted in decreased glycosylation. Moreover, GTDC2-modified GlcNAc epitopes are localized in the endoplasmic reticulum (ER). These data suggested that GTDC2 is a novel glycosyltransferase catalyzing GlcNAcylation of *O*-mannosylated α-DG in the ER. CTD110.6 antibody may be useful to detect a specific form of GlcNAcylated *O*-mannose and to analyze defective *O*-glycosylation in α-dystroglycanopathies.

1. Introduction

Congenital muscular dystrophies (CMDs) are a heterogeneous group of inherited neuromuscular disorders characterized by hypotonia and weakness at birth or in early infancy with variable clinical manifestations of the eye and brain. A common subgroup within the CMDs is the α-dystroglycanopathy characterized by decreased functional glycosylation of α-dystroglycan (α-DG) [1,2]. After post-translational glycosylation in the endoplasmic reticulum (ER) and Golgi apparatus, α-DG is cleaved into two non-covalently associated proteins, namely, α-DG and β-dystroglycan (β-DG) [3–5]. α-DG binds to several extracellular matrix components, including laminin-α2 and other ligands harboring laminin-G domain, [5,6] whereas β-DG is a transmembrane protein and is anchored to the cytoskeleton by the dystrophin complex [4]. Precise glycosylation of α-DG is critical for its function as an extracellular matrix receptor in the skeletal muscle as well as in many tissues such as the epithelia, eye, and central nervous system [7–9].

α-Dystroglycanopathy includes a broad spectrum of clinically related diseases such as Walker–Warburg syndrome (WWS), muscle–eye–brain disease, Fukuyama-type congenital muscular dystrophy, congenital muscular dystrophy type 1C, and limb-girdle muscular dystrophy type 2I. Hypoglycosylation of α-DG is a hallmark of these disorders [10]. α-dystroglycanopathies have been linked to mutations in the following 15 genes: Protein *O*-mannosyltransferase 1 (POMT1) [11], Protein *O*-mannosyltransferase 2 (POMT2) [12], Protein *O*-mannose β-1,2-*N*-acetylglucosaminyltransferase (POMGNT1) [13], Fukutin [14], Fukutin-related protein (FKRP) [15], like-acetylglucosaminyltransferase (LARGE) [16], Dolichyl-phosphate mannosyltransferase 2 (DPM2) [17], Dolichyl-phosphate mannosyltransferase 3 (DPM3) [18], Dolichol Kinase [19], Isoprenoid Synthase Domain Containing (ISPD) [20–23], Glycosyltransferase-like domain containing 2 (GTDC2) [24], β-1,3-*N*-acetylgalactosaminyltransferase 2 (B3GALNT2) [25], β-1,3-*N*-acetylglucosaminyltransferase 1 (B3GNT1) [26], SGK196

Abbreviations: DG, dystroglycan; EGF, epidermal growth factor; GlcNAc, *N*-acetylglucosamine; EOGT, EGF domain *O*-GlcNAc transferase; ER, endoplasmic reticulum.

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[27], and Transmembrane protein 5 (TMEM5) [23,27]. Although, the functions of all of the above genes have not been elucidated, currently the known function is associated to the biosynthesis of *O*-mannosyl core structure, and repeating units of xylose and glucuronic acid mediated by LARGE activity [28,29].

GTDC2 is homologous to the recently identified EGF domain *O*-GlcNAc transferase (EOGT) [30]. In this study, we investigated the biochemical relationship between GTDC2 and EOGT, and our data indicate that GTDC2 generates CTD110.6-reactive epitopes on the *O*-mannosyl glycan in the ER. Together with a recent report showing GTDC2 encoding for *O*-mannose β-1,4-*N*-acetylglucosaminyltransferase [31], our results provide a novel methodology for detecting a specific form of GlcNAcylated *O*-mannose and for analyzing defective *O*-glycosylation in α-dystroglycanopathies.

2. Materials and methods

2.1. Materials

A plasmid encoding mouse Notch1 EGF repeat was kindly provided by Dr. Stanley [32]. The expression construct for GTDC2 (pCMV-Sport6/GTDC2) was obtained from Invitrogen. The following antibodies were used: rabbit anti-EOGT (Sigma), rabbit anti-GTDC2 (Sigma), mouse anti-6xHis (Genscript), rabbit anti-myc (Santa Cruz), mouse anti-calnexin (BD biosciences), mouse anti-GM130 (BD biosciences), mouse anti-*O*-GlcNAc antibodies (CTD110.6; Thermo Scientific), and horseradish peroxidase (HRP)-conjugated anti-human IgG Fc antibody (Rockland). siRNAs for *GTDC2* (s39589; s39591), *POMT1* (s20775; s20776), and *POMT2* (s26787; s229609) were obtained from Qiagen.

2.2. Plasmid constructs

To produce myc-tagged GTDC2 construct (pSectag2/GTDC2:mycHis), the coding region was amplified by PCR using pCMV-Sport6/GTDC2 as a template and cloned into the *Nhe*I and *Xho*I sites of pSectag2 vector (Invitrogen). The following primers were used: *Nhe*I-GTDC2-Fw 5′-CCCCCTGCTAGCGATAGGATGCACCTCTCT-3′ and *Xho*I-GTDC2-Rv 5′- GGTCTGCTCGAGACGTGCTGCACACCAGCA-3′. In vitro mutagenesis for GTDC2 was conducted using KOD-Plus-Mutagenesis Kit (Toyobo) using pSectag2/GTDC2:mycHis as template.

For creating the pFUSE-α-DG-Fc construct (for the expression of α-DG:Fc), mouse α-DG fragment was amplified by PCR and cloned into *Age*I and *Bgl*II sites of pFUSE-SEAP-Fc (Invitrogen) using GeneArt Seamless Cloning and Assembly Kit (Invitrogen). The following primers were used: *Xma*I-DG-Fw 5′-ACCTGAGATCACCGGCCCGGGAGGATGTCTGTGGACAAC-3′ and *Bgl*II-DG-Rv 5′-GGCACTCCACAGATCTGCCCCGAGTGATGTTCTG-3′. Deletion of the α-DG:Fc constructs and alanine-substitution to produce α-DG483[T315A/T317A]-Fc were conducted using the KOD-Plus-Mutagenesis Kit. α-DG[3S]-Fc construct was generated using GeneArt Seamless Cloning and Assembly Kit (Invitrogen) so that all Thr/Ser sites except for T315, T317, and T322 were alanine-substituted. The plasmid encoding alanine-substituted α-DG was synthesized by custom gene synthesis service (Genscript), and the sequence information is available in Supplementary Text.

2.3. Cell culture, transfection, and small interfering RNA (siRNA)

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (DMEM/10% FBS). Expression vectors were transiently transfected into HEK293T cells using polyethylenimine as described in Supplementary method. Co-transfection of siRNA with plasmid constructs was performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's instruction.

2.4. Purification of αDG-Fc and Western blotting

For the isolation of α-DG-Fc from cell lysates, cells were lysed in RIPA lysis buffer (Cell signaling) and the lysates were incubated with dynabeads protein A (Invitrogen) for 1 h at 4 °C. For the purification of secreted α-DG-Fc, the culture supernatant was incubated with the dynabeads protein A for 1 h at 4 °C. The immunoprecipitates were washed extensively with 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 0.25% Triton X-100, and eluted with SDS–PAGE sample buffer containing 2% SDS and 70 mM 2-mercaptoethanol.

For Western blotting, each sample was separated on a SDS–PAGE gel, and transferred onto a PVDF membrane (Millipore). Immunodetection was performed using an appropriate primary antibody, followed by HRP-conjugated secondary antibody, and enhanced chemiluminescence as described previously [33]. HRP-conjugated anti-human IgG Fc antibody was used without secondary antibody for detection.

2.5. Immunostaining

HEK293T cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min and ice-cold methanol for 5 min. Cells were permeabilized with 0.5% Triton X-100 in PBS for 15 min and blocked with PBS containing 5% FBS for 30 min. Immunostaining was performed using an appropriate primary antibody for 2 h followed by incubation with fluorescent dye-conjugated secondary antibody for 1 h. All samples were analyzed using a confocal microscope (Olympus).

2.6. Glycosidase treatment

α-DG-Fc was immunoprecipitated from the culture medium with dynabeads protein A (Invitrogen) as described above. After elution and denaturation of the bound protein using 1× denaturing buffer (NEB), glycosidase digestion was performed for 16 h at 37 °C in 50 mM sodium citrate, pH 6.0, containing 1% Nonidet P-40, protease inhibitor cocktail set I (Calbiochem), and glycosidase mixtures. *O*-Glycosidase, PNGase F, sialidase, β1,4-galactosidase, and β-*N*-acetylglucosaminidase were all obtained from NEB, except for bovine kidney β-*N*-acetylhexosaminidase (A2415; Sigma).

3. Results

3.1. CTD110.6 detects GTDC2-modified O-mannosyl glycans

GTDC2 is highly homologous to EOGT [30,34]. Considering that EOGT specifically *O*-GlcNAcylates Notch1 EGF repeats (Fig. 1A) [30] and GTDC2 is associated with defect in the glycosylation of α-DG [24,27], we initially postulated that GTDC2 might encode *O*-GlcNAc transferase that specifically glycosylates α-DG.

To analyze the glycosylation of α-DG, the heavy-chain constant (Fc) region of human IgG2 was fused with α-DG (α-DG-Fc). HEK293T cells were co-expressed with GTDC2 and α-DG-Fc to isolate glycosylated α-DG-Fc from cell lysates. For immunodetection, CTD110.6 antibody was used because it is widely used for the detection of *O*-GlcNAcylated proteins. Compared to the α-DG-Fc isolated from the control transfectants, immunoreactivity of α-DG-Fc towards CTD110.6 was elevated upon GTDC2 overexpression (Fig. 1A). Conversely, downregulation of *GTDC2* by siRNA resulted in elimination of CTD110.6-reactivity (Fig. 1B), suggesting that GTDC2 acts on α-DG to generate CTD110.6 epitope.

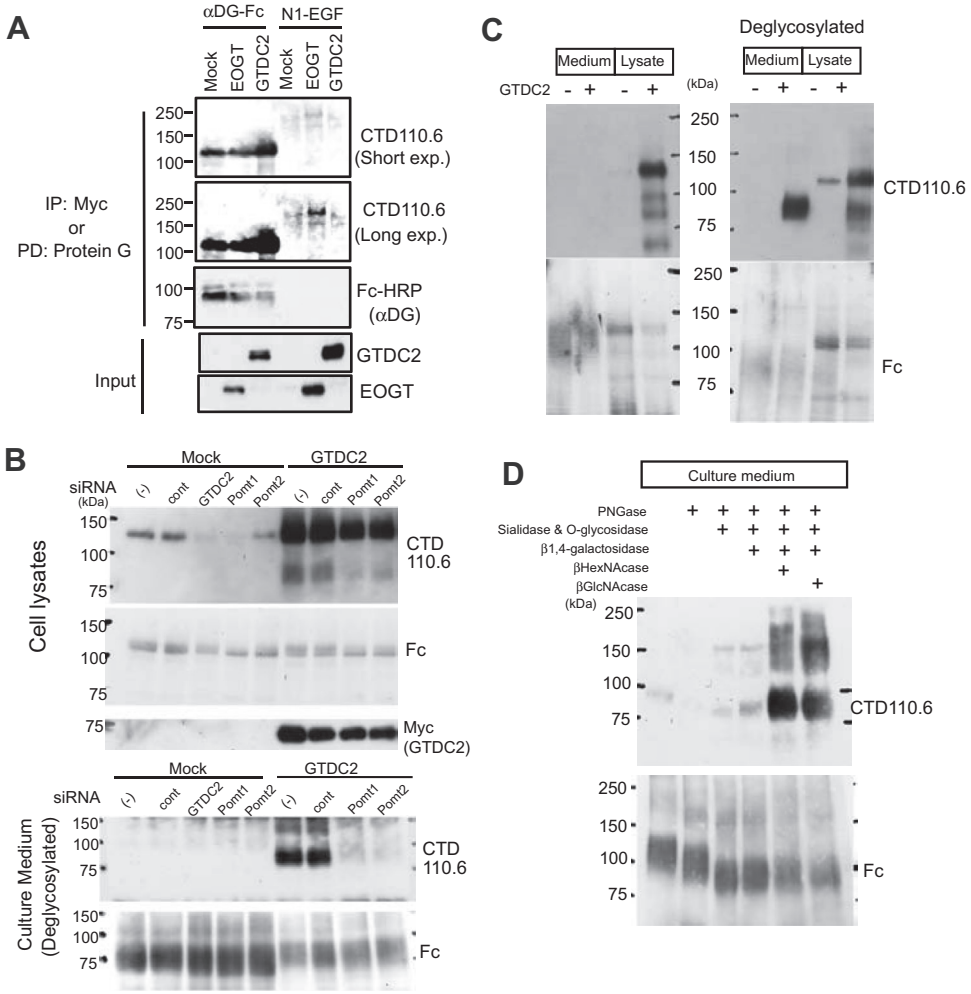

Fig. 1. CTD110.6 detects GTDC2-modified *O*-mannosyl glycans. (A) Immunoblot analysis of the extracellular domain of Notch receptor (mN1-EGF:mycHis) or mouse α-dystroglycan (α-DG-Fc) isolated from cell lysate of HEK293T cells co-transfected with *EOGT*, *GTDC2*, or control vector. Note that α-DG-Fc is detected by CTD110.6 antibody. (B) α-DG-Fc isolated from cell lysate or culture supernatant of HEK293T cells co-transfected with or without *GTDC2* together with indicated siRNA was analyzed by immunoblotting with CTD110.6 antibody or anti-human Fc antibody. α-DG-Fc isolated from the culture supernatant was deglycosylated with glycosidase mixtures (*O*-Glycosidase, PNGase F, sialidase, β1,4 galactosidase, and β-*N*-acetylglucosaminidase). (C) α-DG-Fc was isolated from cell lysate or culture supernatant of HEK293T cells co-transfected with *GTDC2*. Isolated α-DG-Fc was untreated or deglycosylated with glycosidase mixtures (*O*-Glycosidase, PNGase F, sialidase, β1,4 galactosidase, and β-*N*-acetylglucosaminidase), and analyzed by immunoblotting with CTD110.6 antibody or anti-human Fc antibody. (D) α-DG-Fc isolated from culture supernatant of HEK293T cells co-transfected with *GTDC2* was treated with indicated glycosidases and analyzed by immunoblotting with CTD110.6 antibody or anti-human Fc antibody.

The GTDC2-modified structure could indeed be *O*-linked GlcNAc or, alternatively, other epitopes on *O*-mannosyl glycans could display cross-reactivity with CTD110.6 antibody. Therefore, we addressed if *O*-mannosyltransferases are required for the GTDC2 activity. Towards this end, HEK293T cells were transfected with *POMT1* or *POMT2* siRNA along with α-DG-Fc construct. The reactivity of α-DG with CTD110.6 was abolished upon downregulation of *POMT1/2* (Fig. 1B). Similarly, decreased CTD110.6 reactivity was observed in α-DG isolated from GTDC2-overexpressing cells when cells were treated with siRNA for *POMT1* and *POMT2* (Fig. 1B). These results indicate that *POMT1* and *2* are required for the activity of GTDC2, suggesting that GTDC2 modifies the structure on the *O*-mannosyl glycans.

During the course of our study, it was reported that GTDC2 encodes *O*-mannose β1,4-*N*-acetylglucosaminyltransferase [31]. A previous study by Isono [35] suggested that CTD110.6 antibody cross-reacts with GlcNAcβ1,4GlcNAc-Asn structure. Similarly, our results suggest that CTD110.6 antibody cross-reacts with GlcNAcβ1,4Man-Thr/Ser structure on α-DG. In contrast to α-DG isolated from cell lysates, α-DG secreted into the culture medium did not display any immunoreactivity towards CTD110.6 antibody (Fig. 1C). However, after treatment with glycosidase mixtures (*O*-Glycosidase, PNGase F, sialidase, and β1,4 galactosidase) containing β-*N*-acetylhexosaminidase or β-*N*-acetylglucosaminidase, CTD110.6 reactivity could be retrieved (Fig. 1B–D). These results are consistent with that GlcNAcβ1,4Man-Thr/Ser structure is further elongated by β1,3-linked GalNAc and/or mannosyl residues are further branched by β1,2-linked GlcNAc or β1,6-linked GlcNAc [13,36].

3.2. GTDC2-dependent modification occurs on the mucin-like domain of α-DG

Our data suggests that CTD110.6 could serve as an experimental tool to locate GTDC2-modified sites on *O*-mannosylated α-DG. To test this hypothesis, we generated two constructs, α-DG598-Fc and α-DG483-Fc, with deletions in the C-terminal region that end at Gln-598 and Ser-483 (Fig. 2A). As can be seen in Fig. 2B, α-DG598-Fc and α-DG483-Fc were modified by GTDC2 and the level was comparable with that observed for α-DG. In contrast, α-DG[3S]-Fc mutant lacking all the Ser and Thr residues within the mucin-like domain except for the first three Thr residues

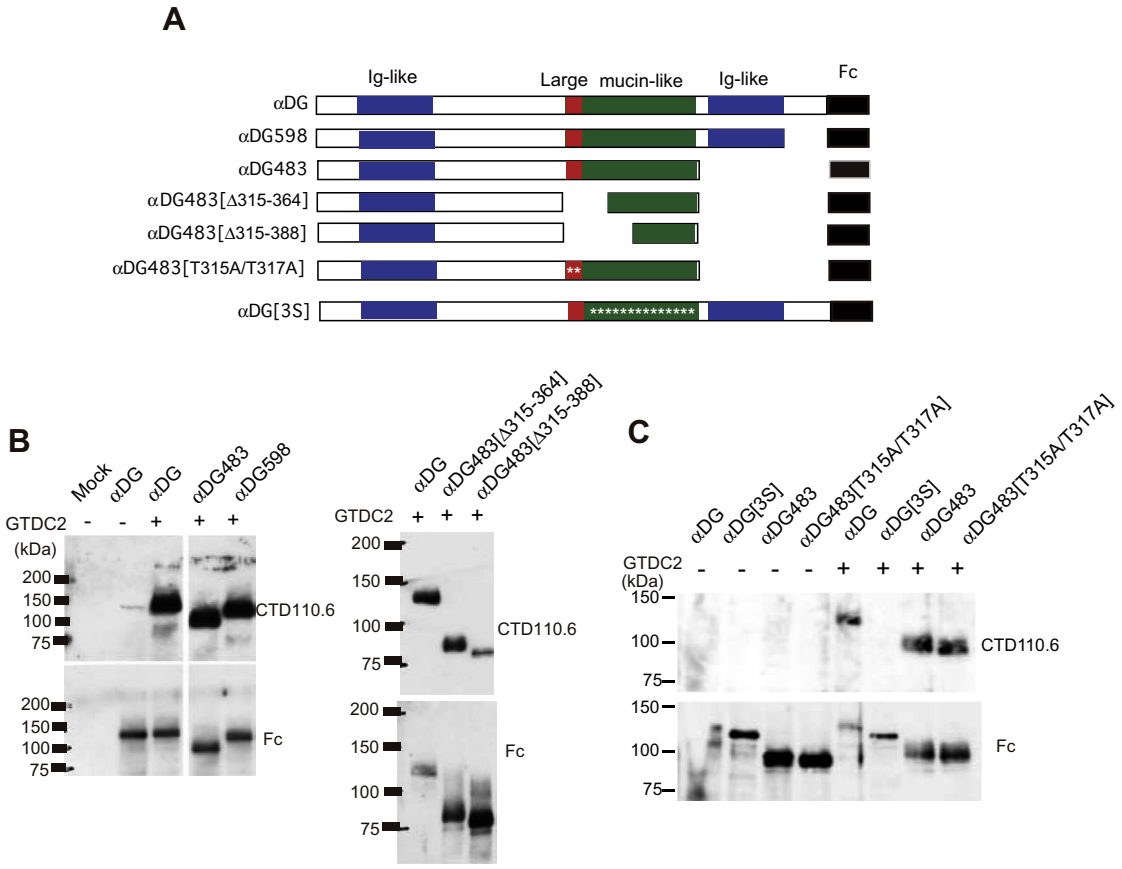

Fig. 2. GTDC2 modifies *O*-mannose on the mucin-like domain of α-DG. (A) Schematic representation of mouse α-DG constructs used in this study. Immunoglobulin (Ig)-like domain is shown in *blue*; Large-targeting sequence in *red*; and the remaining mucin-like domain in *green*. Asterisks indicate alanine-substitution. (B) Immunoblot analysis of α-DG-Fc and the deletion mutants isolated from cell lysate of HEK293T cells. Where indicated, *GTDC2* was co-transfected with each α-DG construct. (C) Alanine-substituted mutants (α-DG483[T315A/T317A]-Fc and α-DG[3S]-Fc) were analyzed as described in (B). (For interpretation of color in Figure legent, the reader is referred to the web version of this article.)

(^{315}TPTPVT^{320}), was not detectable by CTD110.6 antibody (Fig. 2C). These data suggest that the mucin-like domain contains *O*-mannosylation sites modifiable by GTDC2. We further conducted amino-terminal deletion of mucin-like domain in α-DG483 to generate α-DG483[Δ315–388]–Fc and α-DG483[Δ315–364]–Fc (Fig. 2A). The decreased level of modification was observed in α-DG483 [Δ315–364]–Fc whereas the signal was largely diminished in α-DG483[Δ315–388]–Fc (Fig. 2B), suggesting that amino acids 315–388 of α-DG corresponding to the highly *O*-mannosylated region was preferentially modified by GTDC2 [37]. In contrast, alanine-substitution of LARGE targeting the TPTPV sequence (α-DG483[T315A/T317A]) [38] resulted in the CTD110.6 reactivity comparable with that of α-DG483 (Fig. 2C). These data suggest that GTDC2-dependent modification sites are mainly distributed in the *O*-mannosylated region and not limited to the LARGE-targeting sequence.

3.3. Mutation of GTDC2 affects CTD110.6 reactivity

Next, we used CTD110.6 reactivity of α-DG as a measure for the enzyme activity of GTDC2. To this end, we generated mouse GTDC2 carrying mutations found in the patients of WWS (Fig. 3A) [24] and co-expressed with α-DG-Fc in HEK293T cells. Immunoblotting with CTD110.6 antibody revealed that all the reported GTDC2 mutations affect the reactivity: loss of CTD110.6 reactivity was observed in R158H and W197Δ mutants, and decreased reactivity in R445Δ mutant lacking the C-terminal fibronectin III domain (Fig. 3B). These results confirm the contribution of defective

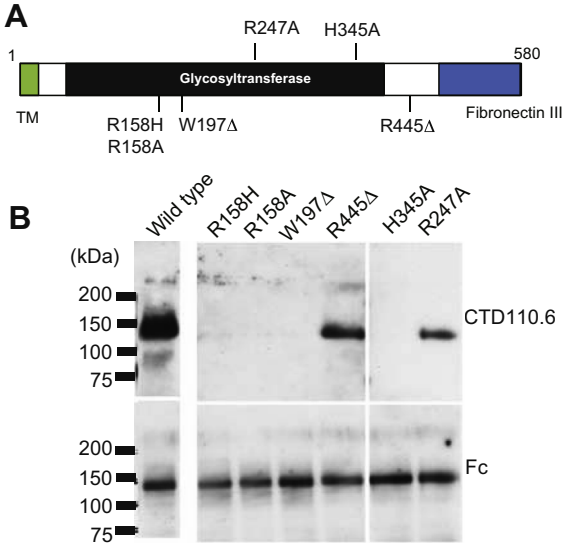

Fig. 3. Mutations of GTDC2 are associated with CTD110.6 reactivity. (A) Schematics of GTDC2 constructs used in this study. Mutant forms of GTDC2 mimicking the mutation in WWS patients are shown below (R158H, W197Δ, and R445Δ). R158A mutation was also analyzed. Alanine substitution of amino acid residues conserved between EOGT and GTDC2 is shown above (R247A, and H345A). (B) α-DG-Fc was co-expressed with wild-type *GTDC2* or its mutant isoforms in HEK293T cells. Immunoblot analysis with CTD110.6 revealed that the glycosyltransferase activity of each mutant is abolished or decreased.

enzymatic activity as a molecular etiology of WWS and further suggest that the fibronectin III domain is not essential for the enzymatic activity of GTDC2.

To further analyze the structural requirement of GTDC2 for its enzymatic activity, conserved residues between GTDC2 and EOGT were alanine-substituted in mouse GTDC2 (Fig. 3A). These were then co-expressed with α-DG in HEK293T cells; our results indicate that H345 and R247 contribute to the enzyme activity of GTDC2 (Fig. 3B).

3. 4. GTDC2-catalyzed glycosylation occurs in the ER

Finally, we attempted to analyze the subcellular localization of GTDC2-modified *O*-mannosyl glycans in cultured cells. Consistent with the recent report [31], subcellular localization of GTDC2 largely overlapped with an ER marker calnexin (Fig. 4A). Next, we asked if GTDC2-dependent glycosylation occurs in the ER or Golgi because of small amounts of GTDC2 localized in Golgi. To this end, α-DG-Fc was co-transfected with GTDC2 and immunostained with CTD110.6 antibody. In control cells expressing α-DG-Fc alone, CTD110.6 staining was observed in the perinucleus. In contrast, co-expression of GTDC2 resulted in immunoreactivity that coincides with the ER marker calnexin (Fig. 4B). These data suggest that GTDC2 is a novel glycosyltransferase catalyzing GlcNAcylation in the ER.

4. Discussion

In this study, we show that GTDC2, a recently reported *O*-mannose β1,4-*N*-acetylglucosaminyltransferase [31], synthesizes carbohydrate epitopes detectable by CTD110.6. The cross-reactivity of CTD110.6 was previously reported in *N*-linked $GlcNAc_2$ modified proteins [35]. Thus, it is tempting to speculate that a common GlcNAcβ1,4-X-(Asn/Ser/Thr) structure would be recognized by CTD110.6. Besides β1,4-linked GlcNAc, *O*-mannose could be modified with β1,2- or β1,6-linked GlcNAc [13,36]. GTDC2-modified β1,4-GlcNAc is further modified to β1,3-linked GalNAc by B3GALNT2 [31]. Consistent with these potential glycosylation processes, CTD110.6-reactive GTDC2-modified structure on the secreted α-DG was retrieved by digestion with glycosidases including β-*N*-acetylhexosaminidase or β-*N*-acetylglucosaminidase. However, the reason for relative resistance of GTDC2-modified structure by these glycosidases is currently not known.

This study also suggests that GTDC2 modifies α-DG in the ER. In parallel experiments, we observed that co-expression of EOGT and Notch1 EGF repeats also results in CTD110.6 reactivity in the ER (Ogawa M, unpublished observation). Thus, GlcNAc transferase activity in the ER is a common characteristic between GTDC2 and EOGT. These enzymes do not show significant homology to other Golgi-resident GlcNAc transferases. In contrast, GTDC2 and EOGT share amino acid residues that contribute to the enzyme activity of GTDC2. Thus, these conserved residues may serve for physical association with the common donor substrate UDP-GlcNAc in a manner distinct from Golgi-resident GlcNAc transferases. Further structural–functional analyses of GTDC2 and EOGT will be required to address this possibility.

Currently, no reagents are available to detect the *O*-mannosyl core structure. Our results provide a novel methodology to detect a specific form of GlcNAcylated *O*-mannose using CTD110.6. Moreover, the capping of *O*-mannose with GTDC2 allows for indirect detection of *O*-mannose monosaccharide and modification sites by CTD110.6 antibody. Thus, the reactivity of α-DG with CTD110.6 would be a valuable measure for the biochemical characterization of α-dystroglycanopathies, especially those lacking B3GALNT2 that acts on the GTDC2-modified enzymatic products.

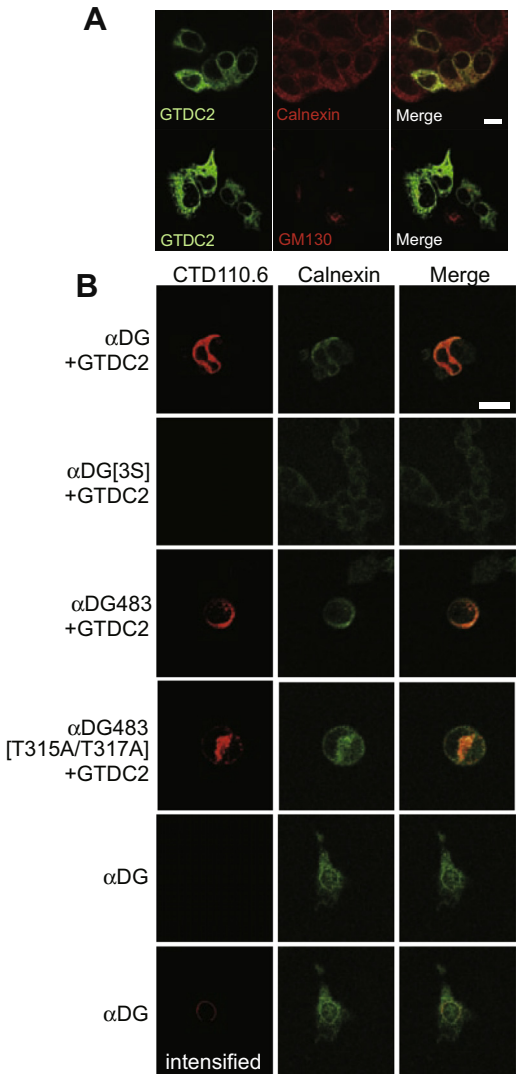

Fig. 4. GTDC2-dependent modification occurs in the ER. (A) Subcellular localization of GTDC2 was analyzed in HEK293T cells using an ER marker (calnexin; red) and Golgi marker (GM130; red). GTDC2 is mainly localized in the ER. Bar, 20 μm. (B) Subcellular localization of GTDC2-modified *O*-mannosyl glycans was analyzed in HEK293T cells transfected with indicated αDG-Fc constructs (α-DG, α-DG[3S], α-DG483, and α-DG[T315A/T317A]) with or without GTDC2. Note that endogenous CTD110.6 staining was observed in the perinucleus (bottom) but did not overlap with the ER marker calnexin (green). Bar, 20 μm. (For interpretation of color in Figure legent, the reader is referred to the web version of this article.)

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.022.

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