HHS Public Access

Author manuscript

J Med Genet. Author manuscript; available in PMC 2024 January 01.

Published in final edited form as:

J Med Genet. 2023 July ; 60(7): 627–635. doi:10.1136/jmg-2022-108821.

Homozygous truncating variant in *MAN2A2* causes a novel congenital disorder of glycosylation with neurological involvement

Sonal Mahajan[1], **Bobby G. Ng**[1], **Lama AlAbdi**[2,3], **Earnest James Paul Daniel**[4], **Paulina Sosicka**[1], **Nisha Patel**[2], **Rana Helaby**[2], **Firdous Abdulwahab**[2], **Miao He**[4], **Fowzan S Alkuraya**[2], **Hudson H. Freeze**[1,*]

[1]Human Genetics Program, Sanford Burnham Prebys Medical Discovery Institute, 10901 North Torrey Pines Road, La Jolla, CA, 92037, United States

[2]Department of Translational Genomics, Center for Genomics Medicine, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia

[3]Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia

[4]Palmieri Metabolic Disease Laboratory, Children's Hospital of Philadelphia, Philadelphia, PA, United States

Abstract

Background—Enzymes of the Golgi implicated in N-glycan processing are critical for brain development, and defects in many are defined as congenital disorders of glycosylation (CDG). Involvement of the Golgi mannosidase, MAN2A2 has not been identified previously as causing glycosylation defects.

Methods—Exome sequencing of affected individuals was performed with Sanger sequencing of the *MAN2A2* transcript to confirm the variant.g N-glycans were analyzed in patient-derived lymphoblasts to determine the functional effects of the variant. A cell-based complementation assay was designed to assess the pathogenicity of identified variants using MAN2A1/MAN2A2 double knock out HEK293 cell lines.

*Correspondence: Hudson H. Freeze, Professor of Glycobiology, Director, Human Genetics Program, Sanford Children's Health Research Center, Sanford-Burnham-Prebys Medical Discovery Institute, 10901 N. Torrey Pines Rd. La Jolla, CA 92037, hudson@sbpdiscovery.org, Phone: 858-646-3142.

Contributors Conceptualization: SM, and HHF; Clinical data collection and analysis: BGN, LA, NP, RH, FA and FSA; Methodology: SM, PS and EJPD; Formal analysis and investigation: SM; Writing, original draft preparation: SM; Writing, review and editing: SM, BGN and HHF; Resources: HHF, FSA and MH. All authors reviewed the final manuscript.

Competing interests

The authors have declared that no competing interests exist.

Patient consent for publication

Informed consent was obtained from all patients themselves or their parents under an approved Sanford Burnham Prebys Medical Discovery Institute IRB protocol or an approved IRB through each family's primary medical physician.

Ethics approval

Informed consent was obtained from the participating family as part of an IRB-approved research protocol (KFSHRC REC# 2080 006).

Supplemental material

Three Data supplement files (1,2 and 3) provided.

Results—We identified a multiplex consanguineous family with a homozygous truncating variant p.Val1101Ter in MAN2A2. Lymphoblasts from two affected brothers carrying the same truncating variant showed decreases in complex N-glycans and accumulation of hybrid N-glycans. Upon testing of this variant in the developed complementation assay, we see the complete lack of complex N-glycans.

Conclusion—Our findings show that pathogenic variants in MAN2A2 cause a novel autosomal recessive CDG with neurological involvement and facial dysmorphism. Here, we also present the development of a cell-based complementation assay to assess the pathogenicity of MAN2A2 variants, which can also be extended to MAN2A1 variants for future diagnosis.

Keywords

MAN2A2-CDG; MAN2A1; complex-type N-glycans; Golgi membrane protein; complementation assay

INTRODUCTION

Congenital disorders of glycosylation (CDG) are a group of rare inherited metabolic disorders caused by defects in the various components critical for glycosylation.[1] The first CDG was identified in 1980, and this group rapidly expanded, particularly after the arrival of whole exome sequencing. The current number of CDG is over 160.[2-4] CDG caused by defects in the assembly of lipid-linked oligosaccharides or their attachment to the nascent proteins in the ER are grouped as type I CDG, whereas type II CDG comprises disorders due to defects in the trimming and remodeling of N-linked oligosaccharides (figure 1A).[5] The first type II CDG to be identified was caused by the deficiency in the Golgi-localized GlcNAc transferase II (MGAT2) which catalyzes the transfer of a GlcNAc residue onto an antennae leading to the formation of complex N-glycans.[6] The presentation of severe multisystemic developmental abnormalities in MGAT2 patients, also known as Alkuraya syndrome, including severe psychomotor retardation, facial dysmorphism and gastrointestinal issues is suggestive of a critical role which complex N-glycans play in human development, particularly in the nervous system.[6-10]

Alpha-mannosidase II (MAN2A1) [OMIM# 154582] and Alpha-mannosidase IIx (MAN2A2) [OMIM# 600988] localized to the medial-Golgi, are two alternative enzymes catalyzing the removal of two, α3 and α6 linked mannosyl residues from hybrid-type N-glycan, GlcNAc1Man5GlcNAc2 to form GlcNAc1Man3GlcNAc2, which is the precursor of complex N-glycans (figure 1A).[11,12] Deficiency of MAN2A1 was reported in a few cases of congenital dyserythropoietic anemia (CDA) type II, also called as HEMPAS (hereditary erythroblastic multinuclearity with a positive acidified serum-lysis test) disease.[13] Later, the molecular basis of HEMPAS was shown to be mutations in the *SEC23B*, encoding the COPII coat protein.[14] However, studies in Man2a1 null mice revealed the similar phenotype as human CDA type II featured by dyserythropoietic anemia and production of abnormal erythrocytes lacking complex N-glycans.[15] The normal production of complex N-glycans in non-erythroid cells in Man2a1 null mice suggested an alternate α-mannosidase enzyme catalyzing this key step of the N-glycan oligosaccharide biosynthesis.[15] Another study in mice identified Man2a2 as an alternative enzyme in the N-glycan processing.[16] Akama et

al. also generated Man2a2 null mice, and studied the involvement of α -mannosidases in spermatogenesis.[17] The Man2a1 null mouse does not show any defect in male reproductive activity, whereas male Man2a2 null mice were infertile with smaller testis and reduced number of spermatogenic cells.[17] N-glycan analysis from Man2a1 / Man2a2 double null mice revealed a complete lack of complex N-glycans and a more severe phenotype than that of single null mice for either of these genes.[16] These studies in mice suggested cell-type-specific roles of Man2a1 and Man2a2 and demonstrated critical roles for at least one of these enzymes for effective N-glycan processing.[15,16]

Here we present a novel type II CDG caused by a defect in MAN2A2. *MAN2A2* encodes a 1150 amino acid type II membrane protein localized in the Golgi membrane (figure 1A, 1B). Both siblings were identified with the same homozygous mutation, c.3301del (p.Val1101Ter) within MAN2A2 and lymphoblasts from both individuals demonstrated an overall decrease in complex N-glycans. Considering the limited availability of patient samples and the possible cell-type specific roles of MAN2A1 vs MAN2A2, we developed an independent complementation assay using MAN2A1/A2 double knockout HEK293 cells which can be used in the future to test the MAN2A1 or MAN2A2 variants of uncertain significance. Our assay demonstrated no rescue of complex N-glycans by the p.Val1101Ter MAN2A2 variant.

MATERIAL AND METHODS

Human subjects

Informed consent was obtained from the participating family as part of an IRB-approved research protocol (KFSHRC REC# 2080 006). Clinical data were collected and analyzed.

Sequencing and variant identification

The details of exome sequencing and analysis pipeline of human disease variants were described elsewhere.[18] Sanger sequencing was used to investigate the segregation of the variant within the family. Lymphoblastoid cell lines (LCL) cell lines were established for two affected siblings and RT-PCR was performed to examine the effect of the variant at the transcript level.

Plasmids

Human *MAN2A2* (NM_006122.4) plasmid used in the complementation assay was purchased from Origene and contained a C-terminus Myc-DDK-tag in a pCMV6-Entry backbone (OriGene Technologies, Inc. CAT#: PS100001). MAN2A2 variant constructs were generated using Q5 Site-Directed Mutagenesis Kit (NEB Biolabs Inc.). For the generation of construct carrying truncating mutation (p.Val1101Ter), we added a linker after Lys1100 and then the Myc-DDK-tag. Plasmid moxBFP used as an internal transfection control was a gift from Erik Snapp (Addgene plasmid # 68064 ; http://n2t.net/addgene:68064 ; RRID:Addgene_68064).[19]

Cell lines

Genetically and biochemically verified MAN2A1/A2 double knockout (DKO) and parental HEK293 cell lines were obtained from Dr. Henrik Clausen.[17] Fibroblasts and HEK293 cells (wild-type and MAN2A1/A2 double knockout) were cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 1 g/L glucose supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Life Technologies, Carlsbad, CA, USA). LCL cell lines were established using standard EBV transformation and were subsequently cultured in RPMI 1640 Medium with 15% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Life Technologies, Carlsbad, CA, USA).

Western Blotting

Lymphoblasts were thoroughly washed with PBS and resuspended in lysis buffer (2% SDS, 62.5mM Tris-HCL pH 6.8, 10% glycerol) supplemented with Protease Inhibitor Tablets (Thermo-Fisher). Cell suspensions were sonicated and then heated at 100°C for 10 minutes to completely solubilize proteins. Protein concentrations were determined using Micro BCA Protein Assay Kit (Thermo-Fisher). Primary antibodies used were MAN2A2 Rabbit pAb (ABclonal, A19323) at 1:1000 dilution and β-Actin (13E5) Rabbit mAb (CST, 4970) at 1:1000 dilution. Secondary antibody used was Anti-Rabbit IgG (H+L) Antibody, Peroxidase-Labeled (sera care, USA) at 1:2000 dilution. Blots were developed using a Bio-Rad Gel Doc system.

Glycan Analysis

N-glycans were extracted from LCL following the method described before.[20] Briefly, the cells were lysed in water by being heated at 95°C for 15 minutes and then isotope labeled internal standard [13C6]-Sialylyl-glycopeptide was added with a final concentration of 10 μg/ml. The glycans from total cellular glycoproteins and the internal standard were released by PNGaseF digestion and then derivatized with modified quinolone attached to the transient amide at the reducing end. The labeled glycans were isolated by a hydrophilic interaction chromatography plate before they were semi-quantified using a flow injection-electrospray ionization-quadrupole time-of-flight mass spectrometry method.[21] Experiment was performed with two biological replicates for each sample.

Complementation Assay

Cells were seeded in 6-well plates (300,000 cells per well) in 2 ml of medium. 48 hours after seeding, cells were transfected with the pCMV6-Entry MAN2A2 Myc-DDK wild-type or variants. Cells were transfected with 500 μg of plasmid DNA using FuGene HD reagent (Promega Corporation, Rockville, MD) according to manufacturer's protocol. After 16h of transfection, cells were split into 60 mm dishes for flow cytometry and into 8-well chambered slides for immunofluorescence.

Immunofluorescence

Poly-Lysine coated plates were seeded with ~25000 cells per well. Next day, cells were fixed with 4% PFA for 15 minutes and washed three times with PBS. Blocking buffer (1% BSA

with 0.1% saponin in PBS) was added and incubated for 1h, followed by lectins/antibodies in blocking buffer for 1h, and three washes with blocking buffer. Cells were incubated with DAPI in blocking buffer for 10 minutes, followed by thorough washes with PBS and a final wash with water. Slides were mounted with coverslips and fluorescence images were acquired using an LSM 710 confocal microscope (Zeiss, Germany) with a 20x objective.

Flow Cytometry

Cells were detached from the dishes using PBS-10 mM EDTA and washed three times with PBS. Cells were fixed with 4% paraformaldehyde-PBS for 15 minutes at room temperature. After three washes with PBS, cells were blocked with 1X Carbo-Free Blocking Solution (Vector laboratories) for 30 minutes at RT. Surface staining with E-PHA was performed by incubating the cells with 0.2 μg/mL of FITC labelled E-PHA (Vector Laboratories Inc., USA) in blocking buffer for 30 minutes at RT followed by three washes with PBS.

After E-PHA staining, intracellular staining was performed using the IntraStain kit (Agilent Technologies Inc.) according to manufacturer's protocol. Briefly, cells were incubated with 100 μl of IntraStain Reagent A (Fixative) for 15 minutes at RT, followed by one 2 ml wash with PBS-1%BSA. Cells were resuspended in 100 μl of IntraStain Reagent B (Permeabilizing solution) containing DYKDDDDK Tag Antibody (Alexa Fluor® 647 Conjugate) for 15 minutes at RT, again followed by one 2 ml wash with PBS-1%BSA. Cells were finally resuspended in 250 μl of PBS and samples were acquired on LSR Fortessa 14-color instrument (BD Biosciences). Experiment was performed three times in duplicate with similar results.

RESULTS

Subjects and clinical report

This study identified two affected brothers (CDG0098 and CDG0097) with a homozygous candidate variant in *MAN2A2* by exome sequencing (figure 2A). Sanger sequencing of the *MAN2A2* transcript (upper band, figure 2F) obtained from RT-PCR, shows a deletion of one nucleotide at the beginning of exon 23 (c.3301del, p.Val1101Ter) in CDG0098 and CDG0097 but not in control (figure 2G).

CDG0098 is an affected male in his mid 20s with intellectual disability, obesity and a dysmorphic face (figure 2B, 2C). He was born to a healthy G1P0AB0 mother in her early 20s following a pregnancy complicated by polyhydramnios. Fetal movements and antenatal scans were otherwise normal. Delivery was via cesarean section at term due to failure to progress and face presentation. Neonatal history is notable for poor sucking and floppiness. He struggled to regain birth weight (3.3kg) until the end of neonatal period and then his feeding and weight gain improved gradually. However, floppiness persisted and manifested as delayed motor milestones. Speech delay was also apparent with only mama and dad at early childhood. He was evaluated for global developmental delay at early childhood with CT brain, *FMR1* repeat analysis and karyotype, all of which were normal. He joined school at middle childhood but was immediately noted to have major challenges. IQ testing revealed intellectual disability. He struggled between regular and special ed classes and

finally left school after 9th grade. He also struggled socially with frequent aggression and violent episodes. Evaluation by psychiatry revealed severe bipolar disorder but his response to Risperdal has been satisfactory. Review of systems revealed normal vision and hearing. Although he achieved toilet training by his middle childhood, he continues to have enuresis. His medical history is notable for an episode of lower urinary tract infection at late adolescence and obesity. Obesity was fully investigated as possibly related to Prader-Willi syndrome, but testing was negative. As part of his endocrinological investigation, he had a normal growth hormone and thyroid hormone level. He achieved puberty at early adolescent age.

Physical examination (PE) revealed weight 103kg (98th percentile (+2.1 SD)), height 172cm (26th percentile), BMI 34.8 (obese), OFC 56cm (73rd percentile), thick eyebrows, synophrys, epicanthus, broad nose with thick overlying skin, brachycephaly, full lips, severe dental caries, tapering fingers, marked truncal obesity, normal adult axillary and pubic hair but small genitalia (short phallus and small testicles), and active acne in the back.

CDG0097 is the younger brother of CDG0098, and he is in his early 20s, presented with intellectual disability and facial dysmorphism (figure 2D, 2E). He was born to a healthy G3P2AB0 mother in her late 20s following an uneventful pregnancy. Delivery was normal spontaneous vaginal delivery at term with birth weight of 3kg. Neonatal history is unremarkable. There is no history of floppiness. However, he did not crawl and even though he achieved walking when he was 10-15 months old, his gait was noted to be clumsy, and this was thought to be due to mild inversion abnormality of the feet. Speech was delayed and when he was ready to join school in his middle childhood, his teachers noted learning difficulties. An IQ test revealed a score of 78. Although he finished K-12 (high school), it was with extensive tutoring. He achieved vocational training and is now employed as a manual worker at a hotel. Unlike his brother, he never had psychiatric issues and is described as quiet and a bit withdrawn. His limited social engagement is usually with younger children. His medical history is notable for an episode of Kawasaki disease around 9-13 years of age with resulting coronary artery complications despite adequate treatment with immunoglobulins. There is no history of obesity. PE revealed weight 74kg (62nd percentile), height 170cm (18th percentile), BMI 25.6 (mildly overweight), OFC 57cm (91st percentile), thick eyebrows, synophrys, epicanthus, broad nose with thick overlying skin, full lips, tapering fingers, normal adult axillary and pubic hair, and normal genitalia.

Decreased levels of alpha-mannosidase IIx in patient-derived lymphoblasts

The identified homozygous variant in *MAN2A2* (p.Val1101Ter) is expected to result in a premature stop codon, and a polypeptide of 125 kDa instead of wild-type 130 kDa polypeptide (figure 1B). To determine whether the p.Val1101Ter variant encodes a stable polypeptide, we used western blot analysis of control and patient's lymphoblast protein extracts using an antibody against human MAN2A2. Both CDG0097 and CDG0098 showed decreased levels of MAN2A2 protein, as compared to control lymphoblasts (figure 2H). For the quantitative analysis, signal intensities of MAN2A2 protein bands were normalized to loading control and MAN2A2 protein levels were represented as relative to control 1 (figure 2I). CDG0097 and CDG0098 showed around 40% and 25% of MAN2A2 protein levels as

compared to controls (figure 2I). The MAN2A2 bands in patient samples were also running slightly faster compared in control samples, consistent with the expected 5 kDa truncation and loss of an N-glycosylation site caused by the variants (figure 2H).

Patient-derived lymphoblasts show decrease in complex-type N-glycans

N-glycan analysis of patient lymphoblasts demonstrated an overall decrease in complex-type N-glycans, compared to control lymphoblasts (figure 3). This observation suggested an effect of defective MAN2A2 on N-glycan processing. Multiple complex N-glycans were significantly reduced in CDG0097 ($p<0.05$, two-tailed t-test) (figure 3). CDG0097 demonstrated a significant increase in pentamannosyl hybrid-type N-glycans with the α1,3 antenna of the core structure variably processed following GlcNAc addition to initiate the formation of hybrid glycans (figure 3). In addition, the hybrid structures with the processed μ1,6 antenna did not accumulate in CDG0097. However, CDG0098 did not show the expected accumulation of the hybrid-type N-glycans but did show a significant decrease over different classes of complex-type N-glycans ($p<0.05$, two-tailed t-test) (figure 3). There was no change in high-mannose type N-glycans in lymphoblasts of both CDG0097 and CDG0098 (figure 3), which further suggests no abnormality in N-glycan processing pathway prior to MAN2A1/A2.

Validation of Complementation Assay

Akama et al. showed that the complementation of mouse embryo fibroblasts from *Man2a1/a2* double null mice either by Man2a1 or Man2a2 could restore the synthesis of complex-type N-glycans, assessed by staining with E-PHA lectin which binds specifically to complex-type N-glycans.[16] We also developed a similar complementation assay using HEK293 MAN2A1/A2 DKO cells. These cells were first tested for the synthesis of complex-type N-glycans. As expected, DKO cells completely lack complex-type N-glycans as demonstrated by lack of E-PHA fluorescence (figure 4A) as compared to wild-type HEK293 cells (figure 4A). The transfection of either MAN2A1 or MAN2A2 plasmid DNA into DKO cells resulted in rescue of E-PHA fluorescence (figure 4A), validating the use of complementation assay to screen the MAN2A1/A2 variants in rescuing the lack of complex-type N-glycans. The assay is further validated using flow cytometry which quantitatively measured the rescue of E-PHA fluorescence in 40 to 50% of the total DKO cells by MAN2A2 and MAN2A1 respectively (figure 4B).

Complementation with MAN2A2 p.Val1101Ter variant does not rescue complex N-glycans in DKO cells

Unlike wild-type MAN2A2, complementation with equal amount of MAN2A2 (p.Val1101Ter) variant plasmid DNA does not rescue E-PHA fluorescence of DKO cells (figure 5A). A catalytically dead mutant of MAN2A2 (p.D289A) also did not restore E-PHA binding (figure 5A). To confirm MAN2A2 expression in wild-type versus p.D289A and p.Val1101Ter complemented cells, Flag tag antibody fluorescence was measured along with E-PHA fluorescence. To rule out the differences in transfection, we used plasmid expressing Blue Fluorescent Protein (BFP) as an internal transfection control. All MAN2A2 transfected samples (wild-type, p.D289A and p.Val1101Ter) demonstrated similar transfection levels with 55-60% of cells expressing BFP and also similar MAN2A2 expression, determined as

around 45% of Flag tag Ab fluorescence for all three conditions (figure 5B). Despite similar MAN2A2 expression levels in p.D289A and p.Val1101Ter as wild-type, both variants showed negligible signal for E-PHA fluorescence (quadrant 4) (figure 5C). However, in case of wild-type MAN2A2, about 31% of cells showed double staining for E-PHA and Flag tag antibody (quadrant 4), confirming the rescue of E-PHA fluorescence by complementation of wild-type MAN2A2 (figure 5C).

To further quantify, DKO cells complemented with wild-type MAN2A2 demonstrated 55 % of E-PHA fluorescence relative to wild-type HEK293 cells (figure 5D) consistent with the 45-50 % transfection efficiency of the MAN2A2 plasmid.

We also screened additional variants of uncertain significance (VUS) in potential MAN2A2-CDG cases, using complementation assay (Supplemental Table 1). Some variants (p.R728C, p.R408Q and p.Q412W) showed less than 40% rescue in E-PHA binding and are likely pathogenic. However, these variants were identified to be compound heterozygous with other benign or likely benign variants in the screened cases, except p.Q412X which was homozygous mutation in one possible case and showed no rescue in complex N-glycan synthesis (Supplemental Table 1).

DISCUSSION

The present study demonstrates that a defect in MAN2A2 is responsible for a new type of CDG characterized by intellectual disability and dysmorphic facial features. The homozygous variant, p.Val1101Ter reported in two MAN2A2-CDG patients results in loss of 50 C-terminal amino acids which also carries a N-glycosylation site leading to an approximately 5 kDa truncation (figure 2B). Patient lymphoblasts verified the truncation and showed the intensity of MAN2A2 protein decreased by 25-40 % compared to control lymphoblasts (figure 2C). This decreased expression could be due to instability of the polypeptide. Analysis of N-glycans in CDG0097 lymphoblasts demonstrated an overall 50% decrease in most of the complex-type N-glycan categories and about 2-fold accumulation of some categories of hybrid-type N-glycans. We assume this residual α–mannosidase activity is due to the compensation by MAN2A1, rather than the truncated protein. This truncated MAN2A2 polypeptide is inactive as overexpression in DKO HEK293 cells to the same levels as wild-type MAN2A2 polypeptide did not restore complex-type N-glycans. Their reduction and accumulation of pentamannosyl hybrid-type structures in MAN2A2 CDG patients is consistent with results seen in ricin-resistant baby hamster kidney cells in culture and HEK293T cell lines mutated at the MAN2A1 locus or in HEK293T cells in the presence of swainsonine.[21,22] Though, CDG0098 lymphoblasts did not show the accumulation of hybrid structures, they do show similar decrease in most of the complex-type N-glycan categories, and western blots also verify the truncation and reduced expression of proteins. We consider the differences in clinical presentation and N-glycan distribution in both patients may be due to variable MAN2A1 content or other factors rather than differential expression of MAN2A2 protein as it is truncated and probably inactive.

The gene encoding MAN2A2 was identified in the human genome by Misago et al. in 1995 by cross-hybridization with a cDNA encoding human MAN2A1, and it was considered

to be an isozyme of MAN2A1.[24] Northern analysis demonstrated the expression of both *MAN2A1* and *MAN2A2* in a wide range of tissues and cell-types.[24] Man2a1 deficiency was studied in mice, which showed the phenotype of dyserythropoietic anemia in these mice with no effects on N-glycosylation in non-erythroid cell types.[15] This mild phenotype of Man2a1 is likely due to partial compensation by Man2a2. *In vitro* assays show both MAN2A1 and MAN2A2 enzymes have the same specificity and double knockout mice show a complete lack of complex-type N-glycans.[16] This study reports, for the first time, MAN2A2 deficiency in two patients and the resulting glycosylation defects. This study further suggested the assumed cell-type specific roles of MAN2A2 and MAN2A1, and the deficiency of one is not compensated by the other in all cell types. The neurological issues in MAN2A2 deficient patients is supported by the fact that MAN2A2 is primarily expressed in brain, with ~4 fold higher expression than MAN2A1 in humans.[25] Neurological involvement in *Man2a2* null mice was not reported. Infertility in male mice may be due to reduced GlcNAc-terminated complex-type N-glycans in testis and the resulting failure of germ cell adhesion to Sertoli cells.[17,26] Both male and female *Man2a2* null mice showed altered growth, but other organs in these mice do not show any obvious abnormalities and were not studied in detail.[17] The lack of phenotypic alignment in MAN2A2 deficiency in mice and humans could be due to limited parameters studied in mice, but nonetheless MAN2A2 deficiency in both species suggested the cell-type specific glycosylation defect.

To overcome the unavailability of patient cells, and eliminate any compensation effects by MAN2A1, we developed an unbiased model system using a double knockout cell line to test future MAN2A1/A2 variants. We validated the complementation assay using wild-type MAN2A1 and A2, and thus confirmed that either of them is sufficient to rescue the lack of complex-type N-glycans tested by E-PHA staining. We also tested a catalytically inactive mutant of MAN2A2 (p.D289A), which like the patient specific p.Val1101Ter variant, was unable to rescue the E-PHA staining in double knockout cells. To quantify the different levels of E-PHA staining in wild-type versus partial or fully pathogenic variants, we used flow cytometric approach and did the double staining to simultaneously check the MAN2A2 expression and E-PHA staining in the cells positive for MAN2A2 expression.

Using our complementation assay, we screened additional variants of uncertain significance (VUS) in potential MAN2A2-CDG cases. In all individuals, either one or both variants identified could significantly rescue the phenotype of DKO cells comparable to wild-type MAN2A2. The lone exception was p.Gln412Ter which showed no rescue in complex N-glycan synthesis (Supplemental Table 1). We identified a single p.Gln412Ter homozygous female with a neurodevelopmental disorder, however the p.Gln412Ter variant was very frequent (allele frequency-1.19e-3) in the gnomAD database. Additionally, within gnomAD there was one individual who was homozygous for this variant. However, because of the unavailability of patient samples/detailed clinical information, we could not conclude if this variant is responsible for any phenotypic effects.

Our study presents the first report of human MAN2A2 deficiency that results in a glycosylation defect. We also highlight a platform to test MAN2A1/A2 variants of uncertain significance and to screen potential drugs or therapies for MAN2A1/MAN2A2-CDG in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank patients, their families, doctors and clinicians for their continued support and for providing valuable biological specimens. The authors thank Dr. Alice Basinger for providing information about other MAN2A2 variants of uncertain significance. The authors thank Dr. Henrik Clausen for providing the MAN2A1/A2 double knockout HEK293 cells and Dr. Kelley Moremen, Complex Carbohydrate Research Center, for many discussions and sage advice.

Funding

This work was supported by The Rocket Fund, R01DK 099551, U54 NS115198 NIH-FCDGC (Frontiers for Congenital Disorders of Glycosylation Consortium, Rare Disease Consortium), King Salman Center for Disability Research through Research Group no RG-2022-011 (FSA), and King Saud University through Researchers Supporting Project number RSP-2021/181 (LA). SM is FCDGC fellow.

Data availability statement

All data relevant to the study are included in the article and can be obtained on request.

REFERENCES

1. Lefeber DJ, Freeze HH, Steet R, Kinoshita T. Congenital Disorders of Glycosylation. Essentials Glycobiol, 2022. 10.1101/GLYCOBIOLOGY.4E.45
2. Ng BG, Freeze HH. Perspectives on Glycosylation and Its Congenital Disorders. Trends Genet, 2018, 34:466–76 [PubMed: 29606283]
3. Ondruskova N, Cechova A, Hansikova H, Honzik T, Jaeken J. Congenital disorders of glycosylation: Still “hot” in 2020. Biochim Biophys Acta - Gen Subj, 2021, 1865:129751 [PubMed: 32991969]
4. About CDG | CDG Hub, n.d. https://www.cdghub.com/about/. (accessed May 11, 2022)
5. Aebi M, Helenius A, Schenk B, Barone R, Fiumara A, Berger EG, Hennet T, Imbach T, Stutz A, Bjursell C, Uller A, Wahlstrom JG, Briones P, Cardo E, Clayton P, Winchester B, Cormier-Dalre V, De Lonlay P, Cuer M, Dupre T, Seta N, De Koning T, Dorland L, De Loos F, Kupers L, Fabritz L, Hasilik M, Marquardt T, Niehues R, Freeze H, Grunewald S, Heykants L, Jaeken J, Matthijs G, Schollen E, et al. Carbohydrate-deficient glycoprotein syndromes become congenital disorders of glycosylation: an updated nomenclature for CDG. First International Workshop on CDGS. Glycoconj J, 1999, 16:669–71 [PubMed: 11003549]
6. Ramaekers VT, Stibler H, Kint J, Jaeken J. A new variant of the carbohydrate deficient glycoproteins syndrome. J Inherit Metab Dis, 1991, 14:385–8 [PubMed: 1770799]
7. Jaeken J, Schachter H, Carchon H, De Cock P, Coddeville B, Spik G. Carbohydrate deficient glycoprotein syndrome type II: a deficiency in Golgi localised N-acetylglucosaminyltransferase II. Arch Dis Child, 1994, 71:123–7 [PubMed: 7944531]
8. Charuk JHM, Tan J, Bernardini M, Haddad S, Reithmeier RAF, Jaeken J, Schachter H. Carbohydrate-deficient Glycoprotein Syndrome Type II: An Autosomal Recessive N-acetylglucosaminyltransferase II Deficiency Different from Typical Hereditary Erythroblastic Multinuclearity, with a Positive Acidified-serum Lysis Test (HEMPAS). Eur J Biochem, 1995, 230:797–805 [PubMed: 7607254]
9. Alkuraya FS. Mental retardation, growth retardation, unusual nose, and open mouth: an autosomal recessive entity. Am J Med Genet A, 2010, 152A:2160–3 [PubMed: 20684000]
10. Alazami AM, Monies D, Meyer BF, Alzahrani F, Hashem M, Salih MA, Alkuraya FS. Congenital disorder of glycosylation IIa: The trouble with diagnosing a dysmorphic inborn error of metabolism. Am J Med Genet Part A, 2012, 158A:245–6 [PubMed: 22105986]

11. Kornfeld R, Kornfeld S. ASSEMBLY OF ASPARAGINE-LINKED OLIGOSACCHARIDES. Https://DoiOrg/101146/AnnurevBi54070185003215, 2003, VOL. 54:631–64
12. Schachter H. The 'yellow brick road' to branched complex N-glycans. Glycobiology, 1991, 1:453–61 [PubMed: 1840403]
13. Fukuda MN, Masri KA, Dell A, Luzzatto L, Moremen KW. Incomplete synthesis of N-glycans in congenital dyserythropoietic anemia type II caused by a defect in the gene encoding α-mannosidase II. Proc Natl Acad Sci U S A, 1990, 87:7443–7 [PubMed: 2217175]
14. Bianchi P, Fermo E, Vercellati C, Boschetti C, Barcellini W, Iurlo A, Marcello AP, Righetti PG, Zanella A. Congenital Dyserythropoietic Anemia type II (CDAII) is caused by mutations in the SEC23B gene. Hum Mutat, 2009, 30:1292–8 [PubMed: 19621418]
15. Chui D, Oh-Eda M, Liao YF, Panneerselvam K, Lal A, Marek KW, Freeze HH, Moremen KW, Fukuda MN, Marth JD. Alpha-Mannosidase-II Deficiency Results in Dyserythropoiesis and Unveils an Alternate Pathway in Oligosaccharide Biosynthesis. Cell, 1997, 90:157–67 [PubMed: 9230311]
16. Akama TO, Nakagawa H, Wong NK, Sutton-Smith M, Dell A, Morris HR, Nakayama J, Nishimura SI, Pai A, Moremen KW, Marth JD, Fukuda MN. Essential and mutually compensatory roles of α-mannosidase II and α-mannosidase IIx in N-glycan processing in vivo in mice. Proc Natl Acad Sci U S A, 2006, 103:8983–8 [PubMed: 16754854]
17. Akama TO, Nakagawa H, Sugihara K, Narisawa S, Ohyama C, Nishimura SI, O'Brien DA, Moremen KW, Millan JL, Fukuda MN. Germ cell survival through carbohydrate-mediated interaction with Sertoli cells. Science (80-), 2002, 295:124–7
18. Monies D, Abouelhoda M, AlSayed M, Alhassnan Z, Alotaibi M, Kayyali H, Al-Owain M, Shah A, Rahbeeni Z, Al-Muhaizea MA, Alzaidan HI, Cupler E, Bohlega S, Faqeih E, Faden M, Alyounes B, Jaroudi D, Goljan E, Elbardisy H, Akilan A, Albar R, Aldhalaan H, Gulab S, Chedrawi A, Al Saud BK, Kurdi W, Makhseed N, Alqasim T, El Khashab HY, Al-Mousa H, Alhashem A, Kanaan I, Algoufi T, Alsaleem K, Basha TA, et al. The landscape of genetic diseases in Saudi Arabia based on the first 1000 diagnostic panels and exomes. Hum Genet, 2017, 136:921–39 [PubMed: 28600779]
19. Costantini LM, Baloban M, Markwardt ML, Rizzo M, Guo F, Verkhusha VV, Snapp EL. A palette of fluorescent proteins optimized for diverse cellular environments. Nat Commun, 2015, 6
20. Polla DL, Edmondson AC, Duvet S, March ME, Sousa AB, Lehman A, Niyazov D, van Dijk F, Demirdas S, van Slegtenhorst MA, Kievit AJA, Schulz C, Armstrong L, Bi X, Rader DJ, Izumi K, Zackai EH, de Franco E, Jorge P, Huffels SC, Hommersom M, Ellard S, Lefeber DJ, Santani A, Hand NJ, van Bokhoven H, He M, de Brouwer APM. Bi-allelic variants in the ER quality-control mannosidase gene EDEM3 cause a congenital disorder of glycosylation. Am J Hum Genet, 2021, 108:1342–9 [PubMed: 34143952]
21. Chen J, Li X, Edmondson A, Meyers GD, Izumi K, Ackermann AM, Morava E, Ficicioglu C, Bennett MJ, He M. Increased Clinical Sensitivity and Specificity of Plasma Protein N-Glycan Profiling for Diagnosing Congenital Disorders of Glycosylation by Use of Flow Injection-Electrospray Ionization-Quadrupole Time-of-Flight Mass Spectrometry. Clin Chem, 2019, 65:653–63 [PubMed: 30770376]
22. HUGHES RC, FEENEY J. Ricin-resistant mutants of baby-hamster-kidney cells deficient in α-mannosidase-II-catalyzed processing of asparagine-linked oligosaccharides. Eur J Biochem, 1986, 158:227–37 [PubMed: 3732270]
23. Crispin M, Chang VT, Harvey DJ, Dwek RA, Evans EJ, Stuart DI, Jones EY, Lord JM, Spooner RA, David SJ. A Human Embryonic Kidney 293T Cell Line Mutated at the Golgi α-Mannosidase II Locus. J Biol Chem, 2009, 284: 21684 [PubMed: 19465480]
24. Misago M, Liao YF, Kudo S, Eto S, Mattei MG, Moremen KW, Fukuda MN. Molecular cloning and expression of cDNAs encoding human α-mannosidase II and a previously unrecognized α-mannosidase IIX isozyme. Proc Natl Acad Sci U S A, 1995, 92:11766–70 [PubMed: 8524845]
25. Fagerberg L, Hallstrom BM, Oksvold P, Kampf C, Djureinovic D, Odeberg J, Habuka M, Tahmasebpoor S, Danielsson A, Edlund K, Asplund A, Sjostedt E, Lundberg E, Szigyarto CAK, Skogs M, Ottosson Takanen J, Berling H, Tegel H, Mulder J, Nilsson P, Schwenk JM, Lindskog C, Danielsson F, Mardinoglu A, Sivertsson A, Von Feilitzen K, Forsberg M, Zwahlen M, Olsson I, Navani S, Huss M, Nielsen J, Ponten F, Uhlen M. Analysis of the human tissue-specific

expression by genome-wide integration of transcriptomics and antibody-based proteomics. Mol Cell Proteomics, 2014, 13:397–406 [PubMed: 24309898]

26. Fukuda MN, Akama TO. In vivo role of α-mannosidase IIx: Ineffective spermatogenesis resulting from targeted disruption of the Man2a2 in the mouse. Biochim Biophys Acta - Gen Subj, 2002, 1573:382–7
27. Freeze HH, Eklund EA, Ng BG, Patterson MC. Neurological aspects of human glycosylation disorders. Annu Rev Neurosci, 2015, 38:105–25 [PubMed: 25840006]

Key messages

What is already known on this topic

- Deficiencies in many Golgi enzymes involved in N-glycan processing, lead to congenital disorders of glycosylation with multisystem effects, particularly affecting the brain.
- Golgi enzymes, MAN2A1/A2 are two alternative enzymes catalyzing a key step in the processing of hybrid glycans to precursors for complex-type N-glycans.

What this study adds

- This study reports that MAN2A2 deficiency results in glycosylation defect with neurological involvement.
- This study suggests the cell-type specific roles of MAN2A2 and MAN2A1, and the deficiency of one is not compensated by the other in all cell types.

How this study might affect research, practice or policy

- This study highlights a platform to screen MAN2A1/A2 variants of uncertain significance.

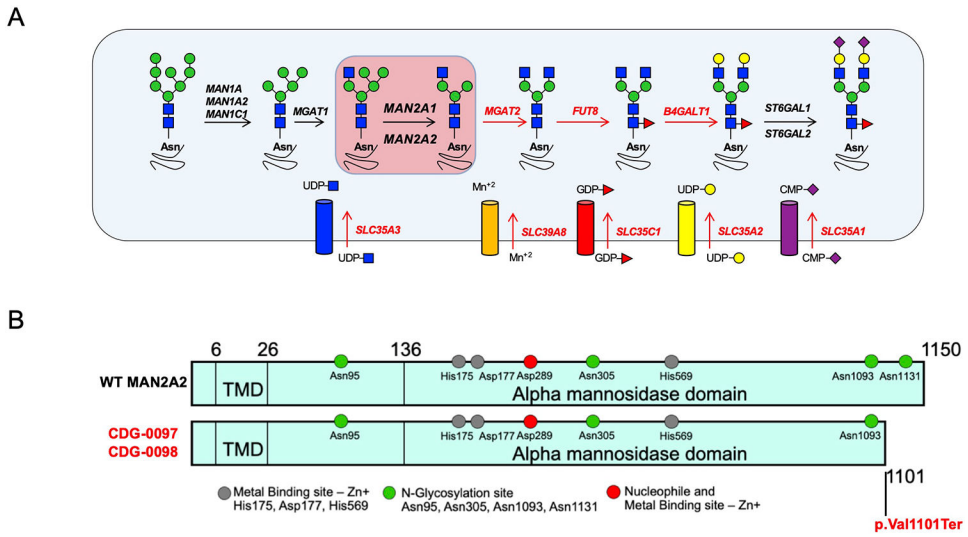

Figure 1.

(A) Schematic showing the N-glycan processing pathway in Golgi, including the step catalyzed by MAN2A1/A2 (highlighted in red box). This figure is adopted and modified version from Freeze, H.H. et al. 2015.[27] The genes highlighted in red are known to cause glycosylation disorders. (B) Domain architecture of the MAN2A2 wild-type protein (Uniprot P49641) and representation of the truncating mutation, p.Val1101Ter.

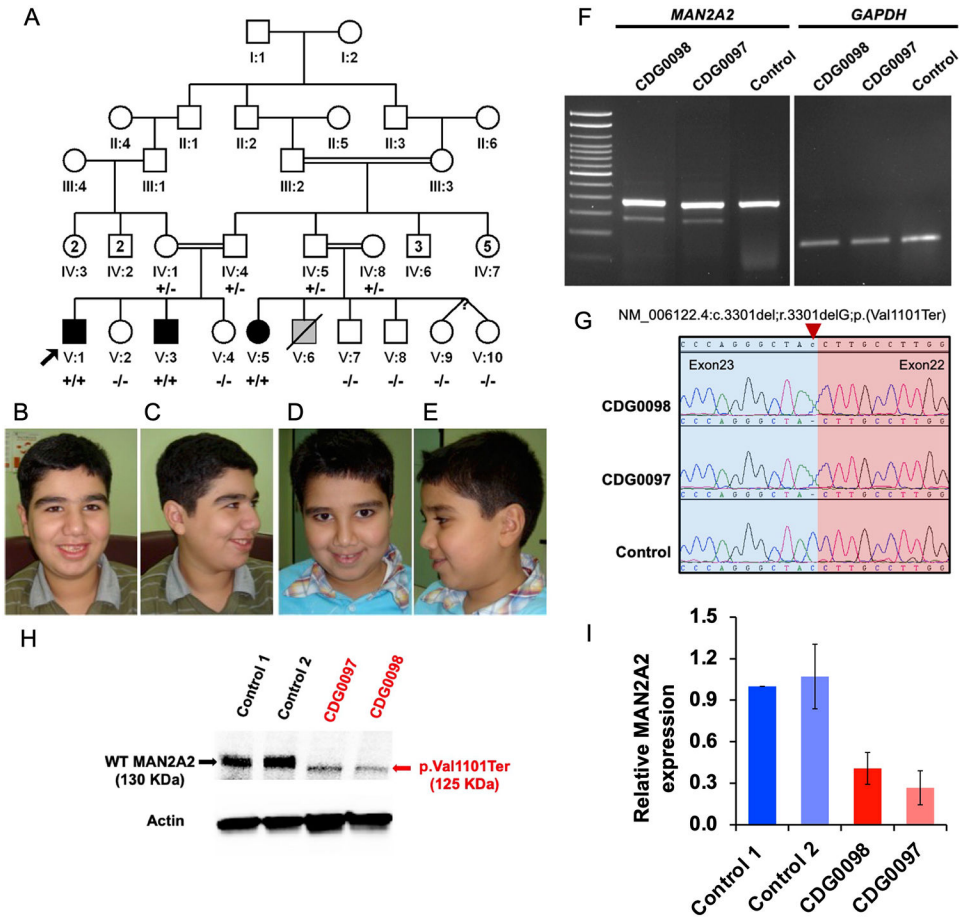

Figure 2.

Pedigree and clinical data for index individuals included in this study.

(A) Pedigree of two branches of the family included in the study. Individual (V:6) was not tested for MAN2A2 variant as he passed away with cerebral palsy and epilepsy due to lack of Oxygen at birth. (B and C) Frontal and lateral photos of the index individual CDG0098, respectively. (D and E) Frontal and lateral photos of affected brother CDG0097, respectively. (B-E) illustrate subtle facial dysmorphism observed in the two affected brothers. (F) Gel electrophoresis image of RT-PCR products generated for *MAN2A2* and *GAPDH* (loading control). (G) Sanger sequencing for the upper band of the RT-PCR product. Sequencing shows a deletion of one nucleotide at the beginning of exon 23 in the two patients and not in control. (H) Western blots showing expression of MAN2A2 in control versus patient lymphoblasts. (I) Quantification of MAN2A2 protein levels (mean ± SD, n = 3). Signal intensities of MAN2A2 bands are first normalized to loading controls and then represented as relative levels to Control 1.

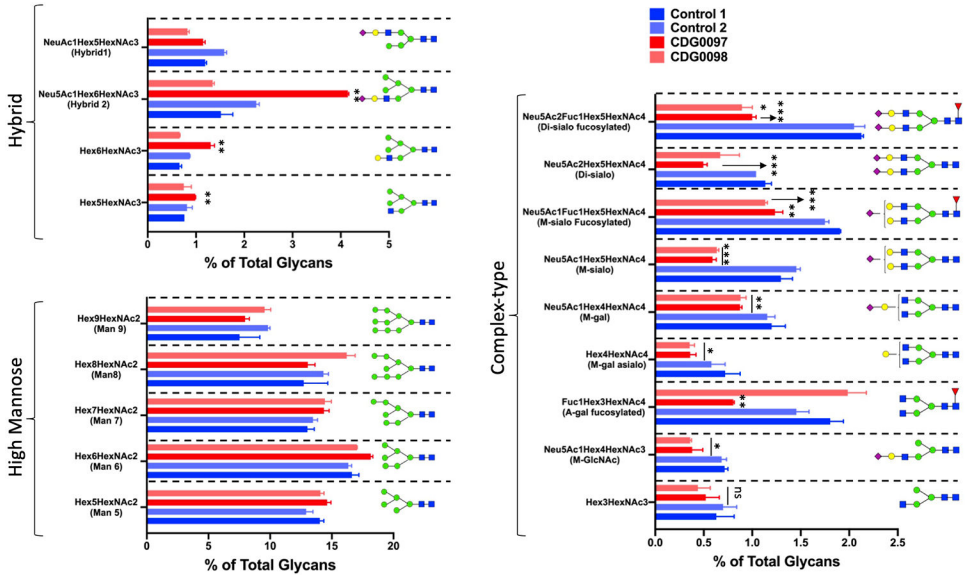

Figure 3.

N-glycan profiles of lymphoblasts from CDG0097 and CDG0098 versus control lymphoblasts. Glycans are represented in three different classes- high-mannose, hybrid-type and complex-type N-glycans. Data were presented as bar graph with mean and SD. Experiments were performed in duplicate with similar results. Statistical significance of the changes (controls versus CDG0097 and controls versus CDG0098) was calculated using unpaired two-tailed *t*-test (±SD, n = 2, *P<0.05, **P<0.01, ***P<0.001).

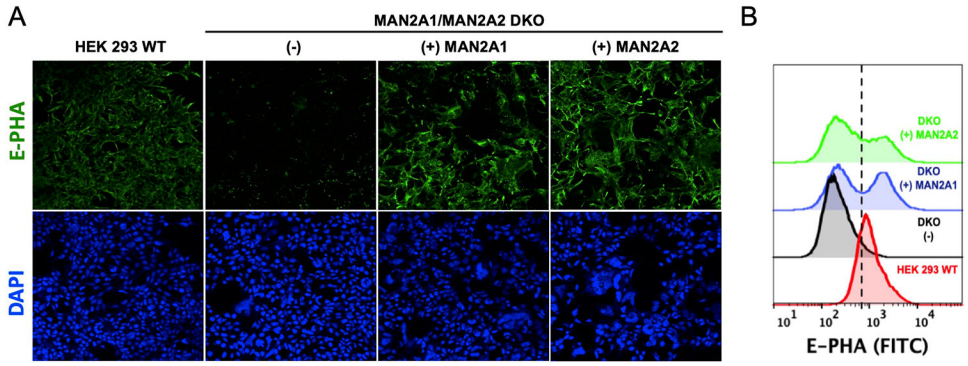

Figure 4.

(A) Representative Immunofluorescence images demonstrating the complementation assay using MAN2A1/MAN2A2 double knock out HEK293 cells. (B) Flow cytometry image showing E-PHA staining in non-transfected DKO cells versus DKO cells complemented with wild-type MAN2A1/MAN2A2. Experiments were performed in triplicate with similar results.

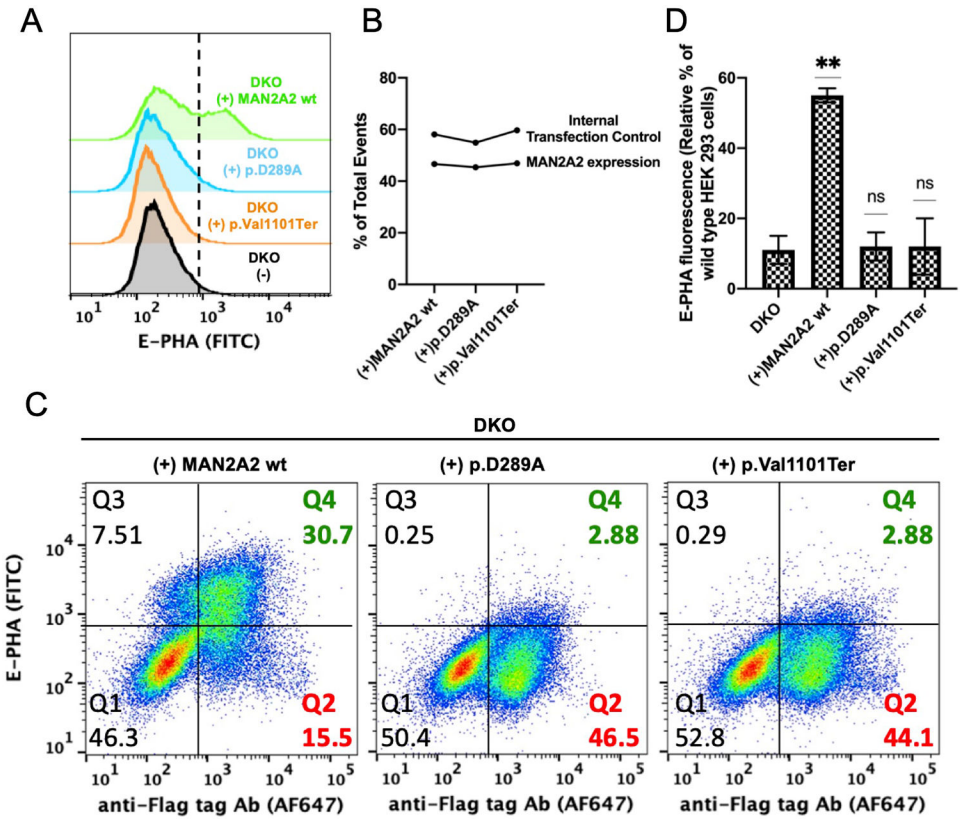

Figure 5.

(A) Flow cytometry image showing E-PHA staining in wild-type MAN2A2 versus MAN2A2 variants. (B) Plot showing levels of MAN2A2 expression and Internal transfection control in different conditions used in complementation assay. (C) Double staining plots of complemented double knock out cells showing Flag-tag positive cells (showing expression of transfected MAN2A2 plasmid) versus E-PHA staining for complex N-glycans. Experiments were performed in triplicate with similar results. (D) Histogram showing quantified E-PHA fluorescence represented as relative percent of wild-type HEK293 cells (mean ± SD, n = 3).