Supporting Online Material

Materials and Methods

cDNAs. All Fc-tagged α-DG recombinants used in this study (Wt construct DGFc5, deletion construct DGFc4 (DGEKFc4), and Fc-Ctrl (IgG1Fc-pcDNA3) have been described previously, in (*S1-3*).

Antibodies. Both the AP83 rabbit polyclonal and IIH6 mouse monoclonal antibodies, which recognize β-DG and the unidentified laminin-binding modification of α-DG, respectively, were described previously (*S4*). CORE is from a sheep polyclonal antiserum raised against the entire dystrophin-glycoprotein complex, and was purified against a hypoglycosylated DGFc5 produced in TSA cells. Biotinylated anti-human IgG was obtained from Vector Laboratories (CA, USA).

Protein enrichment, immunoblots, overlay assays, and solid-phase assays. Frozen skeletal muscle and cultured cells were processed as described (*S4*). Immunoblots, laminin overlay assays, and solid-phase assays were conducted as described (*S4*). Virus overlay assays were conducted as described (*S5*). Protein enrichment with IMAC-beads was carried out using PHOS-beads (Sigma) according to the manufacturer's protocol. Both void and bound samples correspond to 100% of input. Prior to the enrichment, samples were desalted using Amicon-Ultra (Millipore) filters.

Treatment with HFaq. Samples were incubated with ice-cold 48% HFaq (Aldrich) at 0°C for 20 h. The reagent was removed under a stream of nitrogen on ice. Control samples were prepared by the same procedure, except that ice-cold water was used instead of 48% HFaq.

Treatment with enzymes. Alkaline phosphatase from calf intestine (NEB), α(1-2, 3, 6)-mannosidase from jack bean (Glyko), β(1-2, 3, 4, 6)-N-acetylhexosaminidase from jack bean (Glyko), α(1-2, 3, 4, 6)-fucosidase from bovine kidney (Sigma), and the Enzymatic Protein Deglycosylation Kit: including PNGaseF, *O*-glycosidase, α-2(3,6,8,9) neuraminidase, β(1-4)-galactosidase, and β-N-Acetylglucosaminidase (Sigma) were used in this study accoding to the manufacturer's protocol. The samples were desalted using Amicon-Ultra (Millipore) filters prior to treatment.

Quantification of phosphate residues on α-DG. α-DG purified from rabbit skeletal muscle as described elsewhere (*S6*) was treated with Antarctic Phosphatase (NEB) or was hydrolyzed by applying 6N HCl at 110°C for 2 h, conditions under which phosphoamino acids remain intact (*S7*). Released inorganic phosphate was quantified using the Malachite Green Phosphate Assay Kit (BioAssay Systems). For controls, α-DG was incubated under the same conditions, with the heat-inactivated phosphatase or in the absence of HCl. We

subtracted the amount of inorganic phosphate in the control from that in the treated samples.

[^{32}P] orthophosphate labelling of cells. Cells were cultured in DMEM with 0.5% penicillin-streptomycin and 2 mM L-glutamate supplemented with fetal bovine serum (FBS) at 10% (HEK293 cells) or 15% (fibroblasts). Prior to ^{32}P-labeling, HEK293 cells were transfected with DGFc4 or Fc-Ctrl using FuGENE6 (Roche Applied Science). Fibroblasts were infected with the E1-deficient recombinant adenovirus Ad5-CMV-DGFc5-WT, which encodes DGFc5 under the CMV-promoter, at an MOI of 1000. At 24 h post-infection/transfection, the cells were starved in phosphate-free DMEM (Gibco) with 10% dialyzed FBS (Gibco) for 1 h, and then labelled with 0.7 mCi/ml of ^{32}P-orthophosphate (Amersham Code PBS11; 10 mCi/ml) in phosphate-free medium for one hour. Cells were cultured in the usual medium for another 4 days, after which recombinant DG was enriched from the medium using Protein-A agarose (Santa Cruz). The beads were boiled with 5x Laemmli sample buffer and applied to 3–15 or 4-12 % SDS-PAGE. The gels were stained with Coomassie brilliant blue (CBB) and then exposed to phosphoimager plates (Fuji).

Phosphoamino acid analyses. HEK293 cell-expressed [^{32}P]-DGFc4 was purified as described above and hydrolyzed by applying 6N HCl at 110°C for 2 h, conditions under which phosphoamino acids remains intact (*S7*). The hydrolysates were cooled at room temperature and dried in a Speedvac (Thermo Savant). The samples were re-suspended in 50 μl of water and dried in the Speedvac. This step was repeated three times. The hydrolysates were finally re-suspended in 10 μl of water and subjected to TLC analysis. The TLC analysis was conducted as described elsewhere (*S8*).

Preparation of *O*-glycan sample. DGFc4 was produced in HEK293H cells, essentially as described elsewhere (*S2*) with minor modification. The suspension cells were cultivated in a CELLine bioreactor (CL1000, Argos). DGFc4 was purified from the medium using a Hitrap Protein-A HP column (GE Healthcare). *O*-glycans were liberated from purified DGFc4 by reductive β-elimination with 0.1 M NaOH and 1 M $NaBH_4$ for 16 h at 45°C. After passage over a Dowex 50W-X8 (H^+) column, the solution was co-evaporated with methanol several times using a Speedvac. Phosphorylated oligosaccharide-alditol was captured using PHOS-beads (Sigma), eluted in modified PBS in which PO_4^{3-} was at 200 mM, and desalted using a porous graphitic carbon column (GlycoClean H Cartridges, Glyko) according to the manufacturer's protocol.

LTQ XL liner ion trap mass spectrometery and HPAEC-PAD analyses. The glycan samples were dissolved in 50% acetonitrile/water (v/v) and directly infused into an LTQ XL liner ion trap mass spectrometer (Thermo Scientific, Inc.) at 5 μl/min, using a syringe pump. The samples were ionized using negative nanospray ionization. Capillary temperature and needle voltage were 200°C and -4.5-5 kV, respectively. Collisions for MS^n were carried out with an isolation window of 3.0 u and a normalized collision energy of 20-35%. Compositional

sugar analysis using HPAEC-PAD was conducted by the Glycotechnology Core Resource at the University of California, San Diego.

NMR study. After exchanging hydroxyl hydrogens of the *O*-glycan for deuteriums by repeated dissolution in 99.9% D_2O and lyophilisation, the *O*-glycan sample was dissolved in 99.9% D_2O and used directly in the NMR studies. NMR spectra were recorded at 25°C on a Bruker Avance II 800 MHz spectrometer equipped with a cryoprobe or a Bruker Avance II 500 MHz spectrometer. ^{1}H homonuclear two-dimensional DQF-COSY (*S9*), TOCSY (*S10, 11*), and ROESY (*S11, 12*) experiments and $^{1}H/^{13}C$ two-dimensional heteronuclear HMQC, HMBC, and selective-TOCSY-HSQC experiments (*S13*) were carried out using the 800 MHz NMR spectrometer. The $^{31}P/^{1}H$ COSY spectrum (*S14*) was acquired at the 500 MHz NMR spectrometer. The ^{1}H and ^{31}P chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate and external 2% H_3PO_4 in D_2O (*S15*), respectively. NMR spectra were processed with the NMRPipe package (*S16*), and analyzed using NMRView (*S17*). The 2D $^{13}C/^{1}H$ HMQC spectrum of the reduced O-glycan was assigned using combinations of ^{1}H homonuclear COSY, TOCSY, and ROESY, and heteronuclear HMQC, HMBC, and selective-TOCSY-HSQC techniques. A GlcNAc (subunit B) spin system was assigned using DQF-COSY (Fig. S7A) and TOCSY spectra with a series of mixing times (Fig. S7B-D). At a long mixing time of 120 ms, the GlcNAc (subunit B) H1 proton gave TOCSY cross peaks all the way to the H6 protons. A GalNAc (subunit C) spin system was partially assigned based on a selective-TOCSY-HSQC spectrum. When the GalNAc H1 proton was irradiated with a very selective excitation pulse, selective TOCSY transfer reached to its C3/H3 and C4/H4 cross peaks as detected by an HSQC spectrum (Fig. 3B).

Human and mouse cultured cells and patient biopsies. The MEB fibroblasts and FCMD myoblasts used in this study were described previously (*S18*). POMT1-deficient WWS fibroblasts were derived from a patient as described elsewhere (*S19*). Fibroblasts derived from Mucolipidosis II patients were obtained from the Coriell Institute (GM01586). The GlcNAc-phosphotransferase activity was less than 0.1% of that in normal fibroblasts (specific activity in cell lysate). Wt and *Large*^{myd} mouse fibroblasts were generated from the skin biopsies. The MEB muscle biopsy was obtained from a patient (age 6 months) with two heterozygous mutations in POMGnT1: c. 1539+1G>A, which causes a splice-site defect that affects the processing of exon 17, as reported elsewhere (*S20*); and at c. 704G>A, which is predicted to cause the amino acid substitution G235E. The FCMD muscle biopsy was obtained from a patient (age 7 months) homozygous for retrotransposon insertions in the fukutin gene (*S21*). Control muscle was obtained from a 47 year-old individual. All tissue was obtained and tested in compliance with the Human Subjects Institutional Review Board of the University of Iowa.

Mice and adenovirus infection. *Large*^{myd} mice (JAX) were backcrossed to C57BL/6 mice for 5 generations, and then maintained by the mating of heterozygous pairs. All mice were housed at the University of Iowa Animal Care Unit in accordance with animal usage guidelines. Infectious particles (2×10^{10}) of the E1-deficient recombinant adenovirus Ad5-CMV-LARGE, which encodes human LARGE under the CMV-promoter, were diluted in 0.9% NaCl to a final volume of 100 μl, and were injected percutaneously into quadriceps and calf muscles of *Large*^{myd} mice (12-18 weeks old) using an insulin syringe. At 10 days post-infection, the infected muscle was harvested and processed as described above. All mice were maintained at the University of Iowa Animal Care Unit in accordance with animal usage guidelines.

Assignment of the phosphorylated *O*-Mannose residue by LC-MS/MS. Approximately 2mg of DGFc4 produced in HEK293 cells was reduced, alkylated, and digested with 20 μg of sequence-grade trypsin (Promega), resuspended in 40mM NH_4HCO_3 and incubated at 37°C overnight. The reaction was quenched by adding 1% TFA, resulting in a final concentration of ~0.1% TFA. Enrichment of the digested material was carried out using agarose-bound *Wisteria Floribunda* agglutinin (WFA, Vector Laboratories, CA, USA), essentially as described previously (*S22*). Samples on the WFA-agarose column were eluted using the lectin-column buffer containing 200 mM N-acetylgalactosamine (Toronto Research Chemicals Inc.). Eluted glycopeptides were then desalted using C-18 spin columns (Nest Group) and dried.

The resulting material was reconstituted in 48.5 μl of 0.1% formic acid (mobile phase A) and 1.5 μl of 80% acetonitrile/0.1% formic acid (mobile phase B). The solution was loaded onto a tapered-tip nanospray capillary column (75 μm x 8.5 cm) and separated via a 160 min linear gradient of increasing mobile phase B at a flow rate of ~200nl/min. LC-MS/MS/MS analysis was performed on a LTQ Orbitrap XL mass spectrometer (ThermoFisher) equipped with nanospray. A full mass spectrum at 60,000 resolution was acquired from 400-2000 m/z, followed by three data-dependent MS/MS spectra of the most intense ions. When a neutral loss corresponding to a monosaccharide or phosphate was detected as one of the three most intense ions in the MS/MS spectra, this fragment ion was fragmented again by CID to yield an MS^3 spectrum.

The acquired spectra were searched against a non-redundant rabbit database (NCBI) that included a contaminant database, using BioWorks (3.3.1SP1; ThermoFisher). DTA files were generated for spectra with a threshold of 5 ions, a precursor ion tolerance of 1.4 amu, and a range of MH^+ 400-6000 m/z. The search parameters were set to allow for precursor ion tolerance of 50 ppm and fragmented ion tolerance of 0.5Da with strict trypsin cleavage. Peptides that were assigned as being modified by the O-mannosyl phosphorylated trisaccharide were confirmed manually.

Separation of the ER and Golgi organelles of DGFc4-overexpressing HEK293 cells, and immunoprecipitation of the immature DGFc4 with IMAC-

beads. The ER and Golgi in DGFc4-expressing HEK293 cells were separated by two steps of gradients. The methods were adopted from previous reports (*S23, 24*) with minor modification. The pellet of DGFc4-expressing HEK293 cells was washed one time with PBS and resuspended in homogenization buffer containing 0.25M sucrose, 1mM EDTA, and proteinase inhibitors in 10mM HEPES-NaOH (pH 7.4). The cells were dounce homogenized by hand with 6-7 strokes. The homogenate was spun down at 3,000 x g to obtain the post nuclear supernatant (PNS). The PNS was layered on a sucrose cushion of 8 ml of 1.2M Sucrose, 1mM EDTA, and proteinase inhibitors in 10mM HEPES-NaOH (pH 7.4) in a Beckman ultraclear tube (25 x 89mm). The tubes were spun on a SW 32 Ti rotor at 25,000 rpm (106,750 x g) for 98 minutes. A white band just above the 1.2M sucrose/sample interface was collected through the side of the tube, using a syringe with a 23-gauge needle. The sample was pelleted by diluting 1:1 with ddH_2O and spinning on MLA-80 at 70,000rpm (339,000 x g) for 1 hour. The pellet was resuspended in 1 ml of homogenization buffer, and loaded onto a multi-step discontinuous iodixanol gradient using Optiprep™ (Sigma). A multi-step discontinuous iodixanol gradient was made using 4 ml of 2.5%, 5.0 %, 7.5%, 10.0%, 12.5%, 15%, 17.5 % and 3 ml each of 20% and 30% iodixanol in 42 mM sucrose, 1mM EDTA, 10mm HEPES-NaOH (pH 7.4). The gradients were spun in an SW 32 Ti rotor for 5 hours at 32,000 rpm (174,899 x g) using acceleration and deceleration setting 9. At 5,000 rpm, the brake was taken off and the rotor was left to come to a stop on its own. The tube was punctured at the bottom with a 23-gauge needle, and 3ml fractions were collected, with fraction 1 being 30% iodixanol and fraction 12 being the lightest of 2.55 % iodixanol.

The ER and Goigi-enriched fractions were solubilized in 0.5% CHAPS, and the buffer was exchanged to 0.25 % CHAPS in water using Amicon Ultra (Milipore). The resulting solution was analyzed usign IMAC-beads as described above.

FACS study. HEK293 cells stably expressing LARGE were cloned and analyzed by FACS, using the CORE or IIH6 antibody, or biotinylated lectins (1:200 dilution, Vector Laboratories, CA, USA), as described previously (*S25*).

Supporting figures

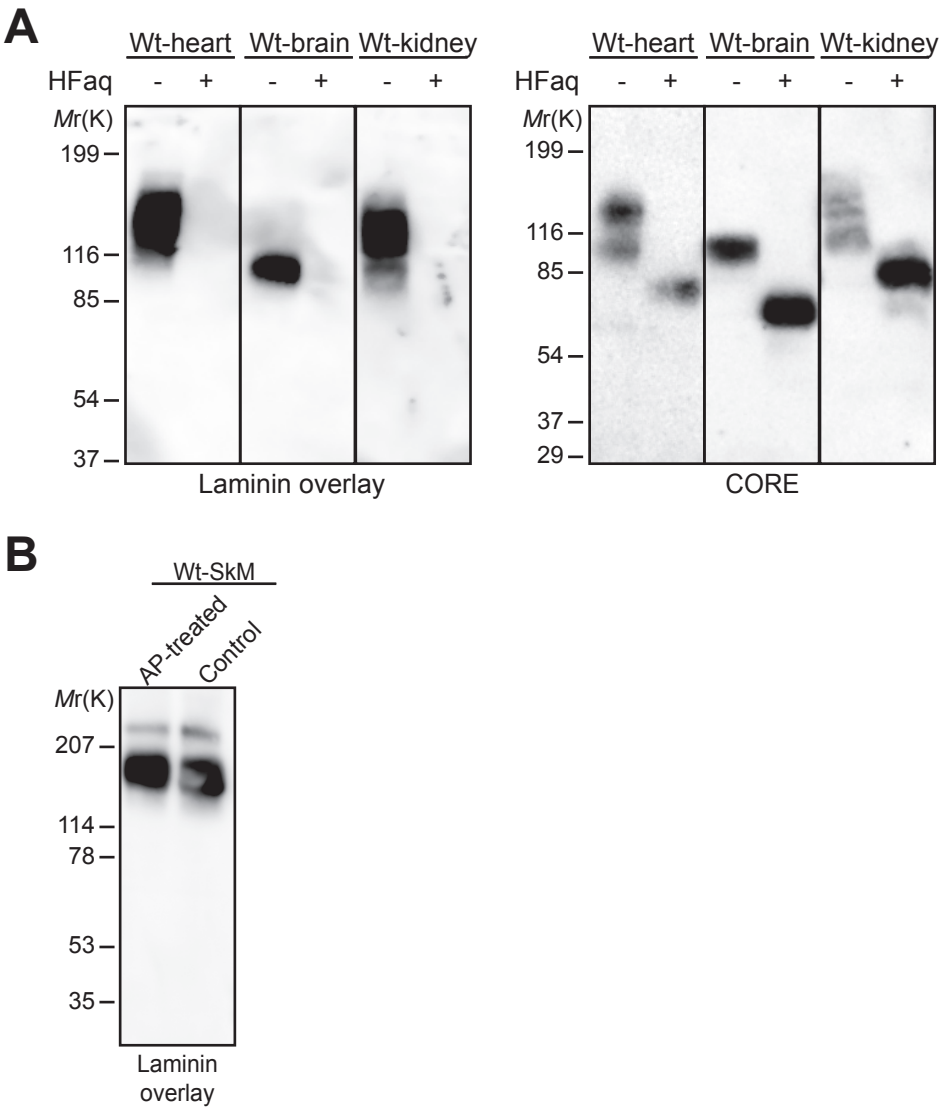

fig. S1. Enzymatic and chemical dephosphorylation of Wt mouse glycoproteins. **(A)** Treated glycoproteins prepared from heart, brain, and kidney of Wt mice were subjected to laminin overlay assay or immunoblotting with CORE antibody. **(B)** Laminin-overlay assay on Wt-muscle α-DG treated with alkaline phosphatase. In contrast to dephosphorylation by HFaq, digestion with alkaline phosphatase did not abolish the laminin-receptor activity of α -DG, suggesting that functional modification of α-DG involves an internal phosphoryl linkage rather than a monoester-linked phosphate.

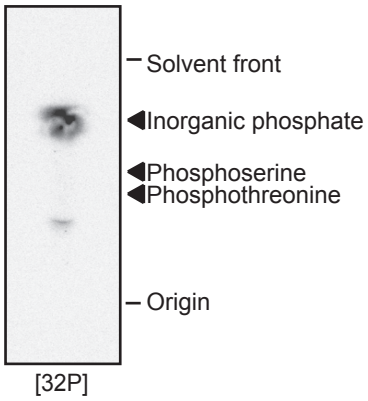

fig. S2. Phospho-amino acid analysis by thin-layer chromatography (TLC). [^{32}P]-labeled DGFc4 produced by HEK293 cells was hydrolyzed under conditions in which phospho-amino acids remain intact. The hydrolysate was separated by cellulose TLC. Arrowheads indicate the spot of unlabeled phospho-amino acid standards. None of [^{32}P]-phospho-amino acid was detected in the hydrolysate, suggesting that phosphorylation does not occur directly on the polypeptide.

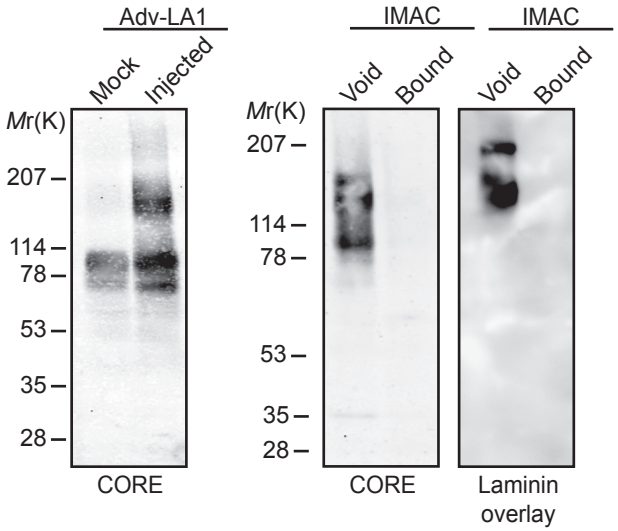

fig. S3. IMAC-binding assay testing glycoproteins from *Large*^{myd} mouse muscle expressing LARGE ectopically (adenovirus-mediated expression). The α-DG regained laminin-receptor activity and concomitantly lost its affinity for IMAC beads, suggesting that LARGE participates in assembly of the laminin-binding moiety on the muscle
α-DG phosphoryl residue.

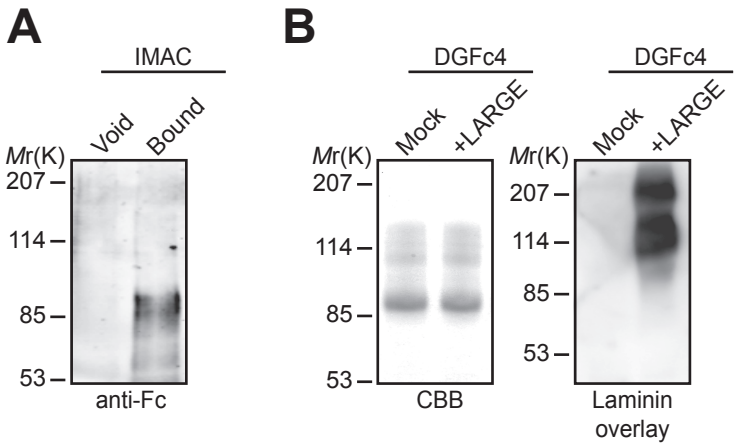

fig. S4. Characterization of DGFc4 produced by HEK293 cells. **(A)** IMAC-binding assay testing DGFc4 produced by HEK293 cells. **(B)** Laminin-overlay assay with DGFc4 produced by HEK293 cells, in the presence and absence of LARGE overexpression.

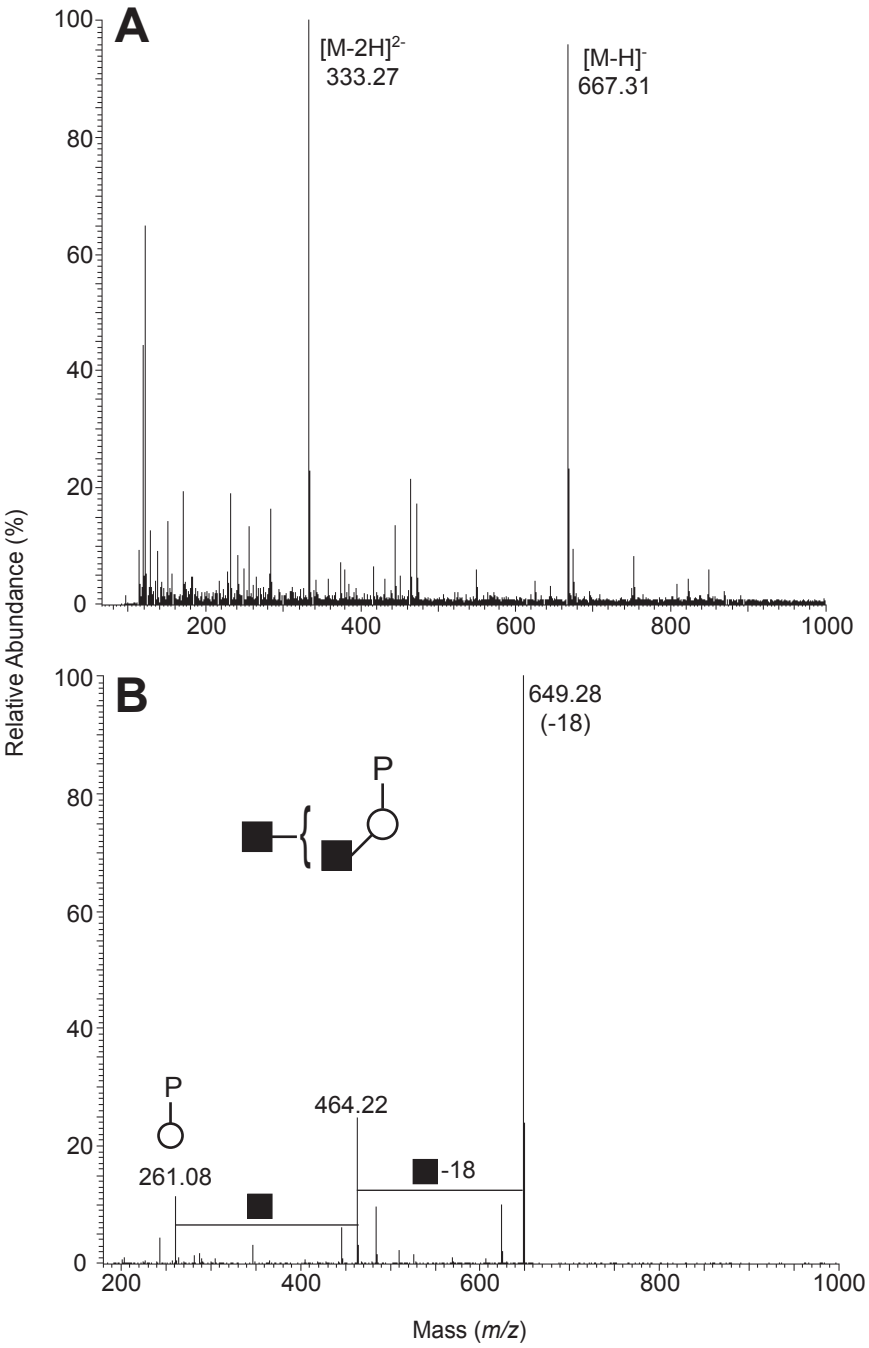

fig. S5. Nanospray ionization-linear ion trap mass spectrometry in negative-ion mode. **(A)** Full mass spectrum of IMAC-enriched *O*-glycan prepared by reductive β-elimination, from HEK293-produced DGFc4. **(B)** CID MS^2 spectrum derived from the precursor ion $[M-H]^-$ (m/z 667.3). Assignments of the fragment ions are shown on the schematic illustration. Filled square: HexNAc; open circle: Hexitol.

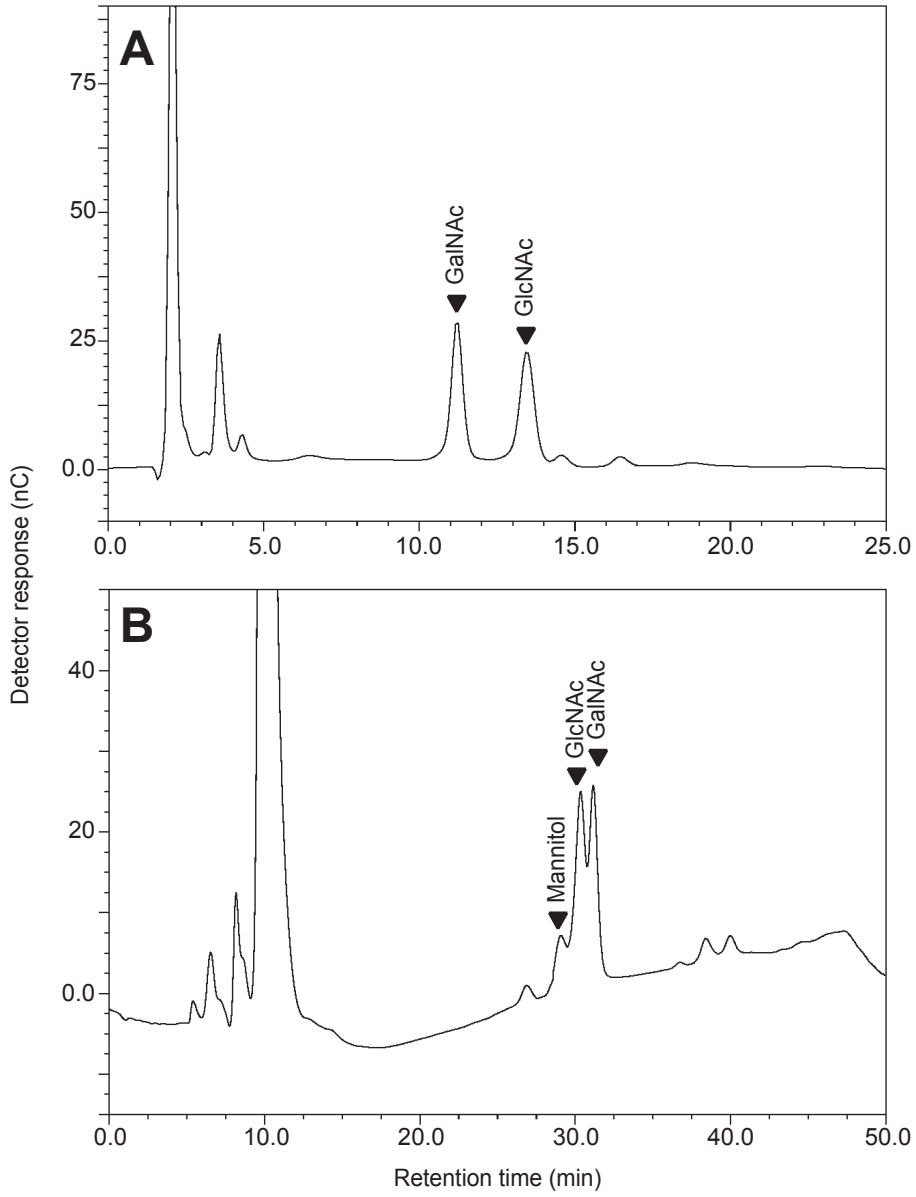

fig. S6. Compositional sugar analysis with HPAEC-PAD. IMAC-enriched *O*-glycan sample prepared by reductive β-elimination from HEK293-produced DGFc4 was acid-hydrolyzed to cleave glycosidic linkages. After hydrolysis, the sample was re-N-acetylated and analyzed by HPAEC-PAD using either a CarboPac PA-1 **(A)** or an MA-1 **(B)** column. GlcNAc, GalNAc and mannitol were detected as the compositional sugars.

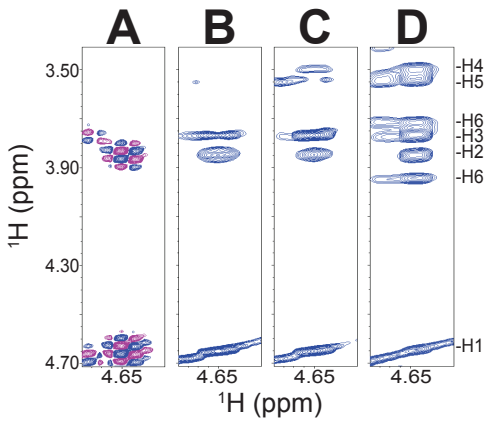

fig. S7. DQF-COSY (A) and TOCSY (B-D) spectra for the assignment of subunit B, with the assigned protons indicated along the right-side panel. TOCSY mixing times of 34, 60, and 120 ms were used for spectra (B), (C), and (D), respectively. At a long mixing time of 120 ms, the GlcNAc (subunit B) H1 proton gave TOCSY cross peaks all the way to the H6 protons.

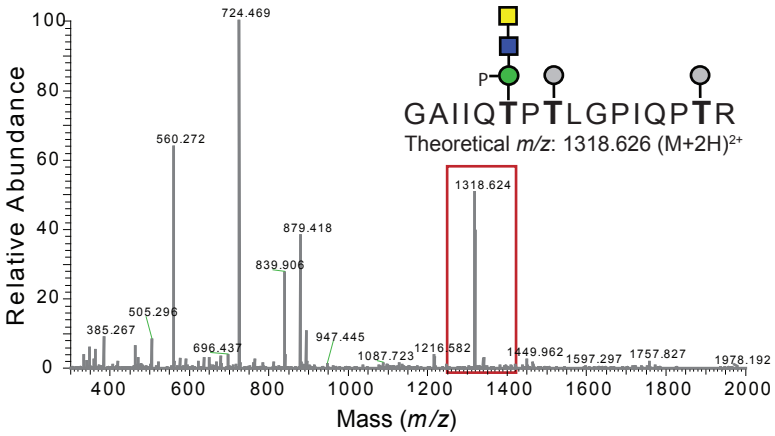

fig. S8. The full FT mass spectrum of the peptide (*m/z* 1318.624) modified by the phosphorylated trisaccharide. The full mass spectrum yielded a mass accuracy of 1.4 ppm for the precursor ions at *m/z* 1318.624. The CID-MS/MS spectrum is shown in Fig. 4A.

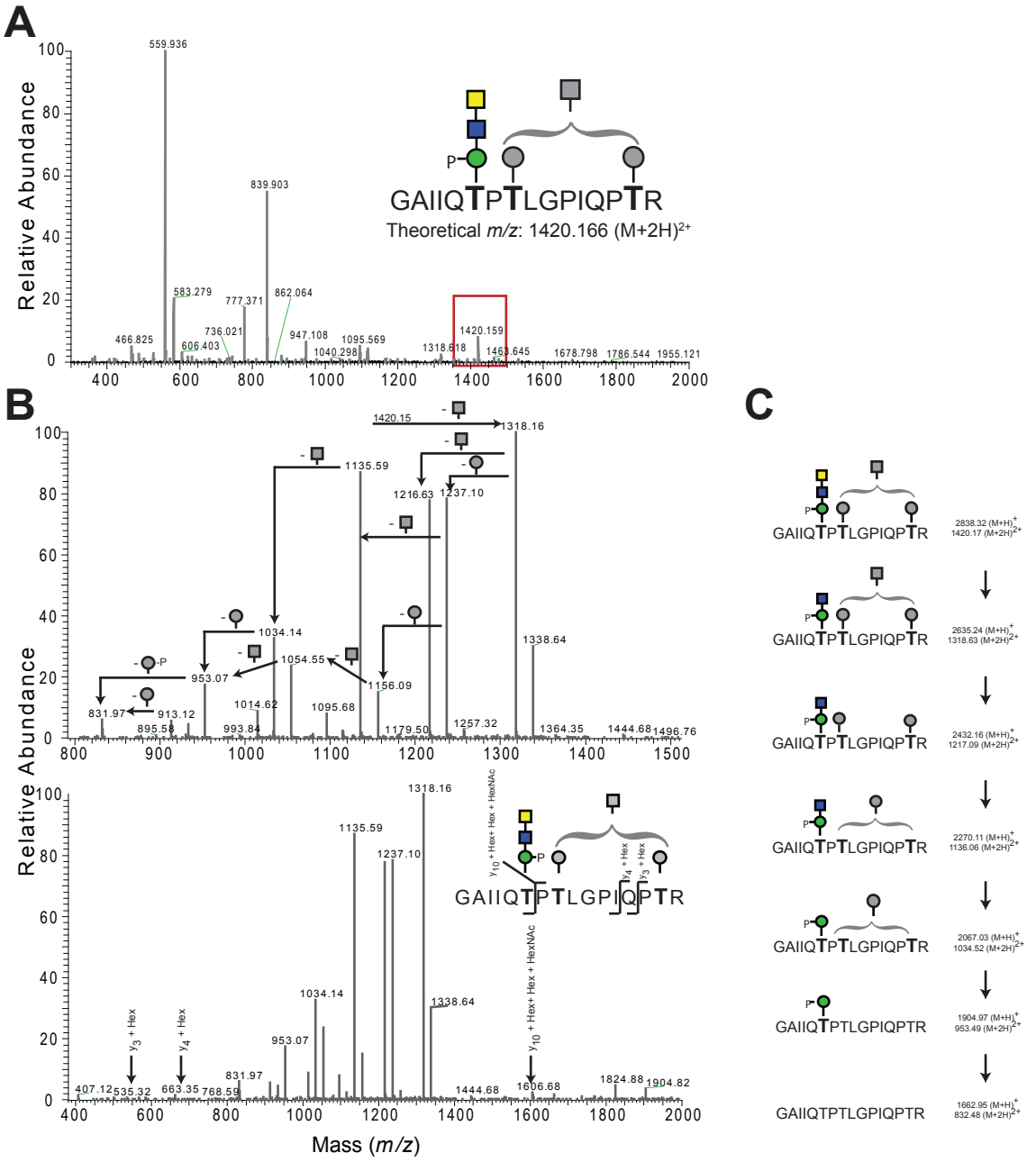

fig. S9. Mass analysis of the peptide modified by phosphorylated trisaccharide (*m/z* 1420.166). **(A)** The full FT mass spectrum of the peptide containing the phosphorylated trisaccharide. The full mass spectrum yielded a mass accuracy of 4.4 ppm for the precursor ion at *m/z* 1420.166. **(B)** The CID-MS/MS spectra from 780-1320 *m/z* (upper panel) and 375-2000 *m/z* (lower panel) show the neutral loss pattern (upper panel) and the peptide-derived b and y ions (lower panel) of the selected precursor ions at *m/z* 1420.17. Square: HexNAc; circle: Hexose. **(C)** The fragmentation of the phosphorylated glycopeptide allowed for identification of the peptide and mononsaccharide units as illustrated. The observation of ions corresponding to the non-phosphorylated hexoses on Thr_{381} and Thr_{388} revealed that Thr_{379} was modified by the phosphorylated trisaccharide and that Thr_{381} and Thr_{388} were occupied by a combination of HexNAc-Hex and Hex in this peptide.

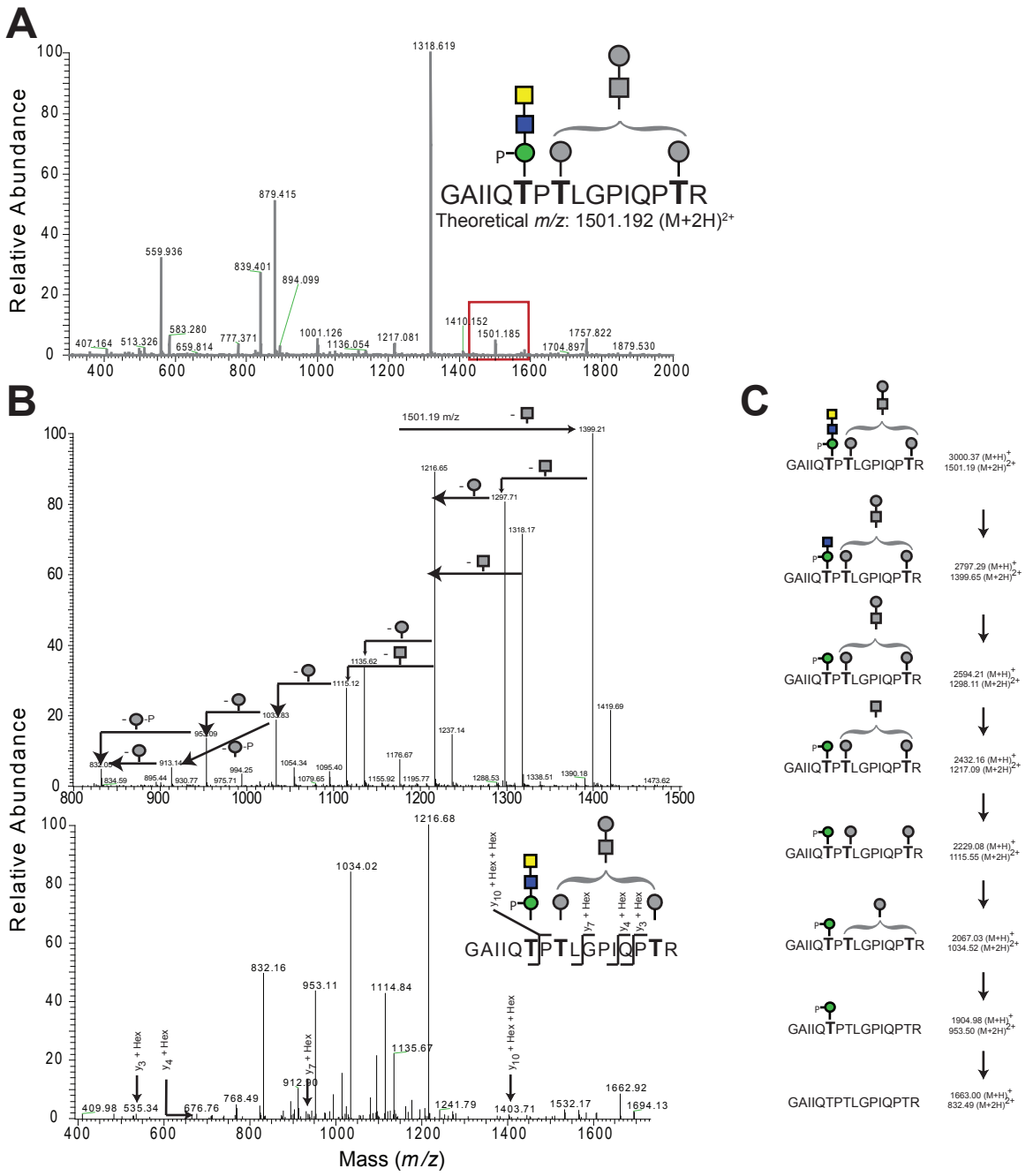

fig. S10. Mass analysis of the peptide modified by phosphorylated trisaccharide (*m/z m/z* 1501.192). **(A)** The full FT mass spectrum of the peptide containing the phosphorylated trisaccharide. The full mass spectrum yielded a mass accuracy of 4.5 ppm for the precursor ions at *m/z* 1501.192. **(B)** The CID-MS/MS spectra from 780-1320 *m/z* (upper panel) and 375-2000 *m/z* (lower panel) show the neutral loss pattern (upper panel) and the peptide-derived b and y ions (lower panel) of the selected precursor ions at *m/z* 1501.19. Square: HexNAc; circle: Hexose. **(C)** The fragmentation of the phosphorylated glycopeptide allowed for identification of the peptide and mononsaccharide units as illustrated. The observation of ions corresponding to the non-phosphorylated hexoses on Thr_{381} and Thr_{388} revealed that Thr_{379} was modified by the phosphorylated trisaccharide and that Thr_{381} and Thr_{388} were occupied by by a combination of Hex-HexNAc-Hex and Hex in this peptide. The Hex-HexNAc-Hex is likely to be Gal-β-1,4-GlcNAc-β-1,2-Man, which was previously shown to modify native and recombinant α-DG in brain, muscle and HEK293 cells (*S26*).

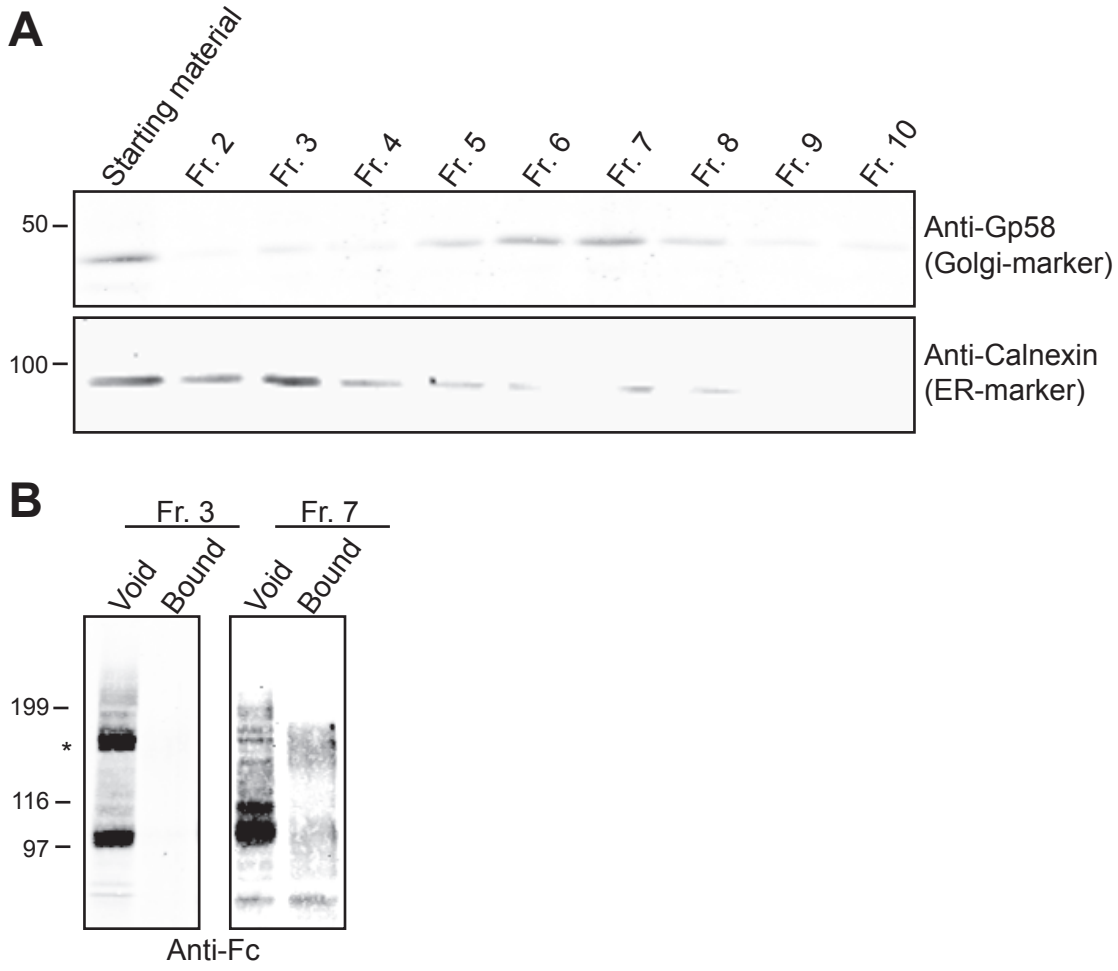

fig. S11. Subcellular fractionation of DGFc4-expressing HEK293 cells and IMAC-binding assay. **(A)** Characterization of subcellular fractions prepared by gradient fractionation. HEK293 cells stably expressing DGFc4 were fractionated by discontinuous iodixanol gradient. The fractions were analyzed by immunoblotting with antibodies against a Golgi marker protein (gp58, upper panel) or an ER marker protein (calnexin, lower panel). **(B)** IMAC-binding assay testing immature DGFc4 in the ER and Golgi-enriched fractions. Asterisk indicates dimerized DGFc4 in the Fc-region.

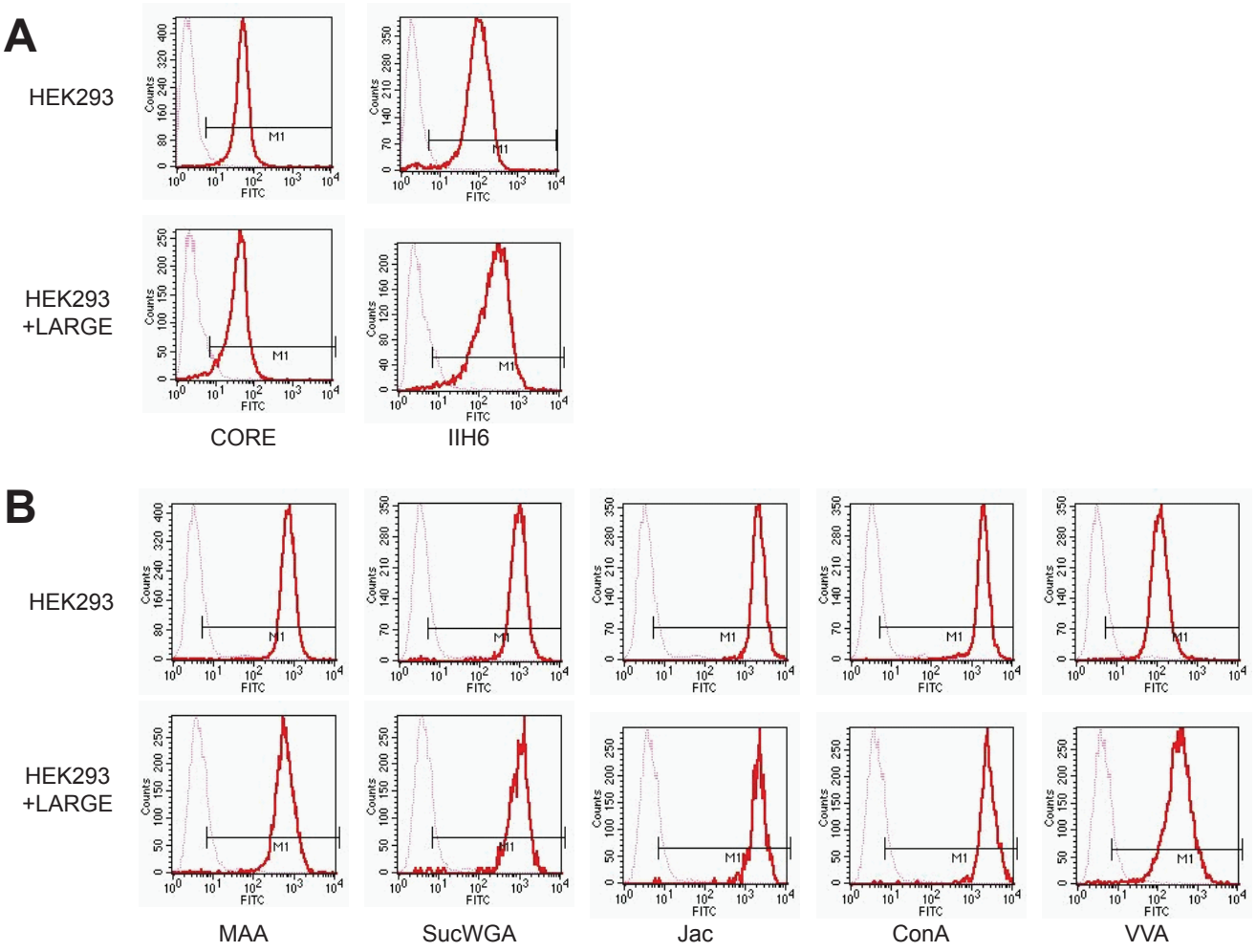

fig. S12. Effects of LARGE expression on glycosylation. HEK293 cells with stable expression of LARGE were generated, and the modified cells were tested by FACS using a CORE or IIH6 antibody **(A)**, or biotinylated lectins **(B)**. LARGE-overexpression increased immunoreactivity of the cell surface against IIH6. Also its overexpression increase the affinity of the cell surface specifically for the VVA lectin, which is known to bind to the laminin-binding form α-DG in muscle and brain (*S27, 28*). Solid line, primary (biotinylated lectins) and secondary antibody (streptavidin); broken line, secondary antibody (streptavidin) only. MAA, Maackia Amurensis Lectin II; SucWGA, succinylated Wheat Germ Agglutinin; Jac, Jacalin; ConA, Concanavalin A; VVA, Vicia Villosa Lectin.

Supporting table

Table S1. Chemical shifts (ppm) of the signals in the ^{1}H and ^{13}C NMR spectra of the *O*-glycan, and inter-residue correlations from NOESY and HMBC spectra.

Sugar residues	$^{1}H/^{13}C$ (ppm)								
	1	2	3	4	5	6	CH_3	NOE	HMBC
→4)-D-Mannitol-6-P	3.87, 3.63	3.92	3.68	4.02	3.89	4.06, 3.92			
A	65.8	73.1	71.9	78.7	71.7	68.3			
→3)-β-D-GlcpNAc-(1→	4.64	3.85	3.76	3.49	3.54	3.94, 3.71	2.10	**A**, H4	**A**, C4
B	103.5	57.11	83.6	71.8	77.5	63.8	25.0		**A**, H4
β-D-GalpNAc-(1→	4.49	3.84	3.74	3.91	3.69	3.77, 3.77	1.99	**B**, H3	**B**, C3
C	104.2	55.21	73.2	70.4	77.7	63.6	25.0		

Supporting references and notes

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