SUPPLEMENTARY DATA

SUPPORTING EXPERIMENTAL PROCEDURES

Identification of mass isotopomers of UDP-HexNAc and CMP-NeuAc

We prepared UDP-HexNAc (UDP-GlcNAc and UDP-GalNAc) and CMP-NeuAc from cellular extracts in Hepa1-6 cells cultured in five 10-cm dishes in order to purify these nucleotide sugars and to identify each isotopic ion in the isotopomers as described in supplemental figures 2 and 3. Cellular extracts were purified by the same procedures described in the “Preparation of labeled nucleotide sugars in cells” section, and analyzed by HPLC where the nucleotide sugars were detected by UV absorption at 254 nm using the same buffers. After the collection of the UDP-HexNAc and CMP-NeuAc fractions, the sample solutions were lyophilized for analysis by direct infusion nanospray ESI-MS/MS. The isotopomers of UDP-HexNAc in the $[M-H]^-$ ion were identified by MS/MS analysis. To detect the fragment ions of the sugar motif in CMP-NeuAc, the isotopomers of CMP-NeuAc were analyzed from the MS/MS of the $[M-2H + Na]^-$ ions, which was generated by the addition of 1 mM NaCl solution to the CMP-NeuAc fractions.

Isolation of total RNA, reverse transcription and RT-PCR

Total RNA from cultured cells was extracted using an RNeasy Mini kit (Qiagen). One microgram of

total RNA was reverse-transcribed using the SuperScript III First-Strand Synthesis System (Life Technology, Japan) with oligo dT primers.

Quantitative real-time PCR

Expression levels of *GNE*, *SLC35A1*, and *GAPDH* were analyzed by using appropriate Assays-on-Demand gene expression products in combination with TaqMan Universal PCR Master Mix in accordance with the instructions provided by the manufacturer (Life Technology, Japan) using an ABI PRISM 7900HT sequence detection system (Life Technology Japan). The levels of *GNE* and *SLC35A1* mRNA were measured in duplicate and normalized to the corresponding GAPDH levels.

Reverse-transcription polymerase chain reaction (RT-PCR)

The semi-quantitative RT-PCR was performed according to the following protocols. PCR cycles were set at 25 cycles for *actin* and ribosomal protein *RPL4*, and 35 cycles for all other genes (*GFAT*, *ST6GAL1*, and *ST3GALs*). PCR, involving a three-step incubation (94°C, 30 s; 58°C, 1 min; 72°C, 30 s), was performed using rTaq DNA polymerase (Takara Bio, Japan). The cycles were set in such a way that the amount of amplified DNA did not reach a plateau.

The oligonucleotide primers used were:

RLP4 (NM_024212, nucleotides 616–949)

5`- CTGGCAAGGGCAAAATGAGAAACC-3` and 5`- GGGCTCTTTGGATTTCTGGGCTTT-3`;

GFAT (NM_013528.3, nucleotides 644–955)

5`-AGAGCCAGGACGTCAGTTTTACCAC-3` and 5`-TATCACGGCACTTGCATCAGAAGC-3`;

ST6Gal1 (NM 145933, nucleotides 1176–1526)

5`-ACCAGGAGTCAAGTTCAGCGTAGA-3` and 5`- CTGTCCTTCAGGAAGCGCTTTTCT -3`;

ST3Gal1 (NM 009177, nucleotides 1191–1479)

5`-CATATTGGTTCGACCAGCGCTTCA-3` and 5`-GCACAAAGTCATGGCTGTCGATCT-3`;

ST3Gal2 (NM 009179, nucleotides 1811–2094)

5`-AAGAACCTGCCTGCCAATGTCA-3` and 5`-CGAACCCATACACGTTCACCTCAT-3`;

ST3Gal3 (NM 009176, nucleotides 1039–1355)

5`-AACAAGTCTCTGGGGTCACGGATT-3` and 5`-AGTATGGGTTGAGGATGCGGATCT-3`;

ST3Gal4 (NM 009178, nucleotides 686–1038)

5`-GTCGGCCCACTTTGACCCTAAAAT-3` and 5`-GTTGGAGGCATCTGGATAGCCAAAT-3`.

Western blotting assay of O-GlcNAc proteins

Cells were washed with ice-cold PBS, harvested and extracted with cell lysis buffer (Cell Signaling Technology, MA). Cell extracts (10 μg) were subjected to 4–15% gradient SDS polyacrylamide gel electrophoresis (Bio-Rad) and transferred to a nitrocellulose membrane. Anti-O-GlcNAc antibody (CTD 110.6) was used as the primary antibody (1:2,000 dilution) and HRP-conjugated anti-mouse IgM (Stressgon, Sab-110) as the secondary antibody (1:2,000 dilution). Horseradish peroxidase activity was detected with enhanced chemiluminescence ECL reagents (GE Healthcare).

Preparation of glycan alditols derived from glycosphingolipids

Total lipids including glycosphingolipids were extracted from cells using the Bligh-Dyer method (1). Cells (5×10^6 cells) were suspended with ice-PBS (100 μl) and extracted by adding a chloroform/methanol solution (1/2 (v/v), 100 μl). The extracts were sonicated five times with a 20 s pulse at 10 s intervals. The cell extracts were centrifuged at 1,200 × *g* for 10 min, and then the upper phase was collected to a new glass centrifuge tube. The lower phase was further extracted by adding a chloroform/methanol/water mixture (1:2:0.8, v/v/v, 400 μl), and then the solution was centrifuged at 1,200 × *g* for 10 min. The upper phase was combined to the first extracts and dried under N_2 gas.

Extracted total lipids were directly subjected to release of glycans by employing endoglycoceramidase (Takara bio, Shiga, Japan). Crude cellular lipids were suspended in 50 mM acetate buffer (pH 5.0) (20 μl), containing 0.4% Triton X-100 followed by the addition of endoglycoceramidase II (4 mU). The enzymatic digestions were performed at 37 °C for 24 h. After enzymatic digestion, water (230 μl) was added and chloroform/methanol = 2:1 (1 ml) was further added. The sample solution was centrifuged at 1,200 × *g* for 10 min, the supernatants were collected to a new glass tube and dried using a speed vac system.

Preparation of disaccharide alditols derived from hyaluronic acids

Culture medium was desalted and the disaccharides were prepared according to the previous report (2). In brief, cells were cultured on 100-mm dishes until 70% confluent, washed with serum free DMEM three times and maintained in the serum-free medium for 24 h. The medium (6 ml) were applied to an Amicon Ultrafree 4 column and washed with 2 ml of water several times. The residues on the filters on the column were collected by water (1 ml). The samples were diluted with 1 M Tris-HCl (pH 8.0, 1 ml) and the mixtures were digested with Pronase (1 mg) at 37 °C overnight. After maintaining the mixture in a boiling water bath, the mixture was centrifuged at 8,000 × *g* and the supernatants were collected. Ninety-five percent ethanol containing 1.3% potassium acetate and 0.27 mM EDTA (3 ml) was added to the mixture, and kept at 0 °C for 2 h. The precipitates were digested with chondroitinase AC-II (10 mU, Seikagaku Corp, Japan) in 20 mM Tris-HCl buffer (pH 8.0, 100 μl) at 37 °C overnight. After boiling and centrifugation, the supernatants were lyophilized.

Graphite carbon LC-ESI-MS and ESI-MS/MS for isotopomers analysis of glycan alditols derived from glycosphingolipids and hyaluronic acids

Disaccharides derived from hyaluronic acids and glycans derived from glycosphingolipids were reduced with 1 M $NaBH_4$/50 mM KOH (20 μl) at 50 °C for 3 h and neutralized with 1 μl glacial acetic acid. These glycan alditols were purified using AG-X8 cation exchange microcolumns and analyzed by the same analytical conditions for O-glycans analysis as mention above.

Supporting references

1. Bligh, E.G., and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol*. 37, 8, 911-917.

2. Matsuno, Y.K., Kinoshita, M., and Kakehi.K. (2004) Electrophoretic analysis of di- and oligosaccharides derived from glycosaminoglycans on microchip format. *J. Pharm. Biol. Anal.* (2004) 36, 9-15.

Supplemental Fig. 1.

Selection of stable isotope labeled-monosaccharides for metabolic labeling of glycans. The Hepa1-6 cell line was labeled for 24 h with (a) 5.5 mM glucose as a control, (b) 5.5 mM $^{13}C_6$-glucose, (c) 5.5 mM $^{13}C_2$-glucose, (d) 5.5 mM glucose and 1 mM $^{13}C_2$-GlcNAc, and (e) 5.5 mM glucose and 1 mM $^{13}C_2$-glucosamine (GlcN).

(a): High mannose type $[Man]_9[GlcNAc]_2$ glycan alditols were detected at $m/z = 941.3^{2-}$ as an unlabeled $[M-H]^-$ ion with doubly charged ions. (b and c): ^{13}C-glucose labeled most of the monosaccharide residues in glycans (6–8 resides). (d): $^{13}C_2$-GlcNAc did not affect the isotopomers. This observation was most likely due to poor uptake by the cells. (e) $^{13}C_2$-glucosamine was specifically incorporated in only two GlcNAc residues.

Supplemental Fig. 2.

The MS/MS spectra of isotopic ions in UDP-HexNAc (UDP-GlcNAc and UDP-GalNAc) isotopomers. (left) Isotopomers of fragment ions, $[HexNAc\text{-}1\text{-}P\text{-}H_2O]^-$, contained a combination of $^{13}C_6$-hexose and $^{13}C_2$-acetyl residues. (right) Isotopomers of fragment ions, $[UDP\text{-}H_2O]^-$, were labeled only at $^{13}C_5$-ribose.

Supplemental Fig. 3.

The MS/MS spectra of isotopic ions in CMP-NeuAc isotopomers. (left) Isotopomers of fragment ions, $[NeuAc\text{-}H_2O]^-$, contained a combination of $^{13}C_6$-hexose, $^{13}C_2$-acetyl and $^{13}C_3$-pyruvate residues. (right) Isotopomers of fragment ions, $[CMP\text{-}H_2O]^-$, were labeled only at $^{13}C_5$-ribose. Some of isotopic ions of CMP-NeuAc comprise several patterns of isotopomers.

Supplemental Fig. 4.

Gene expression levels of several enzymes involved in sialylation. (a) Real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis of *GNE* (UDP-GlcNAc 2-epimerase/ManNAc kinase) and *SLC35A1* (CMP-NeuAc transporter). (b) RT-PCR analysis of *GFAT*, *ST6GAL1*, *ST3GAL1*, *ST3GAL2*, *ST3GAL3*, *ST3GAL4*, *actin* and *RPL4*. Abbreviations: *GFAT*, glucosamine-fructose-6-phosphate aminotransferase; *ST6GAL1*, beta-galactosaminide alpha-2,6-sialyltranferase 1; *ST3GAL1-4*, beta-galactosaminide alpha-2,3-sialyltranferases1-4, *RPL4*, ribosomal protein L4. The mRNAs from Hepa1-6 and Min6 were quantified as described in the supplemental experimental procedures.

Supplemental Fig. 5.

MS/MS spectra of isotopic ions in isotopomers of peak 5 ($Hex_9HexNAc_2$). (Left) MS/MS spectra for the labeled parent ions detected at $m/z = 941.3^{2-}$, $m/z = 943.3^{2-}$ and $m/z = 944.3^{2-}$. (Right) Isotopomers of fragment ions $[M\text{-}Hex]^{2-}$. The isotopic ions were observed as the multiple ions including $^{13}C_2$-GlcN

labeled glycan alditols and $^{13}C_2$-mannose labeled glycan alditols.

Supplemental Fig. 6.

Replacement ratios of labeled ions in UDP-HexNAc and CMP-NeuAc after $^{13}C_2$-GlcN labeling. Hepa1-6 and Min6 cell lines were cultured for 24 h in the same conditions for $^{13}C_2$-GlcN labeling of glycans.

Supplemental Fig. 7.

Comparison of the LC-MS profiles of N-glycan alditols derived from (a) Hepa1-6 and (b) Min6 cells. N-glycan alditols released from whole cellular proteins were analyzed by LC-MS as described in experimental procedures. Glycan structures corresponding to each base peak chromatogram are assigned in Table 1.

Supplemental Fig. 8.

Reversed-phase HPLC chromatograms of the neutral PA-N-glycans fractionated with anion-exchange chromatography derived from (a) Hepa1-6 and (b) Min6. Numbered arrowheads indicate the elution position of PA-maltooligosaccharides with the corresponding degree of polymerization. High mannose type N-glycans (Man_6-Man_9, peak 2-5 assigned in Table 1) were eluted around 15–35 min and asialo

complex type N-glycans eluted later.

Supplemental Fig. 9.

Western blot analysis of O-GlcNAc modified proteins in cell extracts derived from Hepa1-6 and Min6 using the O-GlcNAc antibody (CTD110.6). The cell extracts derived from mouse embryonic fibroblasts (OGT wild-type) and the inducible OGT knock-out cell line (OGT Null cells) were also analyzed as control samples. OGT: O-GlcNAc transferase.

Supplemental Fig. 10.

Time courses of the ratios of unlabeled ions, +6 mass units and +11 mass units in the isotopomers of UDP-Glc and UDP-GlcA. The signal for the +6 mass reflects the labeling at $^{13}C_6$-glucose, and that of +11 mass reflects the labeling at $^{13}C_6$-glucose and $^{13}C_5$-ribose motifs. The peak ratios were calculated for the sums of isotopomers, except for native abundance, as described in the experimental procedures. Symbols are defined in the figure.

Supplemental Fig. 11.

Comparison of the LC-MS profiles of glycan alditols derived from glycosphingolipids in (a) Hepa1-6 and (b) Min6 cells. Glycan alditols were prepared by enzymatic release of endoglycoceramidase II from

total lipid fractions and then analyzed under the same conditions for O-glycan analysis, as described in the experimental procedures.

Supplemental Fig. 12.

The isotopomers of CMP-NeuAc in insulinoma cells labeled at 6 and 24 h. Only a few CMP-NeuAc isotopomers labeled with $^{13}C_5$-ribose were detected in Min6. The replacement ratios of the $^{13}C_5$-ribose motif in CMP-NeuAc (indicator of the salvage pathway from cellular NeuAc pool) are lower in Min6 cells when compared with Hepa1-6 cells (data not shown).

Supplemental Table 1. Nakajima

The distribution of isotopic ions after the $^{13}C_6$-glucose labeling, was a result of the incorporation of $^{13}C_6$-hexose and its metabolites ($^{13}C_5$-ribose and $^{13}C_2$-acetyl moieties) into UDP-GlcNAc, in combination with the natural occurrence of X_0 - X_2 mass isotopomers. Natural isotopic ions (X) include ^{13}C, ^{2}H, ^{17}O, ^{18}O,and ^{15}N. For example, the isotopic ion of *m/z*=614 contains $^{13}C_8$-(hexose and acetyl) labeled UDP-GlcNAc, along with isotopic ions of X_2[natural]+$^{13}C_6$-hexose[labeled] and X_1[natural]+$^{13}C_7$-(ribose and acetyl)[labeled] UDP-GlcNAc. We have calculated the intensity of the $^{13}C_8$-(hexose and N-acetyl) labeled ion by subtracting those for the second and third compounds (calculated based on the relative abundance of natural isotopic ions of UDP-HexNAc, i.e., X_0: X_1: X_2 = 1:0.20:0.055).

UDP-GlcNAc

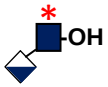
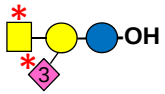
m/z	^{13}C-Labeled isotopic ions	Natural(X) isotopic ions
606	$[M-H]^-$	-
607 (+1)	-	$^{13}C_1$-$[M-H]^-$
608 (+2)	$^{13}C_2$-acetyl	$^{13}C_2$-$[M-H]^-$
609 (+3)	-	X_1+$^{13}C_2$-acetyl
610 (+4)	-	X_2+$^{13}C_2$-acetyl
611 (+5)	$^{13}C_5$-ribose	-
612 (+6)	$^{13}C_6$-hexose	X_1+$^{13}C_5$-ribose
613 (+7)	$^{13}C_7$-(ribose + acetyl)	X_2+$^{13}C_5$-ribose, X_1+$^{13}C_6$-hexose
614 (+8)	$^{13}C_8$-(hexose + acetyl)	X_2+$^{13}C_6$-hexose X_1+$^{13}C_7$-(ribose + acetyl)
615 (+9)	-	X_2+$^{13}C_7$-(ribose + acetyl) X_1+$^{13}C_8$-(hexose + acetyl)
616 (+10)	-	X_2+$^{13}C_8$-(hexose + acetyl)
617 (+11)	$^{13}C_{11}$-(hexose + ribose)	-
618 (+12)	-	X_1+$^{13}C_{11}$-(hexose + ribose)
619 (+13)	$^{13}C_{13}$-(hexose + ribose + acetyl)	X_2+$^{13}C_{11}$-(hexose + ribose)
620 (+14)	-	X_1+$^{13}C_{13}$-(hexose + ribose + acetyl)
621 (+15)	-	X_2+$^{13}C_{13}$-(hexose + ribose + acetyl)

CMP-NeuAc

m/z	^{13}C-Labeled isotopic ions	Natural(X) isotopic ions
613	$[M-H]^-$	-
618 (+5)	$^{13}C_5$-ribose	-
619 (+6)	$^{13}C_6$-hexose	X_1+$^{13}C_5$-ribose
620 (+7)	$^{13}C_7$-(ribose + acetyl)	X_1+$^{13}C_6$-hexose
621 (+8)	Max $^{13}C_8$-(hexose + acetyl) / Min $^{13}C_8$-(ribose + pyruvate) *1	X_2+$^{13}C_6$-hexose X_1+$^{13}C_7$-(ribose + acetyl)
622 (+9)	$^{13}C_9$-(hexose + pyruvate)	X_2+$^{13}C_7$-(ribose + acetyl) Max X_1+$^{13}C_8$-(hexose + acetyl) / Min X_1+$^{13}C_8$-(ribose + pyruvate)
623 (+10)	-	X_1+$^{13}C_9$-(hexose + pyruvate) Max X_2+$^{13}C_8$-(hexose + acetyl) / Min X_2+$^{13}C_8$-(ribose + pyruvate)
624 (+11)	$^{13}C_{11}$-(hexose + ribose)	X_2+$^{13}C_9$-(hexose + pyruvate)
625 (+12)	-	X_1+$^{13}C_{11}$-(hexose + ribose)
626 (+13)	$^{13}C_{13}$-(hexose + ribose + acetyl)	X_2+$^{13}C_{11}$-(hexose + ribose)
627 (+14)	$^{13}C_{14}$-(hexose + ribose + pyruvate)	X_1+ $^{13}C_{13}$-(hexose + ribose + acetyl)
628 (+15)	-	X_1+ $^{13}C_{14}$-(hexose + ribose + pyruvate) X_2+ $^{13}C_{13}$-(hexose + ribose + acetyl)
629 (+16)	$^{13}C_{16}$-(hexose + ribose + pyruvate + acetyl)	X_2+ $^{13}C_{14}$-(hexose + ribose + pyruvate)
630 (+17)	-	X_1+ $^{13}C_{16}$-(hexose + ribose + pyruvate + acetyl)
631 (+18)	-	X_2+ $^{13}C_{16}$-(hexose + ribose + pyruvate + acetyl)

*1 Maximum values were considered to be the ions modified with both $^{13}C_6$-hexose and $^{13}C_2$-acetyl residues.
Minimum values were considered to be the ions labeled with $^{13}C_3$-pyruvate and $^{13}C_5$-ribose.

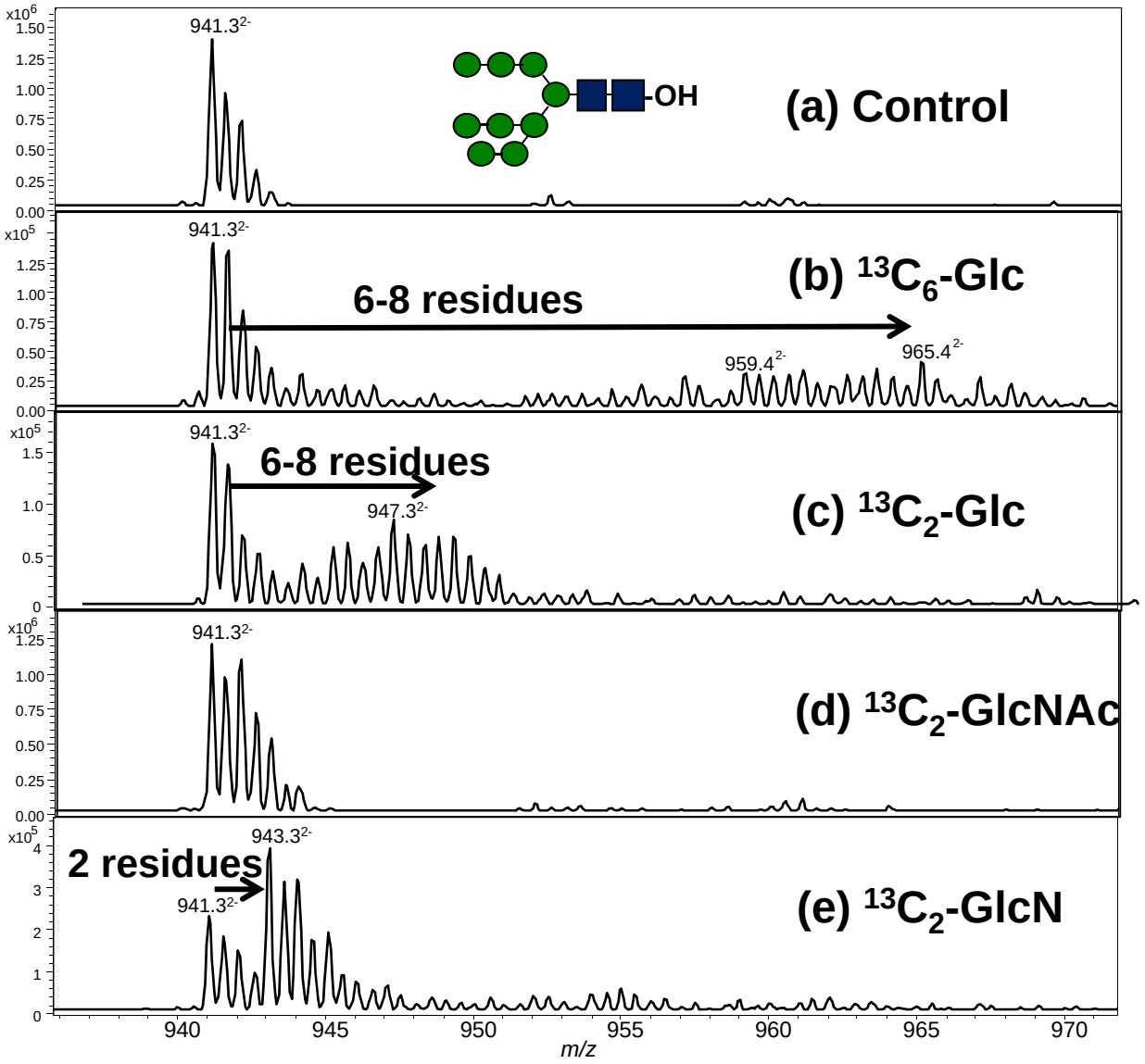
Supplemental Table 2. Nakajima

	[M-H]$^{-}$ *1	Glycan alditols	Numbers of $^{13}C_2$-GlcN *2	Ratio of unlabeled Ions to total isotopomers *3
Hyaluronic acids	380.1		1	22.4
Glycosphingolipids (GM2)	837.3		2	70.6

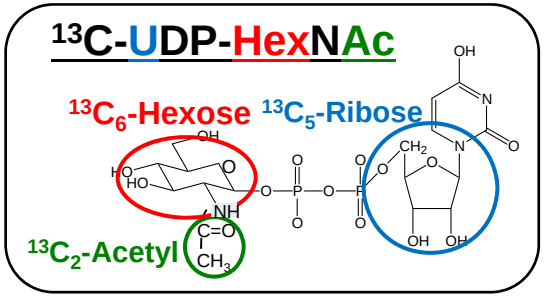
■ : GlcNAc □ : GalNAc, ◇ : Glcuronic acid ○ : Galactose, ● : Glcose, ◇3 : α2-3 NeuAc.

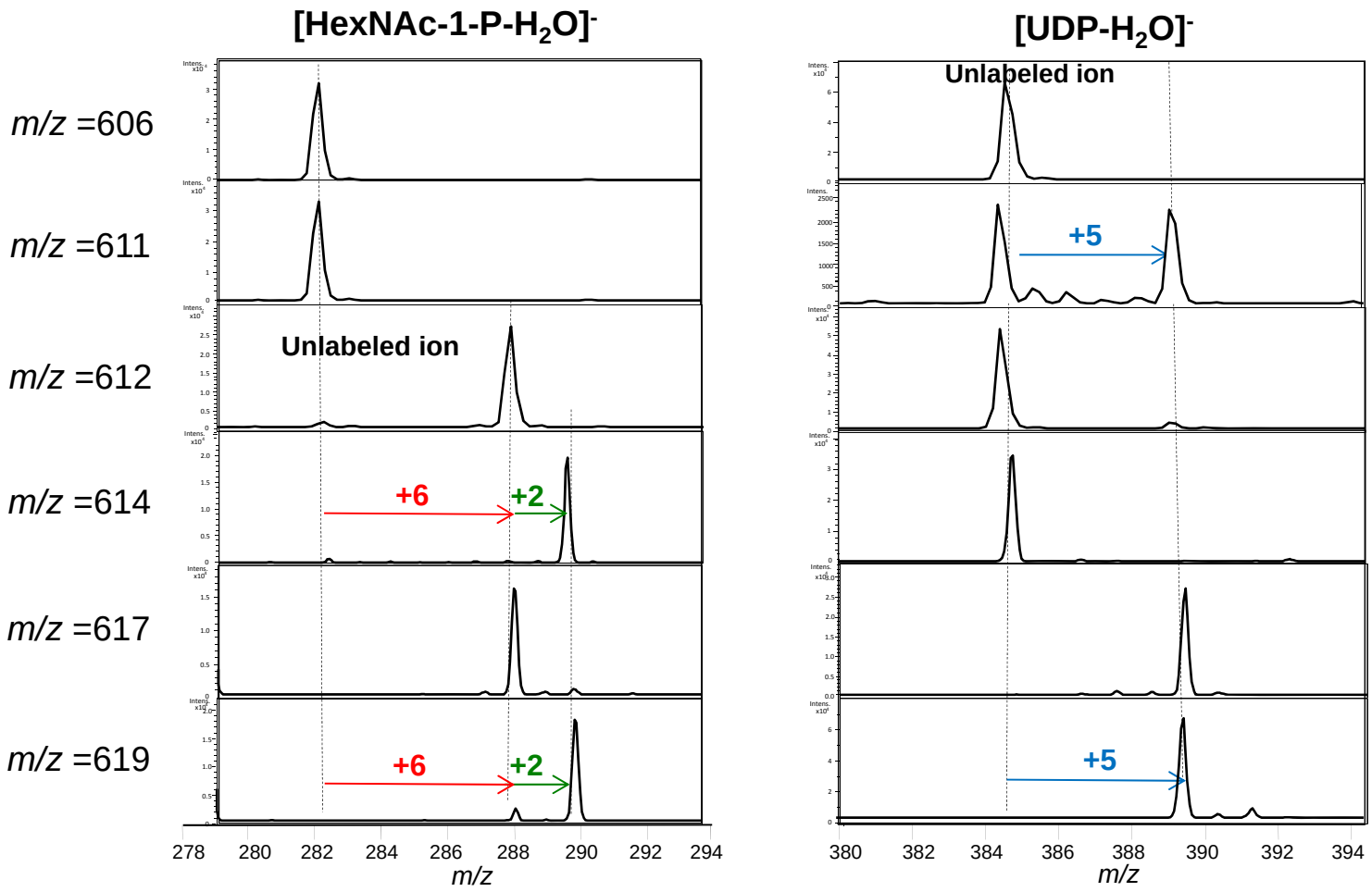
*1, The glycans were detected as single charged ions.
*2, The mass shifts of isotopomers represent the numbers of incorporated $^{13}C_2$-GlcN in each glycan.
*3, Ratio of unlabeled ions to total isotopomers were calculated by the procedure described in “Experimental Procedures” .

Supplemental Figure 1. Nakajima

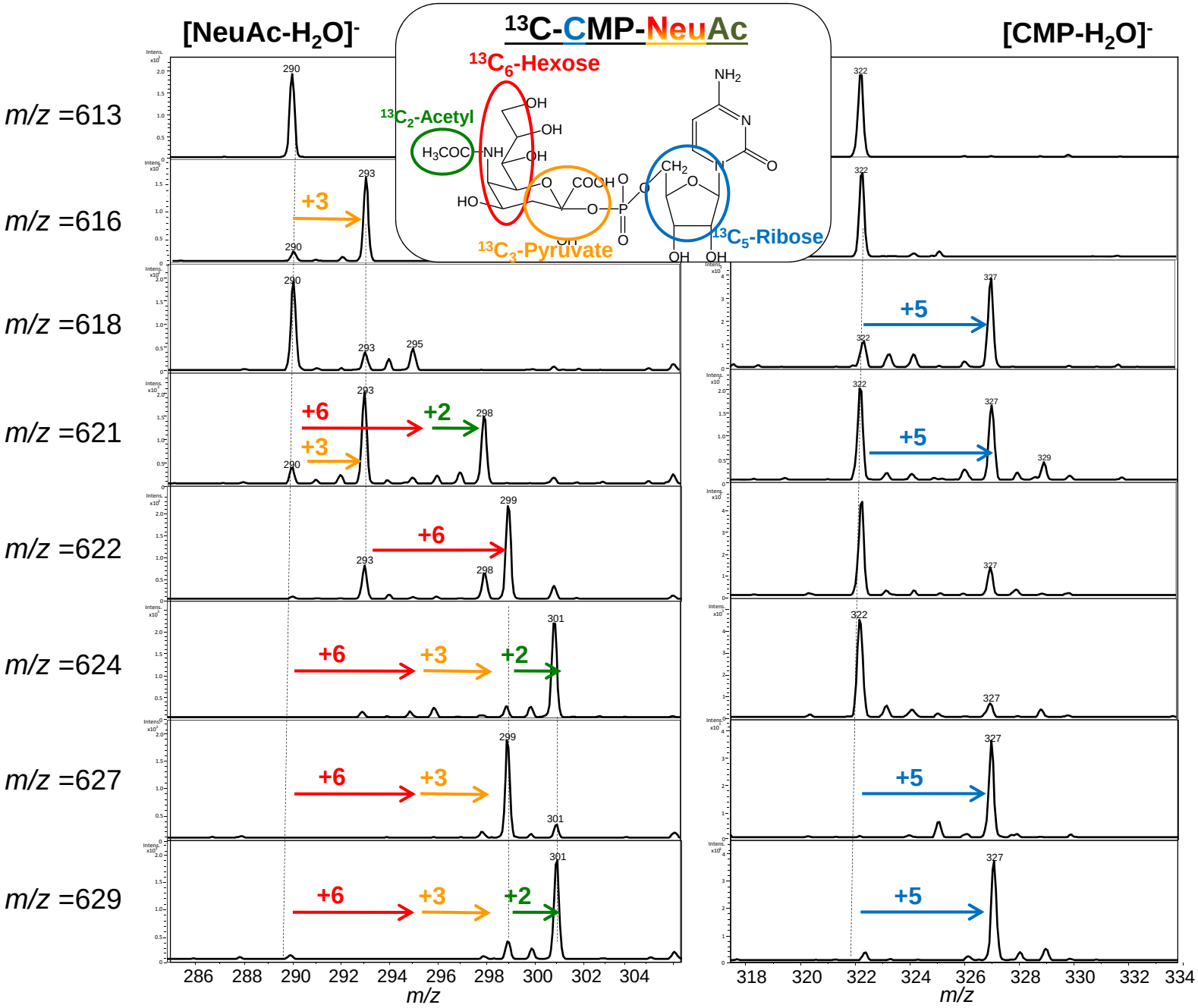


Supplemental Figure 2. Nakajima

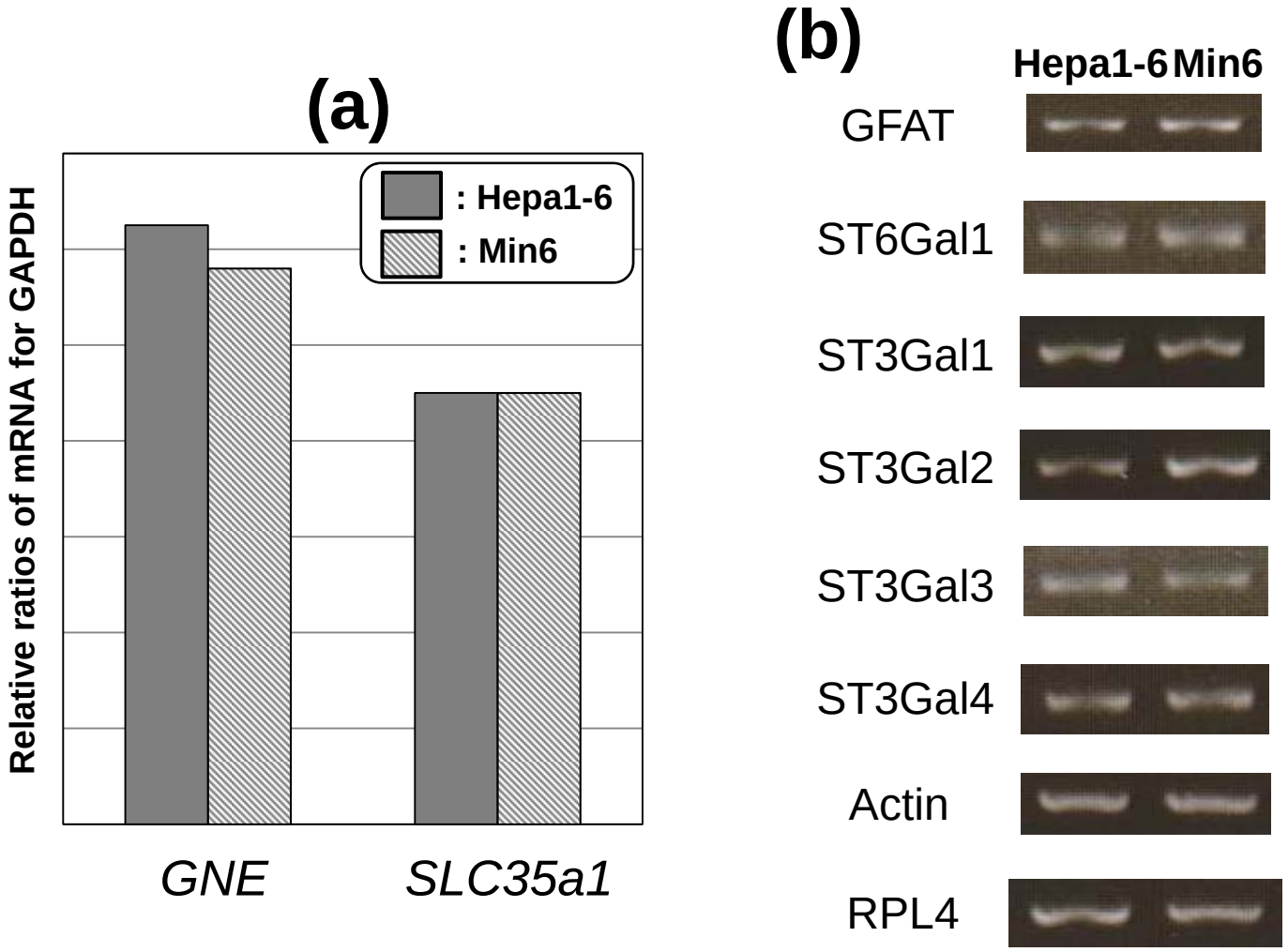




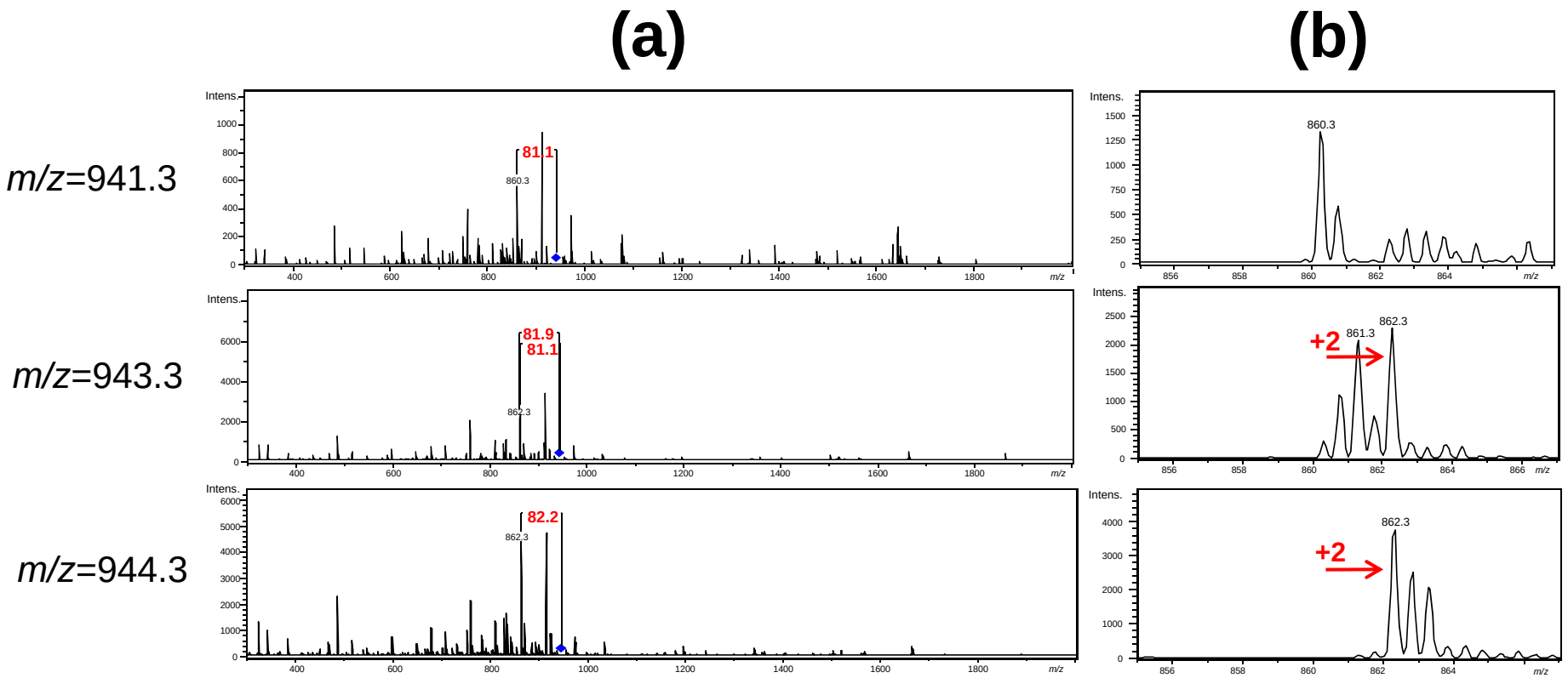
Supplemental Figure 3. Nakajima



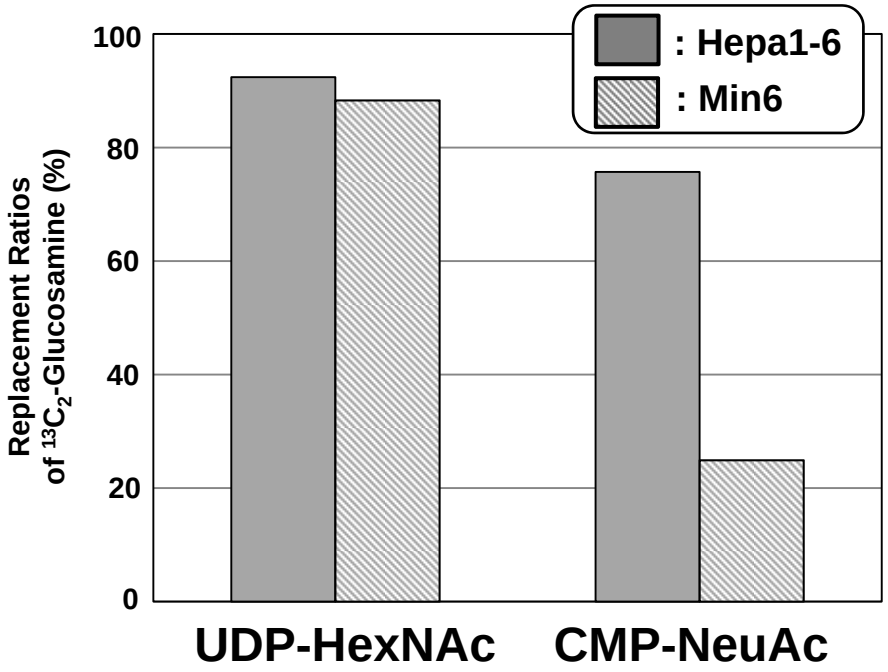
Supplemental Figure 4. Nakajima



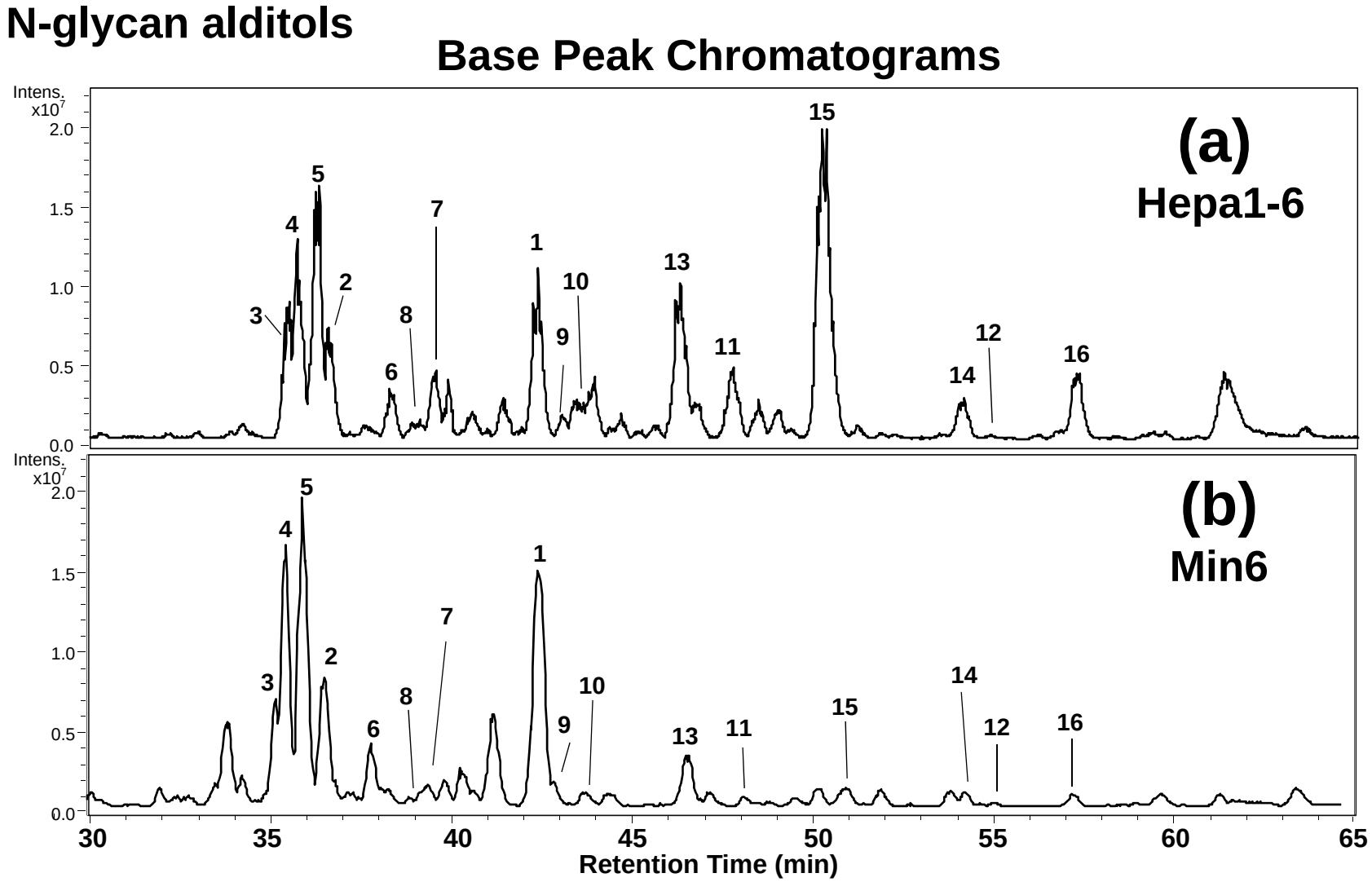
Supplemental Figure 5. Nakajima



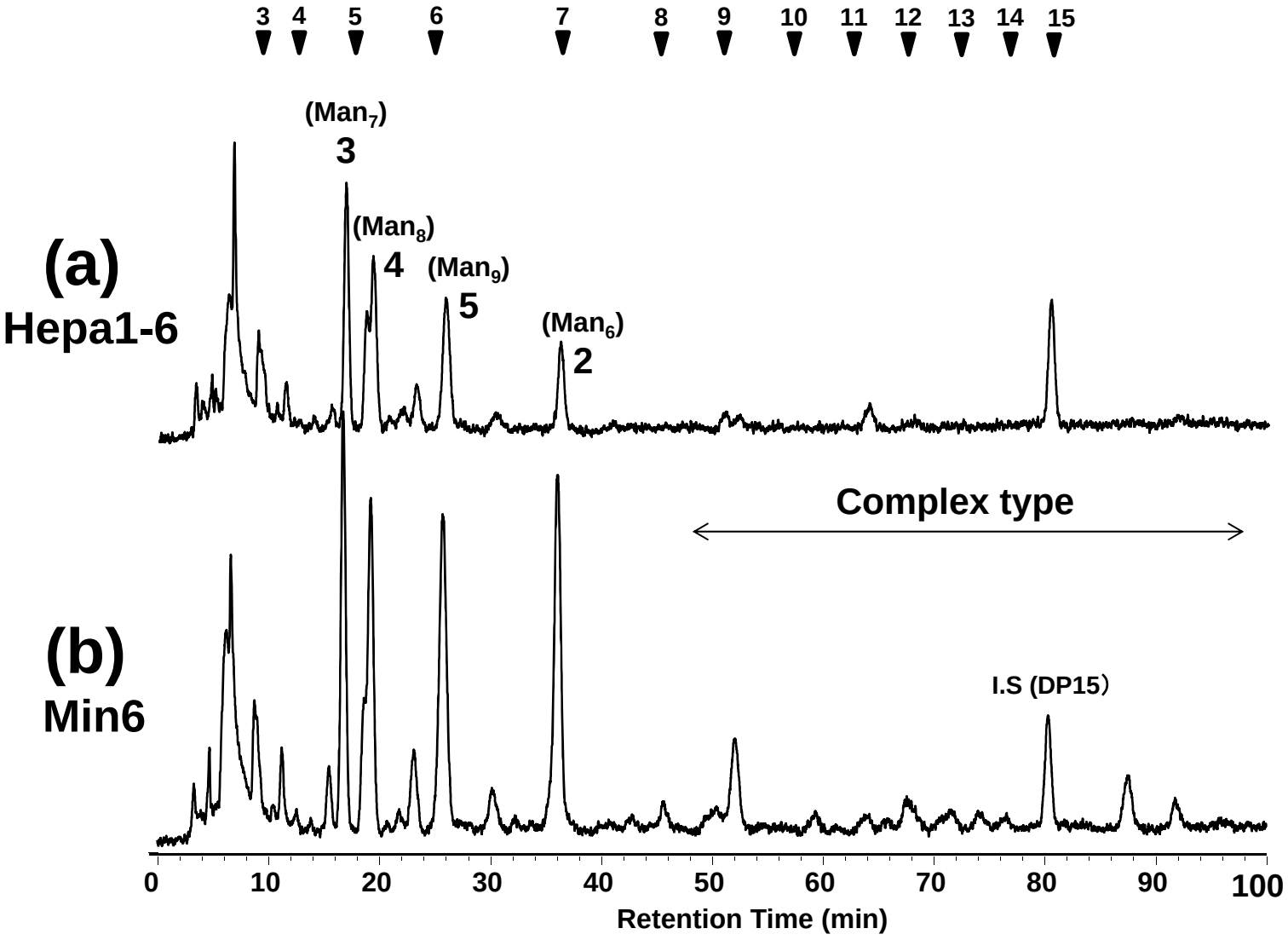
Supplemental Figure 6. Nakajima



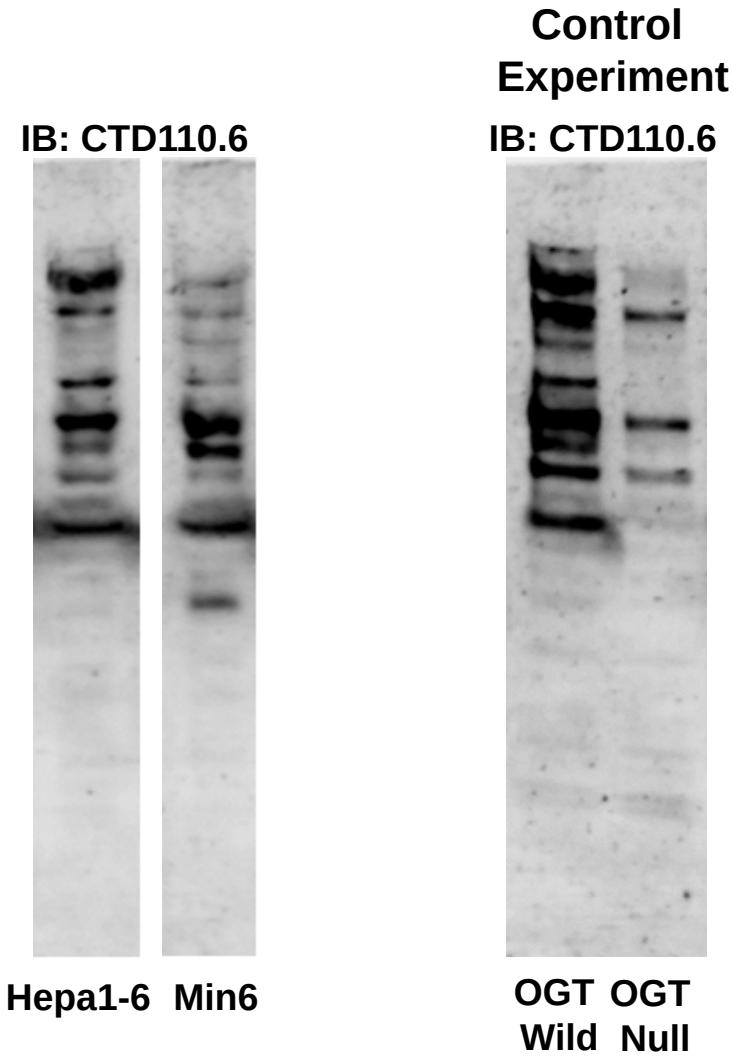
Supplemental Figure 7. Nakajima



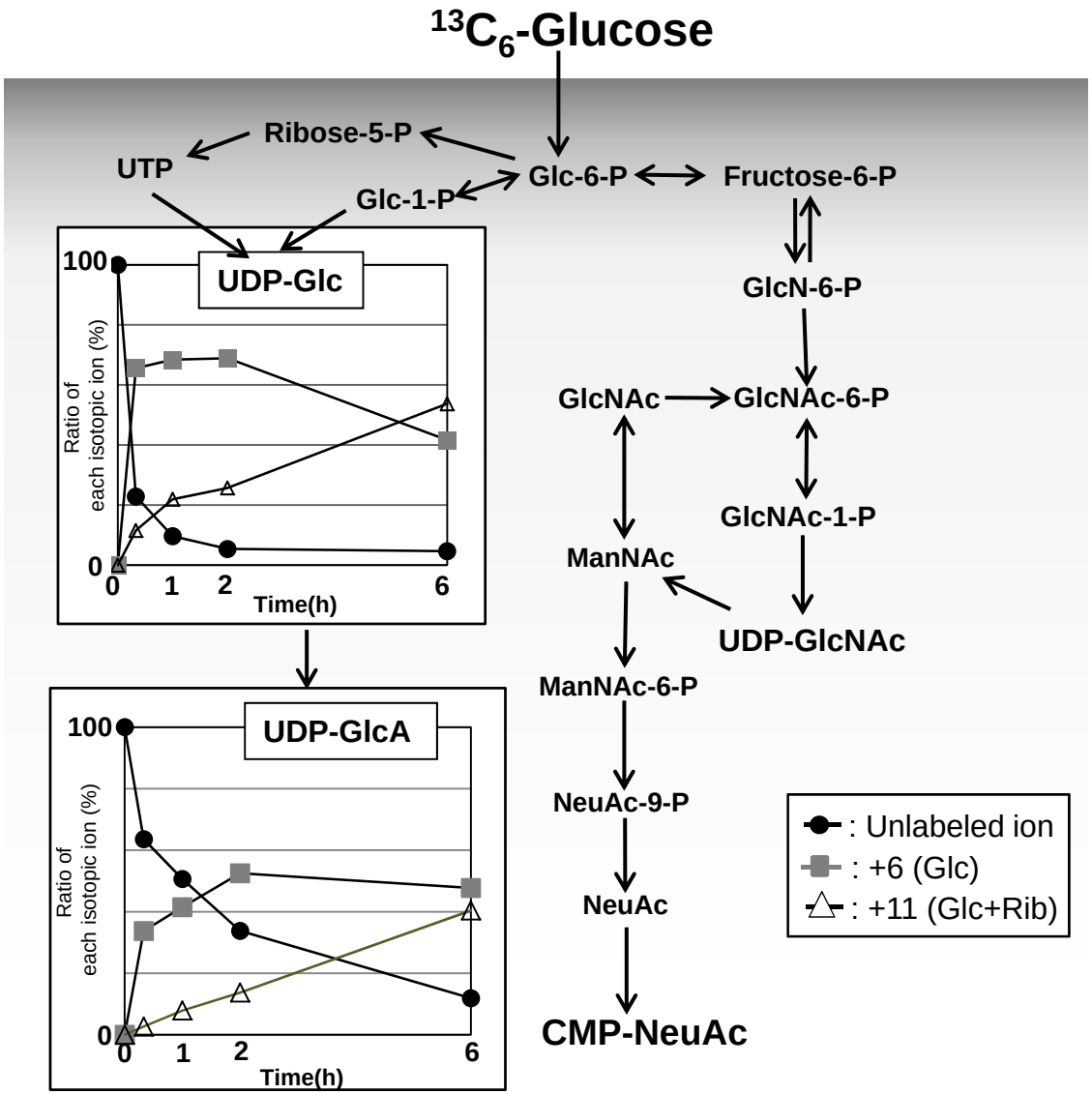
Supplemental Figure 8. Nakajima



Supplemental Figure 9. Nakajima

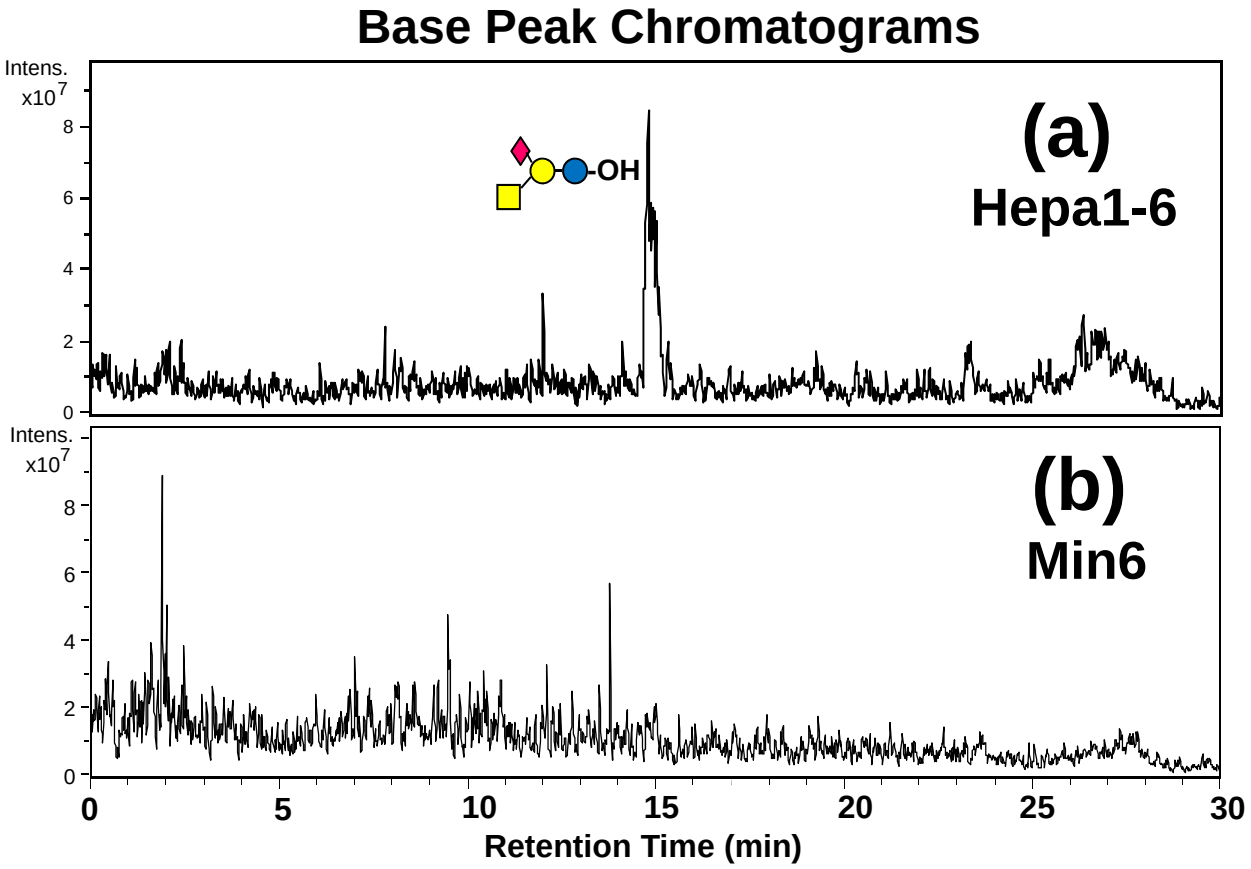


Supplemental Figure 10. Nakajima



Supplemental Figure 11. Nakajima

Glycans derived from glycosphingolipids



Supplemental Figure 12. Nakajima