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Supporting information

One filter, one sample and the whole glycome: towards a system to study disorders of protein glycosylation.

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This supporting information is provided to give more information on the experimental methods employed in this study and to include additional experimental data referred to in the primary manuscript.

1. Experimental details

Extraction, collection of glycoproteins from porcine bladder urothelial tissue

Bladders were obtained from an abattoir near York (A Traves & Son Ltd.) and processed the morning they were collected. Bladders were dissected using forceps and washed with Hank's balanced salt solution (HBSS), containing calcium and magnesium, supplemented with HEPES buffer solution 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (5 mL in 500 mL HBSS), 20 KIU aprotinin (1 mL in 500 mL), Fungizone (2 mL in 500 mL to give a final concentration of 1 μg/mL) and Pen-Strep (5 mL in 500 mL to give a final concentration of 100 μg/mL).

Urothelium was removed from dissected bladders by surface scraping using a scalpel (protocol validated by C-Y Wang (personal communication). The urothelial scrapings were then washed in HBSS medium and centrifuged. The medium was removed and the cell pellet was re-suspended in 750 μL HBSS medium. Aliquots of ~50 μL of cell suspensions were transferred into microfuge tubes (each aliquot around 50 mg), centrifuged and the supernatant was removed and samples were stored at -80 °C until needed.

Cell culture

hTERT mesenchymal stromal cell (MSC) culture

hTERT MSCs are immortalized cell lines generated from primary human mesenchymal stromal cells by lentiviral transduction with a human telomerase gene and subsequent single cell cloning[45]. Cells were cultured in Dulbecco's Modified Eagle Medium (high glucose, pyruvate, no glutamine) supplemented with 10 % fetal bovine serum, 1 % Penicillin/streptomycin and 1 % Gluta-Max-I. Cultures were grown in a humidified incubator at 37 °C in 5 % CO_2 in air in 10 cm Petri dishes and harvested at around 80 % confluency.

One-sample, one-pot protocol

MSC harvest.

A 10 cm dish of MSC cells, just under confluency, was washed gently with warm phosphate buffered saline (PBS) six times. Cells were then scraped with a cell scraper and with 1 mL PBS into a plastic microfuge tube. The tube was centrifuged at 16000 g, 5 min, 4 ˚C. The supernatant was discarded and the cells were ready for lysis.

Cell lysis/glycoprotein sample preparation. using an adapted enhanced filter-aided sample preparation (eFASP)[36] protocol

20 pmol of porcine stomach mucin (Sigma Aldrich, UK) was solubilised in 10 μL eFASP lysis buffer[36] (4 % SDS, 0.2 % deoxycholic acid (DCA), 50 mM Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), 100 mM ammonium bicarbonate, pH 8). (For urothelial and MSC samples, eFASP lysis buffer was added at 10 x volume of the cell pellet). Samples were heated at 90 °C for 10 min. The sample was vortexed to mix and cooled to 37 °C before adding the alkylation stock solution (500 mM 4-

vinylpyridine in ethanol) to give a final concentration of 25 mM 4-vinylpyridine. The sample was incubated at 37 °C, 15 min. Quench buffer (1 M dithiothreitol, 100 mM ammonium bicarbonate, pH 8) was added to give a final concentration of 40 mM DTT. The glycoprotein sample was diluted with exchange buffer (8 M urea, 100 mM ammonium bicarbonate, with or without 0.2 % DCA) solution in either 10:1 or 40:1 ratio of exchange buffer to sample solution by volume. An aliquot of up to 450 µL of the resulting solution was transferred to a filter unit (Amicon® Ultra-0.5 centrifugal filter unit, nominal mass cut off 30 kDa), centrifuged for 5 min, and the filtrate discarded. This was repeated until all the sample solution had passed through the filter membrane. The sample retained above the filter membrane was rinsed three times with 300 µL of exchange buffer (centrifuging after each rinse for 5 min), and the filtrate was discarded. The filter was then rinsed three times with 50 mM ammonium bicarbonate (centrifuging after each rinse for 5 min), and the filtrate was discarded. The filter unit was transferred to a clean collection tube ready for *N*- or O-glycan release.

β-elimination – using overnight incubation

300 µL NH_4OH solution (28-30%) (Sigma-Aldrich, UK) was added to the filter device containing the glycoprotein sample remaining in the filter unit after the solubilisation and washing steps. The glycoprotein sample was incubated in NH_4OH, at 45˚C for 16 h in a heating block. Following incubation, the filter device was centrifuged for 5 min at 14000 x *g*. 150 µL water was added to the filter device and then centrifuged for a further 5 min. This was repeated once. The filtrates containing the released O-glycans were all collected in the same tube and were transferred to a glass tube and dried using a vacuum centrifuge, ready for permethylation. The filter unit was transferred to a clean collection tube ready for *N*-glycan release.

β-elimination – using sonication

300 µL NH_4OH solution (28-30%) (Sigma-Aldrich, UK) was added to the filter device containing the glycoprotein sample remaining in the filter unit after the solubilisation and washing steps. The sample was sonicated at 45 ˚C for 5 min then left in the water bath at 45 ˚C for 10 min to avoid overheating, followed by another 5 min of sonication. The sonication/rest cycle was repeated giving a total sonication time of 20 min and total intervening rest time of 40 min. The filter device was centrifuged for 5 min at 14000 x *g*. 150 µL water was added to the filter device and then centrifuged for a further 5 min. This was repeated once. The filtrate was then transferred to a glass tube and dried using a vacuum centrifuge, ready for permethylation. The filter unit was transferred to a clean collection tube ready for *N*-glycan release.

β-elimination – using microwave irradiation

300 µL NH_4OH solution (28-30%) (Sigma-Aldrich, UK) was added to the filter device containing the glycoprotein sample remaining in the filter unit after the solubilisation and washing steps and gently pipetted to mix. The glycoprotein sample was transferred to a microwave reactor tube and irradiated with microwaves for 10 min, at 20 W, at 45 ˚C, in a CEM discover microwave reactor. Following microwave irradiation, the sample was transferred to a clean filter unit and centrifuged for 5 min at 14000 x *g*. 150 µL water was added to the filter device and then centrifuged for a further 5 min. This was repeated once. The filtrates containing the released O-glycans were all collected in the same tube

and were transferred to a glass tube and dried using a vacuum centrifuge, ready for permethylation. The filter unit was transferred to a clean collection tube ready for *N*-glycan release.

PNGase F treatment – *N*-glycan release

100 µL of 50 mM ammonium bicarbonate solution pH 8 was added to the filter unit, followed by 8 U (4 µL of a 2 U/µL solution in 5 mM potassium phosphate, pH 7.5) of PNGase F. The sample was incubated at 37 °C for 16 h in a heating block. Following incubation, the filter device was centrifuged for 5 min at 14000 x *g*. 150 µL water was added to the filter device and then centrifuged for a further 5 min. This was repeated once. The filtrates containing the released *N*-glycans were all collected in the same tube and transferred to a glass tube and dried using a vacuum centrifuge, ready for permethylation. The filter unit was transferred to a clean collection tube ready for tryptic digestion of the protein remaining above the filter membrane.

Note: *the one pot method can also be carried out by releasing N-glycans first.*

Tryptic digestion

The protein remaining in the filter unit following *N*- and O-glycan release was treated with trypsin at 37 °C for 16 h. Following incubation the filter was centrifuged to collect the tryptic peptides. Samples were acidified with 0.1 % TFA before LC-MS/MS analysis.

Permethylation

The dried released glycans were re-dissolved in approx. 1 mL DMSO (Sigma Aldrich, UK). Approximately 20 mg of NaOH powder was added, immediately followed by 150 µL, dropwise, of iodomethane (Sigma Aldrich, UK) and the solution was left to stand for 10 min. A further 150 µL of iodomethane was added dropwise and the solution was left to stand for 10 min. A final 300 µL of iodomethane was added dropwise to the sample solution and left to stand for 20 min. The reaction was quenched by addition of 1 mL of 100 mg/mL sodium thiosulfate solution, when 1 mL dichloromethane (DCM) was added and the sample solution was mixed using a vortex mixer. The solution was left to stand until the organic/aqueous layers had separated and then the (upper) aqueous layer was removed. The organic layer was washed with about 1 mL water a total of five times, finally removing the aqueous layer and drying the organic layer in a vacuum centrifuge.

The internal standard, maltotetraose, was deutero-permethylated using iodomethane-d3, following the same procedure outlined for permethylation. A stock solution of 400 pmol was prepared and the deutero-permethylated maltotetraose was spiked into the permethylated glycan samples to 4 pmol prior to spotting on the MALDI target plate.

Mass spectrometry/analysis details

MALDI-MS

The dried permethylated glycans were re-dissolved in 10 µL acetonitrile. The matrix was prepared by dissolving 2,5-dihydrobenzoic acid in 50 % aqueous acetonitrile, giving a 20 mg/mL solution. The sample spot was prepared by mixing 4 µL of the matrix solution with 2 µL sample solution. 2 µL of the mixture was transferred to a stainless steel MALDI target plate and dried under vacuum.

Mass spectra were acquired on a Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometer. A 9.4 T solariX FT mass spectrometer (Bruker Daltonics), with a smartbeam-II™ laser was operated in positive ion mode MALDI. The mass spectrometer was calibrated externally using a permethylated maltoheptaose standard. Mass spectra were recorded over an *m/z* range 400-4000, acquiring 12 scans, with each scan being derived from 500 laser shots. The laser power was set at 40 %. Spectra were acquired using ftmscontrol software (2.0) and processed with DataAnalysis 4.0.

Glycan analysis

Relative abundance of glycans was calculated by calculating each glycan MS signal as a percentage of the spiked internal standard signal. Statistical analysis was carried out using a one-way ANOVA with Holm Sidak post-hoc test (* for P = < 0.05, ** for P = < 0.01 and *** for P = < 0.0001).

Tandem mass spectrometry

Product ion spectra were acquired using a Nanospray Flex ion source with off-line borosilicate emitters on an Orbitrap Fusion hybrid mass spectrometer (Thermo Scientific). Positive ESI-MS and MS^2 product ion spectra were acquired in the Orbitrap using Xcalibur software (version 4.0, Thermo Scientific). Fragmentation was induced using either collision induced dissociation (CID) or higher energy collision dissociation (HCD). For CID, collision energy was varied between 25-35 %, and for HCD the collision energy was varied between 32-43 %, depending on the *m/z* of the glycans, and the quadrupole isolation window set to 2 *m/z* units.

Peptide analysis

Samples were loaded onto an UltiMate 3000 RSLCnano HPLC system (Thermo Scientific) equipped with a PepMap 100 Å C_{18}, 5 µm trap column (300 µm x 5 mm Thermo Scientific) and an Acclaim PepMap RSLC, 2 µm, 100 Å, C_{18} RSLC nanocapillary column (75 µm x 150 mm, Thermo Scientific). The trap wash solvent was 0.05 % (v/v) aqueous trifluoroacetic acid and the trapping flow rate was 15 µL/min. The trap was washed for 3 min before switching flow to the capillary column. The separation used a gradient elution of two solvents (solvent A: aqueous 2 % (v/v) acetonitrile containing 1 % (v/v) formic acid; solvent B: acetonitrile containing 1 % (v/v) formic acid). The flow rate for the capillary column was 300 nL/min. Column temperature was 50°C and the gradient profile was: linear 3-10 % B over 7 min, linear 10-35 % B over 30 min, linear 35-99 % B over 5 min then proceeded to wash with 99 % solvent B for 4 min. The column was returned to initial conditions and re-equilibrated for 15 min before subsequent injections.

The nanoLC system was interfaced with an Orbitrap Fusion hybrid mass spectrometer (Thermo) with a Nanospray Flex ionisation source (Thermo). Positive ESI-MS and MS^2 product ion spectra were acquired using Xcalibur software (version 4.0, Thermo). Instrument source settings were: ion spray voltage, 2,200 V; sweep gas, 2 Arb; ion transfer tube temperature; 275 °C. Mass spectra were acquired in the Orbitrap with: 120,000 resolution, scan range: *m/z* 375-1,500; AGC target, $4e^5$; max fill time, 100 ms; data type, profile. MS^2 product ion spectra were acquired in the linear ion trap specifying: quadrupole isolation, isolation window, *m/z* 1.6; activation type, HCD; collision energy, 32%; scan range, normal; scan rate, rapid; first mass, *m/z* 110; AGC target, 5e3; max injection time, 100 ms; data type, centroid. Data dependent acquisition was performed in top speed mode using a 3 s cycle. Dynamic exclusion was performed for 50 s post precursor selection and a minimum threshold for fragmentation was set at 5e3.

Protein identification

Peak lists were generated in MGF format using Mascot Distiller (version 5, Matrix Science), stipulating a minimum signal to noise ratio of 2 and correlation (Rho) of 0.6. MGF files were searched against the porcine subset of the UniProt database (34,316 sequences; 14,316,901 residues), with a decoy database, using a locally-running copy of the Mascot program (Matrix Science Ltd., version 2.5.1). Search criteria specified: enzyme, trypsin; fixed modifications, pyridylethyl (C); variable modifications, deamidated (NQ), oxidation (M), ser->diamino-propanoate (S), thr->Dab (T), Thr-> diamino-butyrate (T); peptide tolerance, +/- 5 ppm; MS/MS tolerance, 0.5 Da; Instrument, ESI-TRAP. Results were passed through Mascot percolator to achieve a false discovery rate of <1% and further filtered to accept only peptides with an expect score of 0.05 or lower.

2. Additional experimental data

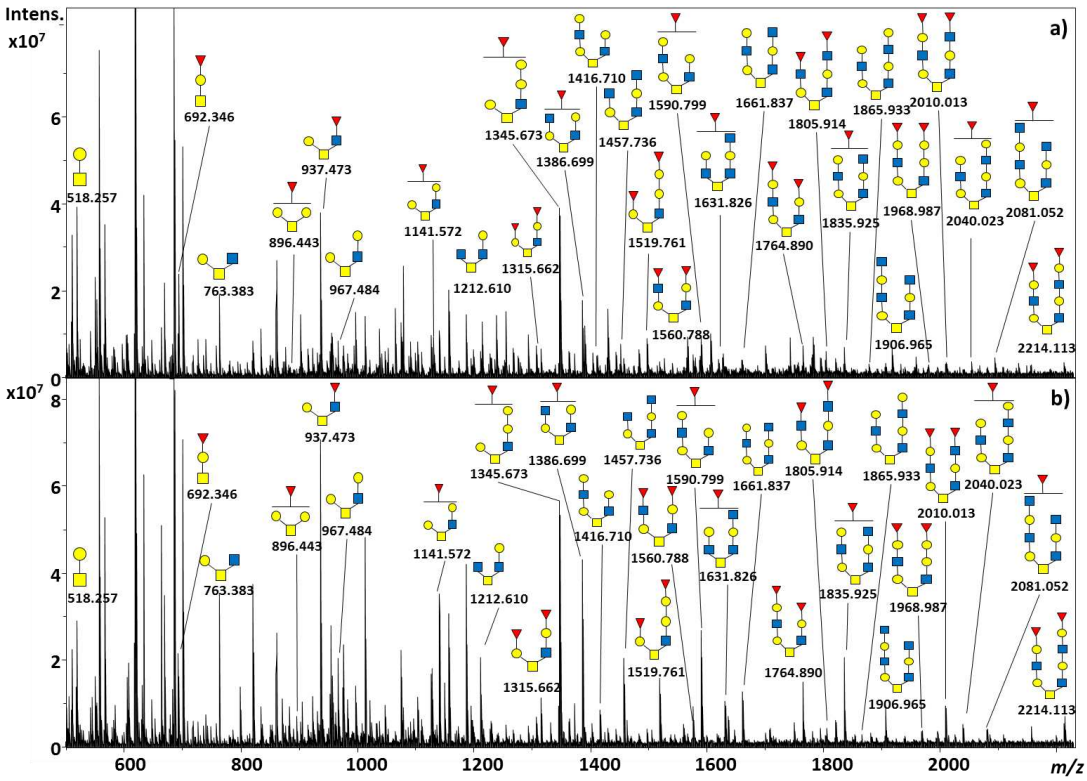

Figure S- 1. MALDI-FT-ICR spectrum of permethylated O-glycans, ionised as $[M + Na]^+$, released from porcine stomach mucin a) following FASP for SDS removal/exchange, b) following eFASP for SDS removal/exchange. Both followed by O-glycan release, in the filter using NH_4OH. Note glycans displayed are the composition only. Other isomers are possible.

Table S- 1: Mass accuracies for porcine stomach mucin O-glycans

Observed *m/z*	Calculated *m/z*	Mass accuracy (ppm)	O-glycan composition
518.257	518.2572	0.39	$Hex_1HexNAc_1$
692.346	692.3464	0.58	$Hex_1HexNAc_1Fuc_1$
763.383	763.3835	0.65	$Hex_1HexNAc_2$
896.443	896.4462	3.57	$Hex_2HexNAc_1Fuc_1$
937.473	937.4727	-0.32	$Hex_1HexNAc_2Fuc_1$
967.484	967.4833	-0.72	$Hex_2HexNAc_2$
1141.572	1141.5725	0.44	$Hex_2HexNAc_2Fuc_1$
1212.610	1212.6096	-0.33	$Hex_2HexNAc_3$
1315.662	1315.6617	-0.23	$Hex_2HexNAc_2Fuc_2$
1345.673	1345.6723	-0.52	$Hex_3HexNAc_2Fuc_1$
1386.699	1386.6988	-0.14	$Hex_2HexNAc_3Fuc_1$
1416.710	1416.7094	-0.42	$Hex_3HexNAc_3$
1457.736	1457.7359	-0.07	$Hex_2HexNAc_4$
1519.761	1519.7615	0.33	$Hex_3Hex\ NAc_2Fuc_2$
1560.788	1560.7880	0.00	$Hex_3Hex\ NAc_3Fuc_1$
1590.799	1590.7986	-0.25	$Hex_2Hex\ NAc_4Fuc_1$
1631.826	1631.8251	-0.55	$Hex_3Hex\ NAc_4$
1661.837	1661.8357	-0.78	$Hex_3HexNAc_3Fuc_2$
1764.890	1764.8878	-1.25	$Hex_3HexNAc_4Fuc_1$
1835.925	1835.9249	-0.05	$Hex_3HexNAc_3Fuc_1$
1865.933	1865.9355	1.34	$Hex_4HexNAc_4$
1906.965	1906.9620	-1.57	$Hex_3HexNAc_5$
1968.987	1968.9876	0.30	$Hex_4HexNAc_3Fuc_2$
2010.015	2010.0141	-0.45	$Hex_3HexNAc_4Fuc_2$
2040.023	2040.0247	0.83	$Hex_4HexNAc_4Fuc_1$
2081.052	2081.0512	-0.38	$Hex_3HexNAc_5Fuc_1$
2214.113	2214.1139	0.41	$Hex_4HexNAc_4Fuc_2$

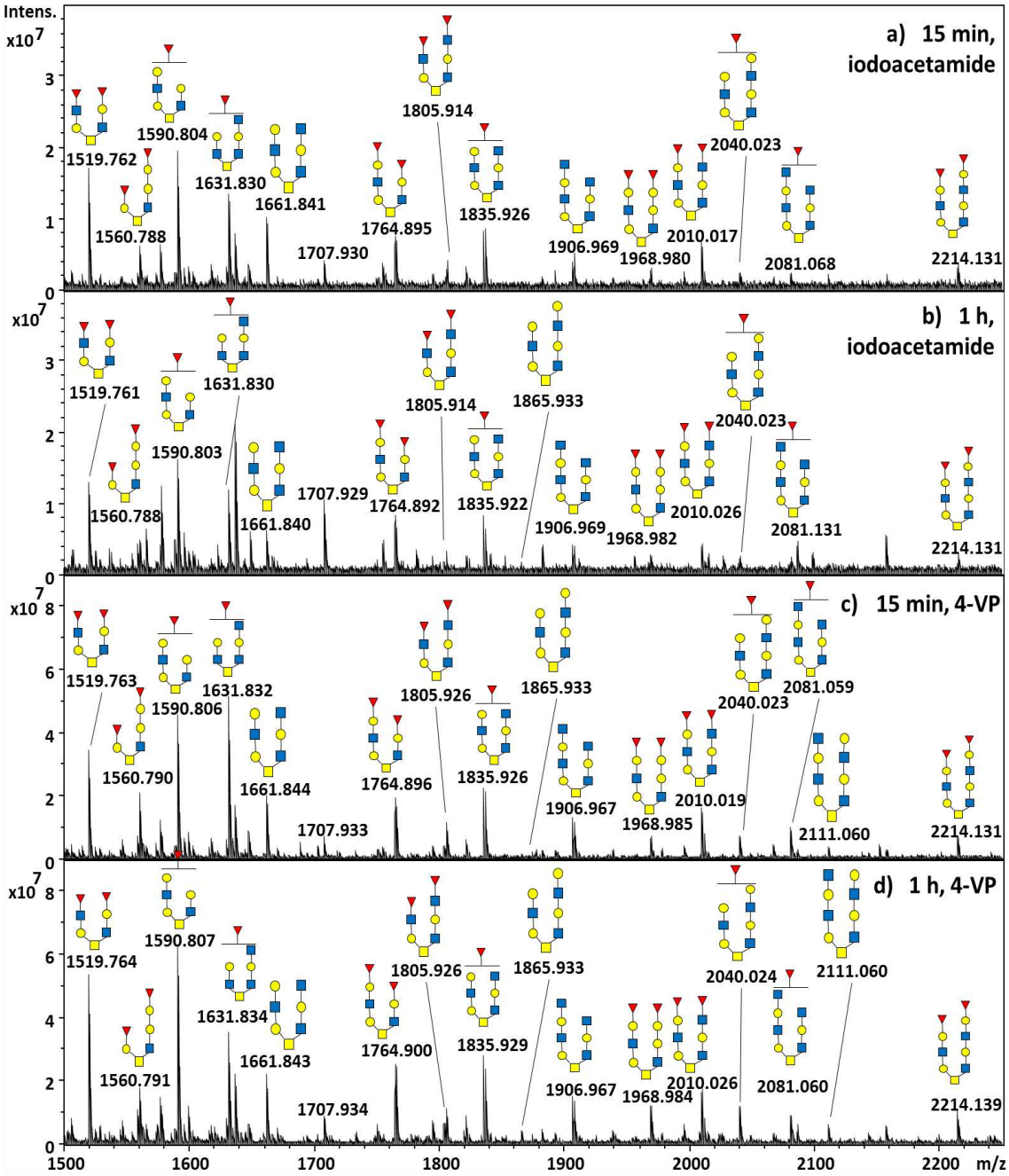

Figure S- 2. MALDI-FT-ICR spectrum (*m/z* 1200-2220) of permethylated O-glycans, ionised as $[M + Na]^+$, released from mucin. a) alkylation using 4-vinylpyridine for 1 h, b) alkylation using iodoacetamide for 1 h, c) alkylation using 4-vinylpyridine for 15 min, d) alkylation using iodoacetamide for 15 min. Glycans displayed illustrate composition only. Other isomers are possible.

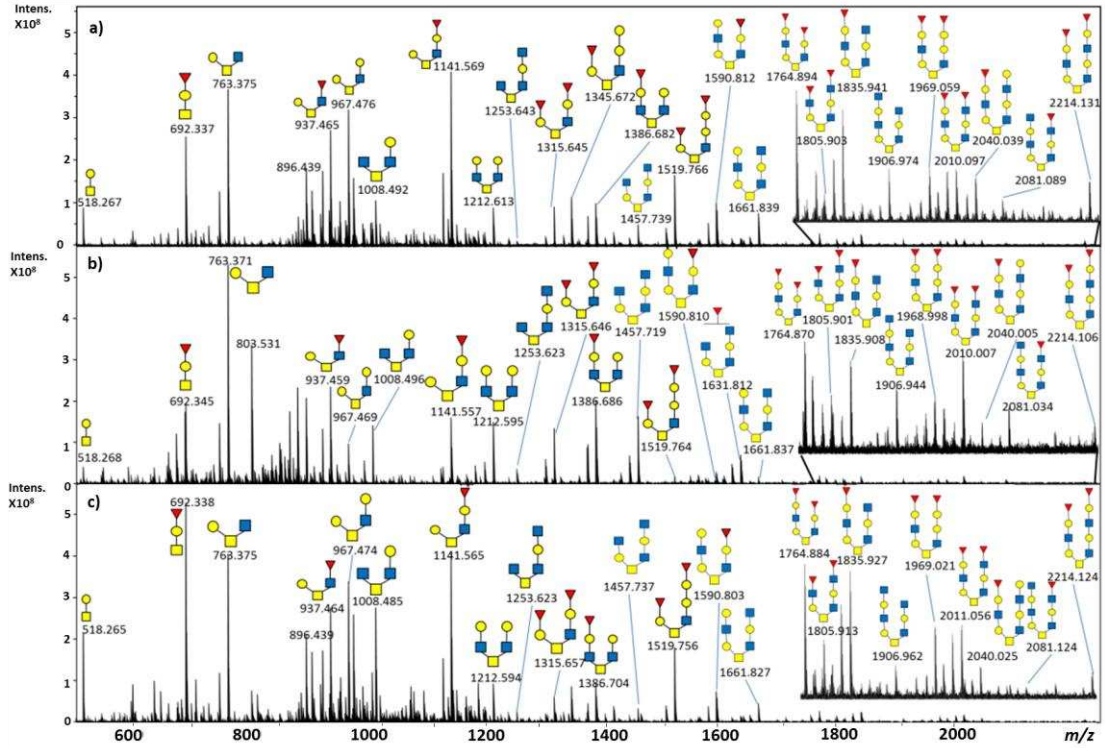

Figure S- 3. MALDI-FT-ICR mass spectrum of permethylated O-glycans released from porcine stomach mucin, ionised as $[M + Na]^+$, using 16 h incubation (top panel), 5 min sonication/10 min rest (x4) (middle panel) and 10 min microwave irradiation (bottom panel). Glycans displayed illustrate the composition only. Other isomers will be possible.

Table S- 2: Mass accuracies of O-glycans identified in porcine urothelial cells.

Observed *m/z*	Calculated *m/z*	Mass accuracy (ppm)	O-glycan composition
518.257	518.2572	0.39	$Hex_1HexNAc_1$
692.346	692.3464	0.58	$Hex_1HexNAc_1Fuc_1$
722.357	722.3569	-0.14	$Hex_2HexNAc_1$
879.430	879.4304	0.45	$NeuAc_1Hex_1HexNAc_1$
896.443	896.4462	3.57	$Hex_2HexNAc_1Fuc_1$
937.476	937.4727	3.77	$Hex_1HexNAc_2Fuc_1$
967.483	967.4833	0.31	$Hex_2HexNAc_2$
1083.530	1083.5306	0.55	$NeuAc_1Hex_2HexNAc_1$
1124.556	1124.5572	1.07	$NeuAc_1Hex_1HexNAc_1$
1141.571	1141.5725	1.31	$Gal_2GalNAc_2Fuc_1$
1154.657	1154.6577	0.61	$NeuGc_1Hex_1HexNAc_2$
1171.582	1171.5831	0.94	$Hex_3HexNAc_2$
1328.656	1328.6569	0.68	$NeuAc_1Hex_2HexNAc_2$
1358.668	1358.6675	-0.37	$NeuGc_1Hex_2HexNAc_2$
1485.733	1485.7308	-1.48	$NeuAc_2Hex_1HexNAc_2$
1573.784	1573.7833	-0.44	$NeuAc_1Hex_2HexNAc_3$
1777.883	1777.8830	0.00	$NeuAc_1Hex_3HexNAc_3$

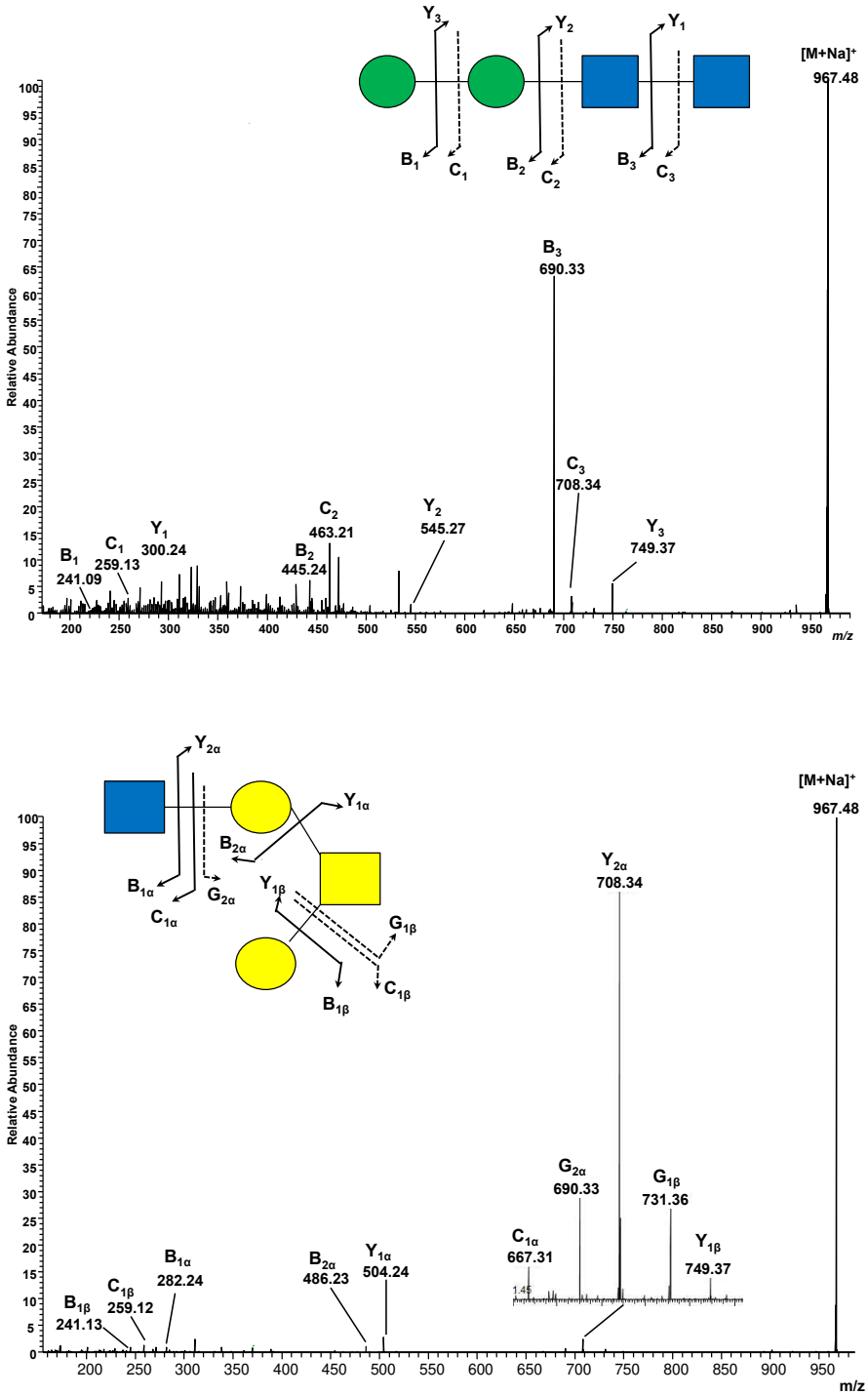

Figure S- 4. HCD spectra of precursor *m/z* 967, [M + Na]⁺ from porcine urothelial *N*-glycans (top) and O-glycans (bottom).

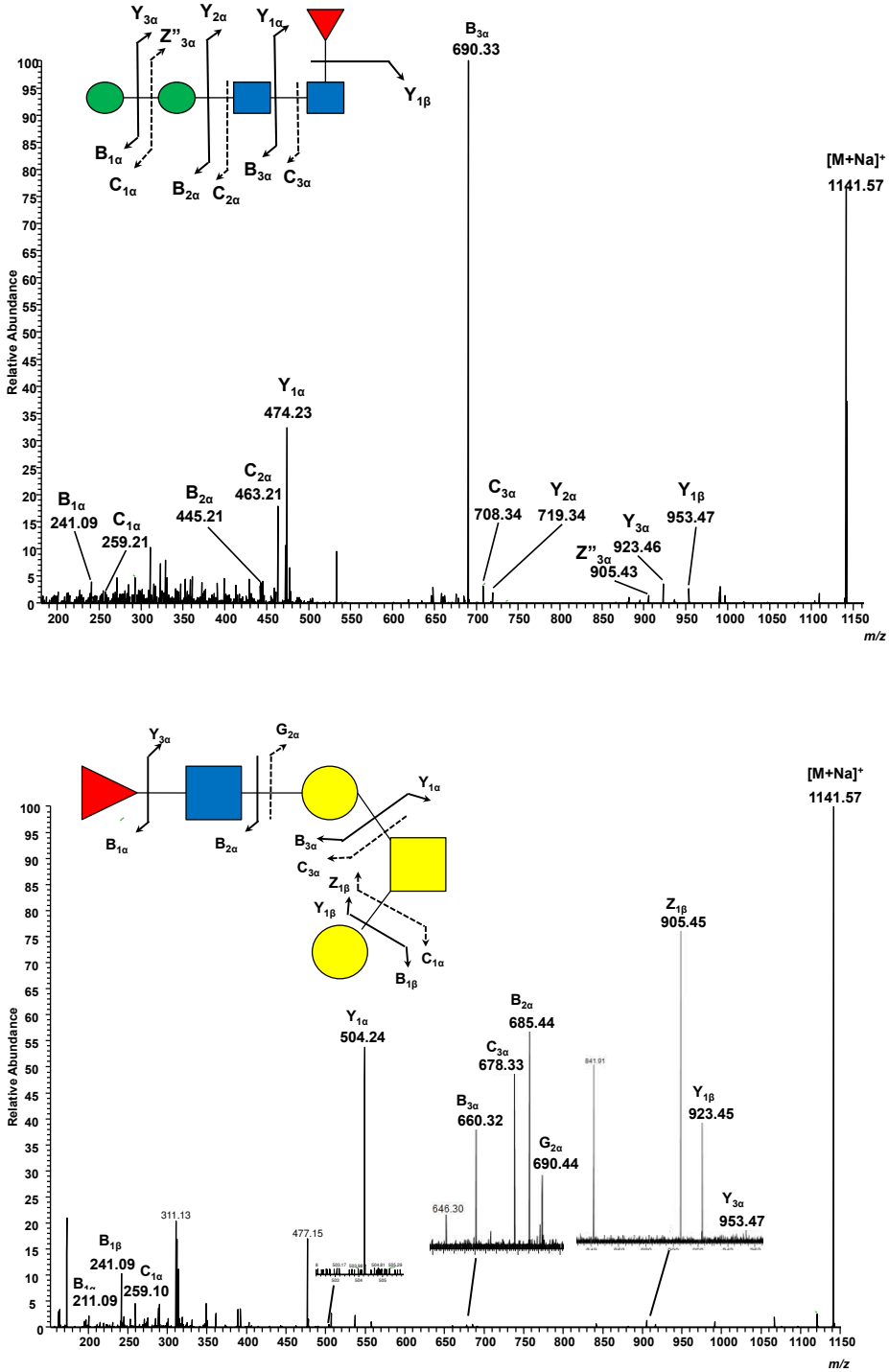

Figure S- 5. HCD spectra of precursor *m/z* 1141, [M + Na]⁺ from porcine urothelial *N*-glycans (top) and O-glycans (bottom).

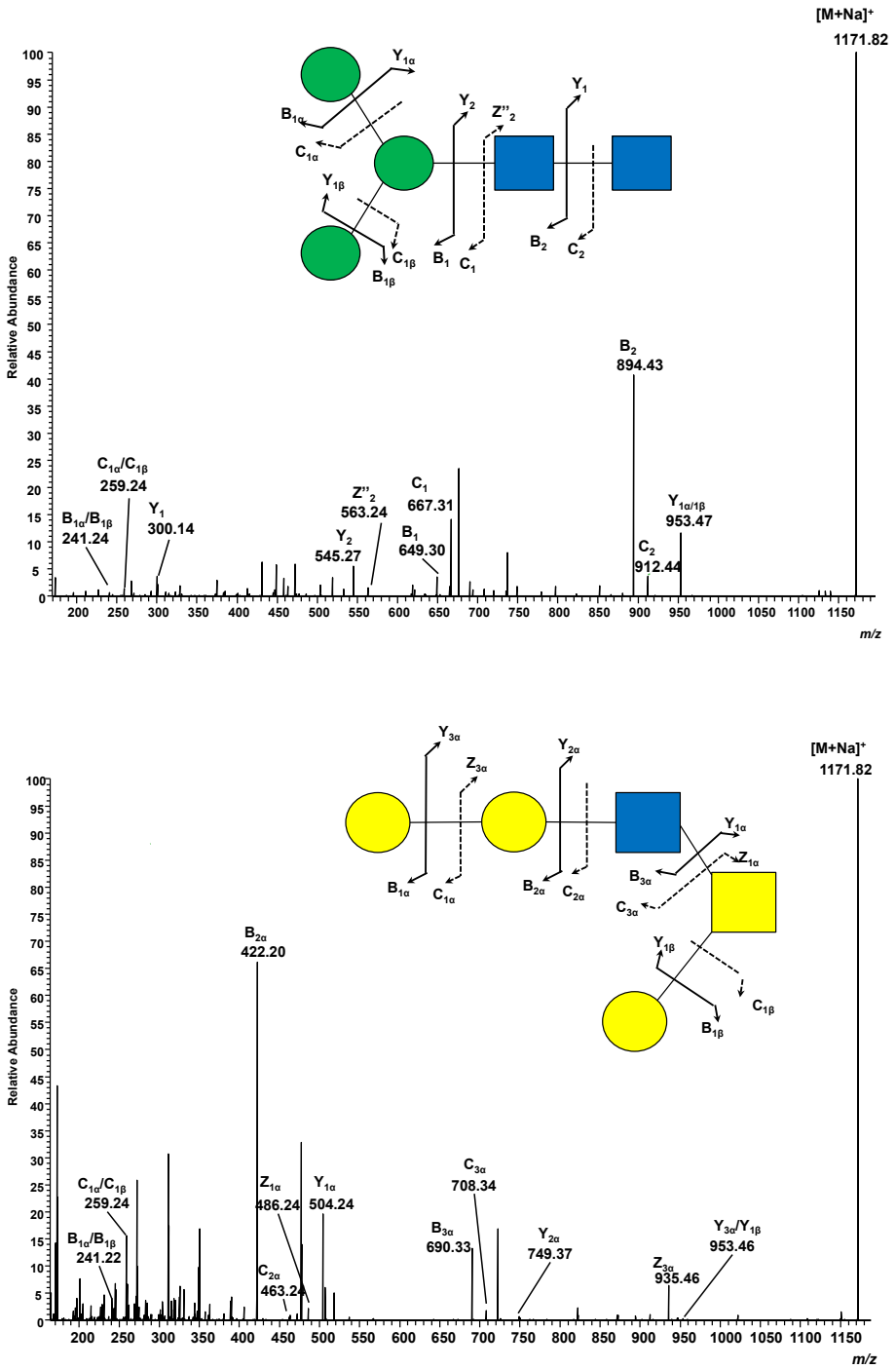

Figure S- 6. HCD spectra of precursor *m/z* 1171, [M + Na]$^+$ from porcine urothelial *N*-glycans (top) and O-glycans (bottom).

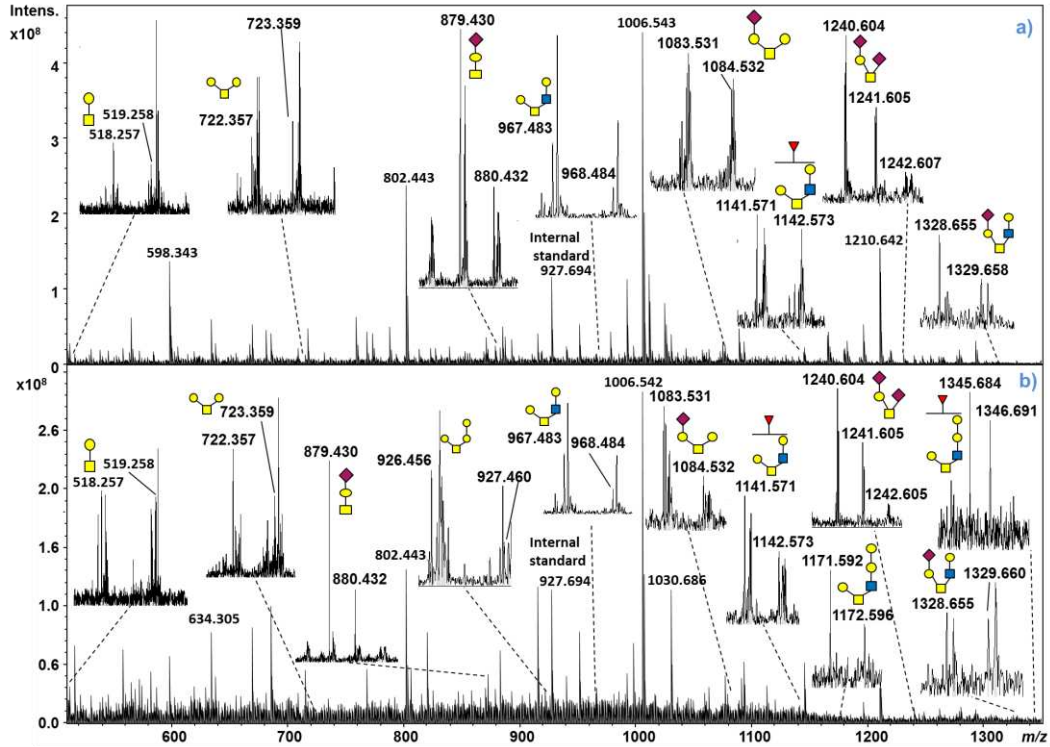

Figure S- 7. MALDI-FT-ICR mass spectrum of permethylation O-glycans, released from a) Y101 and b) Y201 MSC cell lines. All glycans ionised as $[M + Na]^+$.

Table S- 3. List of O-glycans identified from Y201 and Y101 MSC cell lines, showing mass accuracy.

glycan composition	calculated *m/z*	Y201 WT average measured *m/z* (n=5)	Y201 WT mass accuracy (ppm)	Y101 WT average measured *m/z* (n=5)	Y101 WT mass accuracy (ppm)
$Hex_1HexNAc_1$	518.2572	518.2571	-0.12	518.2570	-0.41
$Hex_2HexNAc_1$	722.3569	722.3568	-0.19	722.3567	-0.32
$Hex_1HexNAc_1NeuAc_1$	879.4304	879.4301	-0.40	879.4304	0.05
$Hex_3HexNAc_1$	926.4567	926.4559	-0.87	-	-
$Hex_2HexNAc_2$	967.4833	967.4826	-0.72	967.4826	-0.75
$Hex_2HexNAc_1NeuAc_1$	1083.5306	1083.5299	-0.63	1083.5307	0.12
$Hex_2HexNAc_2Fuc_1$	1141.5725	1141.5714	-0.95	1141.5744	1.64
$Hex_3HexNAc_2$	1171.5831	-	-	1171.5830	-0.12
$Hex_1HexNAc_1NeuAc_2$	1240.6045	1240.6034	-0.88	1240.6032	-1.04
$Hex_2HexNAc_2NeuAc_1$	1328.6569	1328.6535	-2.53	1328.6561	-0.59
$Hex_3HexNAc_2Fuc_1$	1345.6723	1345.6726	-0.22	-	-

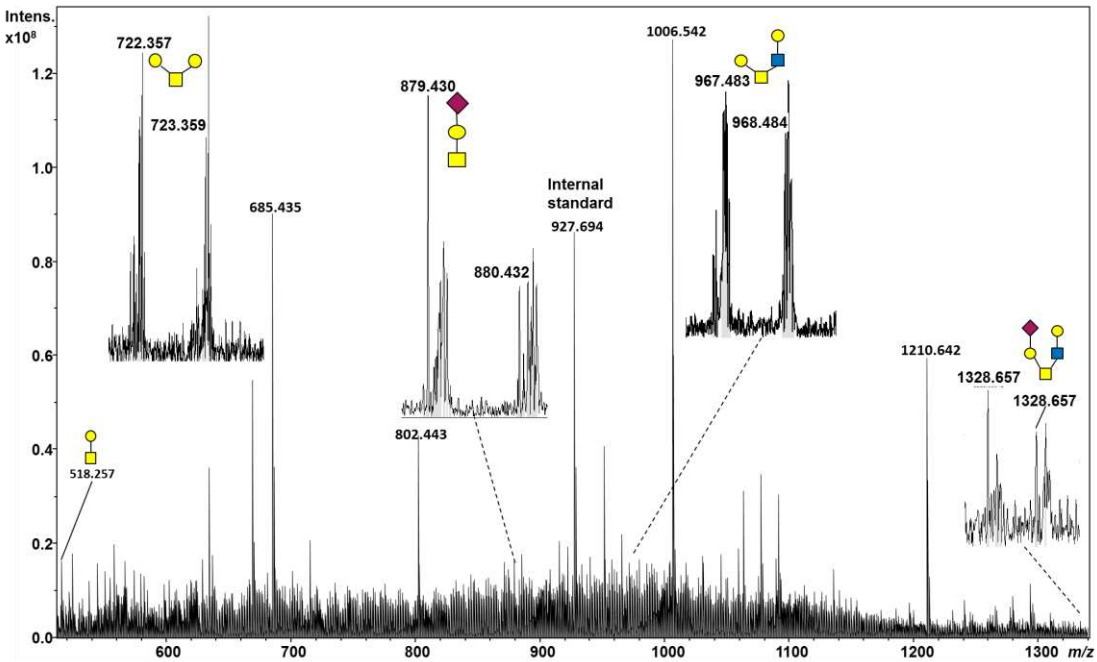

Figure S-8. MALDI-FT-ICR mass spectrum of permethylated O-glycans, released from Y101 Cog4KD MSCs. All glycans ionised as $[M + Na]^+$.

Table S- 4. List of O-glycans identified in Y101 Cog4KD MSC cell line, along with their mass accuracies.

glycan composition	calculated *m/z*	Y101 Cog4KD (av. measured *m/z*, n = 5)	Y101 Cog4KD mass accuracy (ppm)
$Hex_1HexNAc_1$	518.2572	518.2571	-0.28
$Hex_2HexNAc_1$	722.3569	722.3568	-0.15
$Hex_1HexNAc_1NeuAc_1$	879.4304	879.4303	-0.11
$Hex_2HexNAc_2$	967.4833	967.4827	-0.58
$Hex_2HexNAc_2Fuc_1$	1141.5725	-	-
$Hex_3HexNAc_2$	1171.5831	-	-
$Hex_2HexNAc_2NeuAc_1$	1328.6569	1328.6559	-0.76

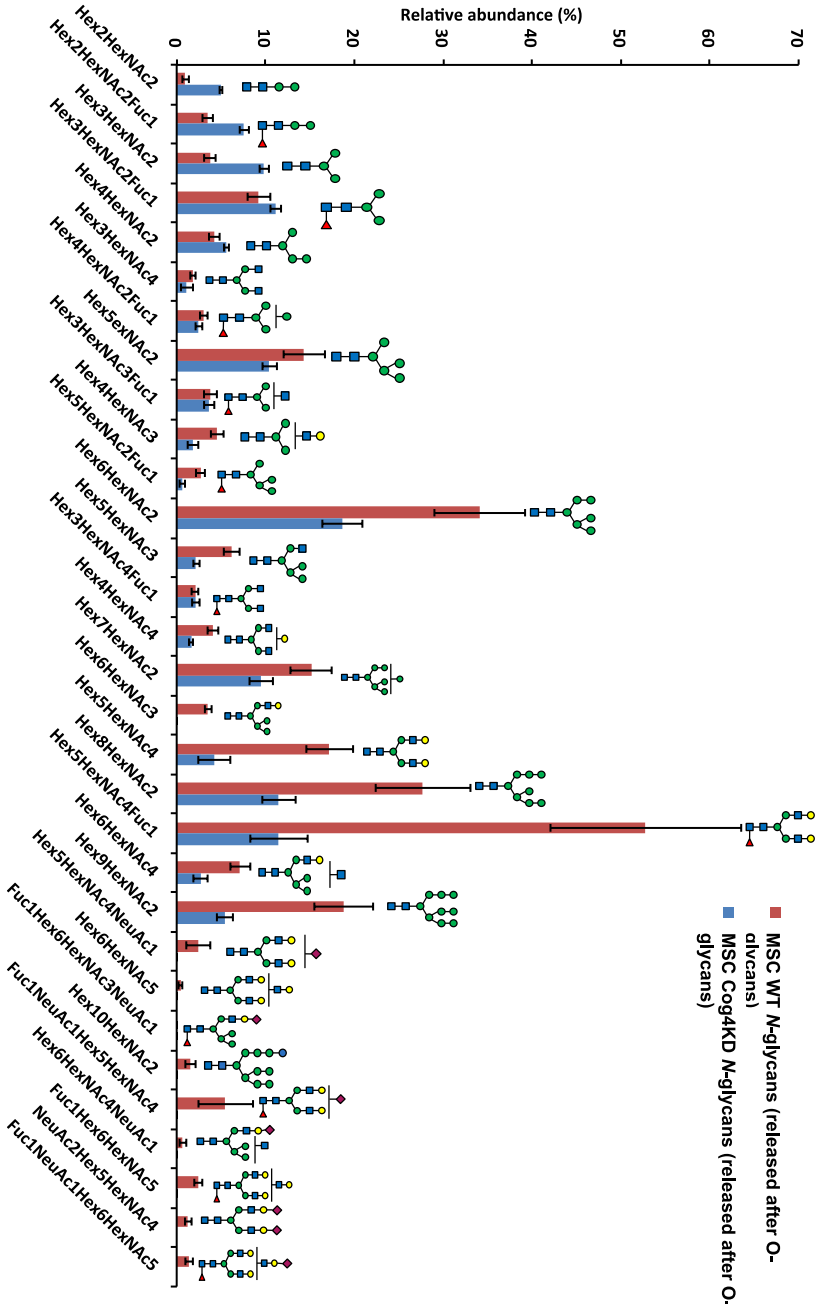

Figure S-9. Comparison of *N*-glycan profiles of the Y101 wild-type MSCs with Cog4KD MSCs. Relative abundance calculated by calculating each glycan signal as a percentage of the spiked internal standard signal.