Glycoconjugate Journal

Supplementary material for

Comparison of analytical methods for profiling *N*- and *O*-linked glycans from cultured cell lines −HUPO Human Disease Glycomics/Proteome Initiative Multi-institutional Study—

Hiromi Ito, Hiroyuki Kaji, Akira Togayachi, Parastoo Azadi, Mayumi Ishihara, Rudolf Geyer, Christina Galuska, Hildegard Geyer, Kazuaki Kakehi, Mitsuhiro Kinoshita, Niclas G. Karlsson, Chunsheng Jin, Koichi Kato, Hirokazu Yagi, Sachiko Kondo, Nana Kawasaki, Noritaka Hashii, Daniel Kolarich, Kathrin Stavenhagen, Nicolle H. Packer, Morten Thaysen-Andersen, Miyako Nakano, Naoyuki Taniguchi, Ayako Kurimoto, Yoshinao Wada, Michiko Tajiri, Pengyuan Yang, Weiqian Cao, Hong Li, Pauline M. Rudd*, Hisashi Narimatsu.

* to whom correspondence should be addressed:

Pauline M. Rudd (pauline.rudd@nibrt.ie.)

National Institute for Bioprocessing Research and Training (NIBRT) Dublin Ireland

Supplementary Method 1

***N*- and *O*-glycan analyses in lyophilized cell pellets by each laboratory**

Laboratory A

Technology from Lab A is based on the GALAXY-based HPLC profiling using 2-aminopyridine (PA)-derivatized glycans (http://www.glycoanalysis.info/galaxy2/index.jsp). This enables quantitative evaluation of the incidence of each oligosaccharide on the basis of the fluorescence intensity of PA-glycans. The elution time expressed in real time or volume can vary depending on the individual column, its age, or the batches of buffers used. In our method, elution time is normalized as the glucose unit (GU), which is daily calibrated with a PA-isomalto-oligosaccharides mixture in order to reduce any variations. Moreover, we use an extensive array of PA-standard oligosaccharides for co-injection into the column with the sample oligosaccharide in order to confirm the identification by observing a single peak. All these controls ensure the robustness and reproducibility of our technologies.

N-glycans

Experimental procedures, including chromatographic conditions and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), were as described previously [1–4].

Cell lysates were proteolyzed with chymotrypsin-trypsin mixture and further digested with glycoamidase A to release *N*-glycans. After removal of the peptides, the reducing ends of the *N*-glycans were derivatized with 2-aminopyridine (PA). The PA derivatives of the *N*-linked oligosaccharides were separated by HPLC on a TSK-gel diethylaminoethyl (DEAE)-5PW column (Tosho) according to their sialic acid content. Each fraction separated from the DEAE column was collected, evaporated, and then applied to a Shim-pack HRC-octadecyl silica (ODS) column. The fractions separated on the ODS column were subjected to MALDI-TOF/MS analysis and the fractions possibly including two or more *N*-glycans were further separated using an amide column (Tosho). Identification of *N*-glycan structures was based on their elution positions on the column and their molecular mass values compared with those of PA-glycans in the GALAXY database (http://www.glycoanalysis.info/galaxy2/ENG/index.jsp) [2].

O-glycans

The experimental procedure used for structural analysis including hydrazinolysis, fluorescence-tagging, and chromatographic conditions have been described previously [5], with slight modification of the separation condition of the anion exchange column.

O-glycans were released from delipidated cell lysates by hydrazinolysis and then labeled with PA. The

PA-glycan mixture was firstly separated on a DEAE-5PW column (7.5 mm i.d.×75 mm; Tosoh, Tokyo, Japan) at 30°C with a flow rate of 1.0 mL/min using two solvents, A and B. Solvent A was aqueous ammonia (pH 9.0) and solvent B was a 50 mM ammonium acetate solution (pH 9.0). The column was equilibrated with solvent A. The gradient elution parameters were 0–3 min, linear gradient 0%–12% B; 3–17 min, linear gradient 12%–40% B; 17–22 min, linear gradient 40%–100% B. Each oligosaccharide was separated according to its anionic charges.

Next, each fraction separated from the DEAE column was collected, evaporated, and then applied to a Decelosil C30-HG-5 (C30) column (Nomura Chemical Co., Ltd.). The HPLC elution times were represented by glucose units (GUs) on the columns calibrated with a PA-derivatized isomalto-oligosaccharides mixture. The structures of the PA-saccharides were characterized by HPLC mapping [5] and MALDI-TOF/MS analysis using a MALDI-TOF/MS spectrometer (AXIMA-CFR; Shimadzu or ABI 4700 Proteomics analyzer; ABI).

Laboratory B

N-glycans

Lyophilized material was suspended in 2 M thiourea, 5 M urea (267 mL), 1 M DTT (16.7 mL), benzonase (125 units), and then incubate at room temperature for 30 min. After centrifugation at 15000 rpm for 10 min, the supernatant was mixed with cold acetone (1.5 mL), and then kept at −20°C for 30 min. After centrifugation at 15000 rpm for 10 min, the pellet was washed with 75% ethanol (1 mL), and then lyophilized to dryness. The lyophilized material was suspended in water/10% SDS/2-ME (210:24:2.4, 250 mL), and then the mixture was kept in a boiling water bath for 5 min. After cooling, 10% NP-40 (25 mL) and 1 M phosphate buffer pH 7.5 (25 mL) were added, after addition of N-Glycanase F (2 unit, 4 mL), the mixture was kept at 37°C overnight. After keeping the mixture in the boiling water bath for 5 min, the mixture was centrifuged at 12000*g* for 15 min. The supernatant was collected and lyophilized to dryness.

The mixture of the released glycans was dissolved in 2-AA (200 mL) solution which was prepared by dissolution of 2-AA (30 mg) and sodium cyanoborohydride (30 mg) in methanol (1 mL) containing 4% sodium acetate and 2% boric acid. The mixture was kept at 80°C for 60 min. After addition of distilled water (200 mL), the mixture was applied to a column of Sephadex LH-20 (1.0 cm i.d., 30 cm length) equilibrated with 50% aqueous MeOH. The earlier eluted fluorescent fractions were collected and evaporated to dryness.

O-glycans

Lyophilized material was digested with pronase (50 mg) in 50 mM Tris-HCl (pH 8.0, 1 mL) at 37°C for 24 hours. The reaction mixture was kept in a boiling water bath for 10 min, the supernatant was collected after centrifugation. The supernatant was passed through an ultrafiltration membrane (MW 5000 cutoff, Ultrafree-MC,

Millipore) at 10000*g*. The mixture of glycopeptides on the membrane was recovered by dissolving in distilled water. The releasing reaction of *O*-glycans was performed with the automatic glycan releasing system according to the method reported previously. An aqueous solution of 0.5 M LiOH was used as the releasing reagent. To the flow of the eluent at 1.0 mL/min, an aqueous solution of the glycopeptides obtained as described above was injected. After the sample solution was mixed with the 0.5 M LiOH in the device. The mixture was moved to the reactor maintained at 60°C, in which a reaction tube (0.25 mm i.d., 10 m length: 700 mL volume) was set. The eluate containing the reaction mixture from the reactor was immediately passed through a cation exchange cartridge (0.7 mL volume), and collected to a fraction collector. The collected solution containing the released *O*-glycans was evaporated to dryness by a centrifugal evaporator.

Fluorescent labeling of N- and O-glycans

The mixture of the released glycans was dissolved in 2-AA (200 mL) solution which was prepared by dissolution of 2-AA (30 mg) and sodium cyanoborohydride (30 mg) in methanol (1 mL) containing 4% sodium acetate and 2% boric acid. The mixture was kept at 80°C for 60 min. After addition of distilled water (200 mL), the mixture was applied to a column of Sephadex LH-20 (1.0 cm i.d., 30 cm length) equilibrated with 50% aqueous MeOH. The earlier eluted fluorescent fractions were collected and evaporated to dryness.

Serotonin-affinity chromatography for group separation of N-glycans based on the number of sialic acid residues.

HPLC was performed with a Jasco apparatus equipped with two PU-980 pumps and a Jasco FP-920 fluorescence detector. The *N*-glycan pool obtained from cancer cells as described above was separated based on the number of sialic acid residues using a serotonin-immobilized column (4.6 × 150 mm) with a linear gradient from water (solvent A) to 40 mM ammonium acetate (solvent B) at a flow rate of 0.5 mL/min. Initially, solvent B was used at 5% concentration as eluent for 2 min, and then linear gradient elution was performed to 30 mM ammonium acetate for 35 min, and finally the eluent was changed to solvent B (40 mM ammonium acetate) during the following 10 min. Peaks were collected and lyophilized to dryness.

After separation of *N*-glycan pool based on the number of sialic acid residues as described above, oligosaccharide fractions containing sialic acids were digested with neuraminidase. Neuraminidase (2 units, 4 mL) was added to the mixture of sialo-N-glycans in 20 mM acetate buffer (pH 5.0, 20 mL), and the mixture was incubated at 37°C overnight. After keeping the mixture in a boiling water bath for 3 min followed by centrifugation, a portion of the supernatant was used for MALDI-QIT-TOF/MS analysis.

Analysis of O-glycans on a polymer-based amino column

The apparatus was the same as described for the analysis of 2-AA labeled *O*-glycans by serotonin affinity chromatography. Separation was done with a polymer-based Asahi Shodex NH2P-50 4E column (Showa Denko, Tokyo, Japan: 4.6 mm i.d., 250 mm length) using a linear gradient formed by 2% acetic acid in acetonitrile (solvent A) and 5% acetic acid in water containing 3% triethylamine (solvent B). The column was initially equilibrated and eluted with 70% solvent A for 2 min, then solvent B was increased to 95% over 80 min and kept at this composition for further 100 min.

MS analysis of O-glycans

The analyses of the *N*- and/or *O*-glycans were performed by a MALDI-QIT-TOF/MS (AXIMA-QIT, Shimadzu, Kyoto, Japan) in negative or positive ion linear mode. Acquisition and data processing were controlled by Launchpad software (Kratos Analytical, Manchester, U.K.). For sample preparation, a 0.5-μL volume of the matrix solution (DHB; 10 mg/mL in 30% ethanol/0.1% trifluoroacetic acid) was deposited on the stainless steel target plate and allowed to dry. Then, a portion (0.5 mL) of the appropriately diluted analyte solution (typically ca. 1 pmol/mL) was used to cover the matrix on the target plate and allowed to dry.

Laboratory C

Reduction and carboxamidomethylation [6]

Each sample was sonicated in extraction buffer (25 mM Tris, 150 mM NaCl, 5 mM EDTA and 1% CHAPS, pH 7.4) and then dialyzed against 50 mM ammonium bicarbonate, pH 8.5, at 4°C for 24 h. After dialysis, the sample was reduced in 50 mM ammonium bicarbonate buffer, containing 10 mg dithiothreitol, at 56°C for 1 h. Carboxamidomethylation was carried out by the addition of iodoacetic acid (20 mg), and the reaction was allowed at room temperature in the dark for 45 min. Carboxamidomethylation was terminated by dialysis against 50 mM ammonium bicarbonate, pH 8.5, at 4°C for 24 h.

Tryptic digest

The reduced carboxymethylated proteins were digested with trypsin (Promega) and lysyl endopeptidase (Wako) for 16 h at 37°C in 50 mM ammonium bicarbonate buffer, pH 8.4.

Peptide N-glycosidase F digestion of glycopeptides [6]

Peptide *N*-glycosidase F (PNGase F, Roche) digestion was carried out in 100 μL of ammonium bicarbonate (50 mM, pH 8.5) for 16 h at 37°C using 3 units of enzyme. The reaction was terminated by lyophilization, and the released *N*-glycans were separated from peptides and *O*-glycopeptides by C18 Sep-Pak (Waters)

Hydrophilic affinity method [7]

There were serious contaminants at this step, probably due to omission of step-wise elution with propanol described in the protocol in the reference. Therefore an additional procedure of glycan enrichment with a hydrophilic affinity method was performed according to our publication.

Reductive elimination [6]

O-glycans were released by reductive elimination in 100 μL of 0.1 M sodium borohydride hydroxide 2 M sodium borohydride solution at 45°C for 16 h. The reaction was terminated by dropwise addition of glacial acetic acid, followed by Dowex 50W-X8 (H) 50–100 mesh (Sigma) chromatography and borate removal.

Solid-phase permethylation[8]

A microspin column was placed in a 2-mL microcentrifuge vial and filled with acetonitrile. Sodium hydroxide beads were prepared in acetonitrile to prevent the absorption of moisture from the atmosphere. Next, the spin columns were packed with sodium hydroxide beads using a 1-mL syringe. A microspin column is commonly filled with sodium hydroxide beads to approximately 1 cm below the top. The spin column was then centrifuged at a medium speed (ca. 5000 rpm) for few seconds. The acetonitrile collected in the 2-mL micro-centrifuge vial was discarded, and 200 mL of DMSO were added to the spin column to wash its contents. Again, the spin column was centrifuged, and DMSO was discarded. A 200-mL aliquot of DMSO was then added, while the column was kept in DMSO. Optimum permethylation was achieved by resuspending the dried *N*-glycan sample dissolved in 90 μL DMSO and 40 μL iodomethane. Sodium hydroxide packed spin columns were centrifuged prior to applying the sample to discard the DMSO, in which the spin column was maintained. Next, the sample was applied through the packed spin column by centrifugation at 800 rpm for 30 s. The sample was then recycled through the packed spin column for at least eight times. The packed spin column was then washed using a 100-mL aliquot of acetonitrile. Finally, the packed spin column was centrifuged at 10 000 rpm for 0.5 min to ensure complete collection of all liquid in the column.

Permethylated samples were extracted from the reaction solution using 200-mL aliquots of chloroform and water. The aqueous layer was discarded, while the chloroform layer was washed again with water. The permethylated samples in the chloroform layer were dried using SpeedVac.

Note

Although permethylation was used for glycan analysis, purity of *N*-glycans was poor as described above probably due to lack of experience in glycan analysis from whole cell components. We are afraid that the

reliability of the data for *N*-glycans was not assured.

Laboratory D

Sonication of L428, SK-N-SH and U937 cells

10^7 cells were dissolved in 500 µL 500 mM sodium phosphate buffer, pH 7.5, containing 1% SDS and 40 mM DTT. The cells were then placed on ice and sonicated for 5 min. The tubes were heated for 10 min at 100°C.

Tryptic digestion

TPCK trypsin (1 mg, Sigma) was added and the tubes were incubated at 37°C for 6 h. The tubes were heated for 5 min at 100°C.

PNGase F digestion

PNGase F (25 U, Roche) was added and the tubes were incubated at 37°C for 4 h. Further 15 U PNGase were added and incubation was continued at 37°C overnight.

Sep-Pak® C_{18} separation

The Sep-Pak® C_{18} cartridge (1 mL) was conditioned by washing successively with 2 mL methanol, 2 mL water, 2 mL 0.1% TFA, 2 mL 40% acetonitrile/0.1% TFA, 2 mL 80% acetonitrile/0.1% TFA and 10 mL water. The sample was loaded onto to the pre-conditioned Sep-Pak cartridge. The cartridge was washed with 4 mL 0.1% TFA to recover released *N*-glycans. Remaining glycopeptides were eluted with 0.5 mL 20% acetonitrile/0.1% TFA, 0.5 mL 40% acetonitrile/0.1% TFA, 0.5 mL 60% acetonitrile/0.1% TFA, and 0.5 mL 80% acetonitrile/0.1% TFA. Eluates were combined and evaporated to dryness.

Reductive elimination

Dried glycopeptides were dissolved in 400 µL 0.8 M $NaBH_4$ in 50 mM NaOH and incubated at 45°C in a Teflon-lined screw capped culture tube overnight for ~16 h. The reaction was terminated by adding glacial acetic acid drop wise until the fizzing stopped (usually 5 drops, pH ~5) and the sample was dried in a speedvac concentrator. The sample was dissolved 2 times in 500 µL methanol and dried down under a stream of nitrogen at room temperature. The sample was applied on a pre-conditioned Sep-Pak cartridge as described above and released *O*-glycans were recovered in the 0.1% TFA washing fraction.

Desalting of released oligosaccharides

Porous graphitic carbon (PGC) cartridges (1 mL) were conditioned by washing successively with 2 mL water, 2 mL 20% acetonitrile/0.1% TFA, 2 mL 80% acetonitrile/0.1% TFA and 10 mL water. Released *N*- and *O*-glycans were loaded onto PGC cartridges. The cartridges were washed with 4 mL 0.1% TFA. Glycans were eluted with 0.5 mL 20% acetonitrile/0.1% TFA, 0.5 mL 40% acetonitrile/0.1% TFA, 0.5 mL 60% acetonitrile/0.1% TFA, and 0.5 mL 80% acetonitrile/0.1% TFA. Eluates were combined and evaporated to dryness.

Reduction of N-glycans

The *N*-glycan fraction was dissolved in 500 µL $NaBH_4$ (10 mg/mL) in 10 mM NaOH and incubated at room temperature overnight. The reaction was terminated by adding glacial acetic acid drop wise until the fizzing stopped (pH ~5) and the sample was dried in a speedvac concentrator. The sample was dissolved 2 times in 500 µL methanol and dried down under a stream of nitrogen at room temperature. The sample was desalted on a PGC cartridge as described above.

Permethylation

Samples to be permethylated were transferred into a round bottom glass vial with a teflon-lined screw cap and completely dried. Five pellets of NaOH were grinded in a dry mortar and a few grains of the powder were added to the dry sample. Then anhydrous DMSO (300 µL) was added. The sample was mixed vigorously and centrifuged at 2500 g. Fifty µL of methyl iodide were added and the sample was again mixed and centrifuged. The reaction mixture was agitated using an automatic shaker for 40 min at room temperature. The vial was placed in an ice bath and the reaction was quenched by drop wise addition of 1 mL 10% acetic acid with constant shaking between additions. Chloroform (1 mL) was added and the sample was mixed thoroughly for 5 min. After centrifugation at 2500 g the upper aqueous layer was discarded. The extraction was repeated once more. The organic layer was washed 4 times with 1 mL of water each and placed into a freezer. After removal of the remaining frozen water the sample was transferred into a glass vial and dried down.

MALDI-TOF-MS

The permethylated sample was taken up in 98 µL 75% aqueous methanol. MALDI MS was performed in positive-ion reflectron mode using a Bruker Ultraflex I MALDI-TOF instrument equipped with a lift facility. Carbohydrate samples (1 µL) were spotted onto a stainless steel target and mixed with 1 µL matrix solution (10mg/mL DHB in 50% acetonitrile/1% phosphoric acid). External mass calibration was performed using a pepmix calibration standard (1-3 kDa; Bruker). Assignment of oligosaccharides was performed using the software tools Glyco-Peakfinder and GlycoWorkbench.

Laboratory E

Reduction and carboxamidomethylation

Each cell line (1×10^7 cells) was lysed with CelLytic™ MEM Protein Extraction Kit (Sigma-Aldrich). The whole cell lysate was precipitated twice with cold acetone (1 mL). The dried precipitated proteins were resuspended in 7 M guanidine hydrochloride, 0.5 M Tris-HCl (pH 8.6), 10 mM EDTA-Na and were reduced with 10 mM dithiothreitol followed by alkylation with 20 mM iodoacetamide. Samples were dialyzed against 10 mM ammonium bicarbonate using a Mini Dialysis Kit (GE Healthcare) with a molecular mass cut-off of 8 kDa.

Release and reduction of N-glycans from reduced carboxymethylated proteins

The dialyzed samples were digested with 50 μg of trypsin (Wako) at 37°C for 12 h, followed by heat inactivation of the trypsin. Subsequently, 8 mU PNGaseF (Takara Bio Inc.) were added to the digested solutions, followed by incubation at 37°C overnight to remove N-glycans. The released *N*-glycans were purified using a reverse-phase column Oasis HLB (30 mg/mL; Waters) and PGC column HyperCarb (25 mg/mL; Thermo). The desalted N-glycans were subjected to reduction. 50μL of 500 mM NaBH4 in 50 mM NaOH were applied to each purified N-glycans and incubated at 45°C overnight. Each reaction solution was neutralized by adding 5 μL of 50% aqueous acetic acid. The reduced *N*-glycans were desalted by a cation-exchange column AG50W-X (H+) (Bio-Rad). Alditols were eluted with 750 μL of distilled water and dried with a Speed-Vac. The remaining borate was removed by the addition of 100 μL of 1% acetic acid in methanol and dried under vacuum three times.

Permethylation of N-glycans from each sample

Permethylation was performed using the solid NaOH technique. Small NaOH pellets (approximately 50 mg; Fluka) were first mixed with 250 μL of DMSO (∞Pure; Wako). The released *N*-glycans (alditols) were dried in a glass tube and approximately 50 μL of the NaOH/DMSO slurry was added to the sample followed by 50 μL of iodomethane (TCI). The reaction mixture was agitated at room temperature for 30 min. The reaction was terminated by addition of 150 μL of ice-cold 50% aqueous acetic acid and diluted with ice-cold distilled water. The diluted reaction solution was applied to a reverse-phase column Oasis HLB (10 mg/mL; Waters), followed by elution of permethylated alditols with 500 μL of 95% acetonitrile and dried with a Speed-Vac.

Release of O-glycans

O-glycans were directly subjected to reductive β-elimination on a reverse-phase column Oasis HLB (30 mg/mL; Waters). 150 μL of 500 mM NaBH4 in 50 mM NaOH were applied to each column and incubated at 45°C overnight. The released *O*-glycans were passed through Oasis HLB column and neutralized by adding 10 μL of 50% aqueous acetic acid. The released and reduced *O*-glycans were desalted by a cation-exchange column AG50W-X (H+) (Bio-Rad). Alditols were eluted with 750 μL of distilled water and dried with a Speed-Vac. The remaining borate was removed by the addition of 100 μL of 1% acetic acid in methanol and dried under vacuum three times.

Permethylation of O-glycans

Permethylation was performed using the solid NaOH technique. Small NaOH pellets (approximately 50 mg; Fluka) were first mixed with 250 μL of DMSO (∞Pure; Wako). The released *N*-glycans (alditols) were dried in a

glass tube and approximately 50 µL of the NaOH/DMSO slurry was added to the sample followed by 50 µL of TCI. The reaction mixture was agitated at room temperature for 30 min. The reaction was terminated by addition of 150 µL of ice-cold 50% aqueous acetic acid and diluted with ice-cold distilled water. The diluted reaction solution was applied to a reverse-phase column Oasis HLB (10 mg/mL; Waters), followed by elution of permethylated alditols with 500 µL of 95% acetonitrile and dried with a Speed-Vac.

MALDI MS and MSn analysis of permethylated N- and O-glycans.

The MS spectra and MSn spectra were obtained in a reflectron positive ion mode with a Reflex IV MALDI-TOF (Bruker-Daltonik GmbH, Bremen, Germany) and an AXIMA-QIT MALDI quadrupole ion trap TOF instruments (Shimadzu Corp., Kyoto, Japan), respectively. For sample preparation, the dried permethylated sample was resuspended in 50 µL of acetonitrile. A matrix solution (0.5 µL: 10 mg of 2,5-DHB dissolved in 1 mL of 50% ethanol) and 0.5 µL of the diluted analyte solution were spotted and mixed on the plate. Finally, the dried matrix-analyte mixture was recrystallized with 1 µL of ethanol. All MS and MSn spectra were obtained from Na+ adduct ions.

Laboratory F

Cell membrane preparation

Cell pellets (1×10^7 cells) were homogenized in 2 mL of lysis buffer containing 50 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 1 mM EDTA and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) using a polytron homogenizer (Omni TH homogenizer, Omni International, Inc., VA; 15 sec, 7 times on ice bath). We performed the following preparation according to the procedure reported by Lee et al [9] with some modifications. The homogenized cell was centrifuged at 2000*g* for 20 min at 4°C to precipitate nuclei and unlyzed cells. The supernatant was diluted with 2 mL of Tris-buffer (20 mM Tris-HCl (pH 7.4), 0.1 M NaCl) and then were sedimented by ultracentrifugation at 120000*g* for 80 min at 4°C. The supernatant was discarded, and the membrane pellet was suspended with 100 mL of the Tris-buffer. After adding 400 mL of the Tris-buffer containing 1% (v/v) Triton X-114, the suspended mixture was homogenized by strong pipetting. The homogenate was chilled on ice for 10 min and was incubated at 37°C for 20 min and then was phase partitioned by centrifugation at 1000*g* for 3 min. The upper aqueous phase was removed. The lower detergent phase was further mixed with 1 mL of ice-cold acetone and was kept at −25°C overnight to precipitate proteins and remove any detergent. After centrifugation at 1000*g* for 3 min, the precipitated cell membrane proteins were stored at −25°C if not used immediately.

PNGase F enzymatic cleavage of N-glycans from cell membrane

The precipitated membrane protein by acetone was dissolved with 10 mL of 8 M urea. The dissolved protein was dotted to PVDF membrane which was activated with ethanol in advance (dotted volume: 2.5 mL, 4 times). After drying the dotted protein on PVDF membrane at room temperature overnight, the PVDF membrane was washed with ethanol for 1 min and then washed with water for 1 min three times. The dotted protein on PVDF membrane was stained with direct blue 71 (the mixture of solution A (800 mL) and solution B (10 mL), solution A: 0.1% direct blue 71 (Sigma-Aldrich) in water, solution B: acetic acid: ethanol: water = 1:4:5) for 5 min. After destaining the background with solution B for 1 min, the PVDF membrane was dried at room temperature for 4 h. We performed the following preparation according to the procedure reported by Wilson et al [10] with some modifications. Proteins stained with blue were cut from the PVDF membrane and placed in separate wells of a 96-well microtiter plate. The spots were then covered with 100 μL of 1% (w/v) poly (vinylpyrrolidone) 40000 in 50% methanol, agitated for 20 min, and were washed with 100 mL of water 5 times. Two units of PNGase F (in 10 μL of 20 mM phosphate buffer (pH 7.3), Roche Diagnostics) were added to the well and the 96-well plate was incubated at 37°C for 15 min. After adding 10 mL of water to the well, the 96-well plate was incubated at 37°C overnight to release *N*-glycans from proteins. During the incubation, the sample wells were properly sealed with amplification tape to prevent evaporation. To collect the released *N*-glycans, the samples were sonicated (in the 96-well plate) for 10 min, and the solution including the released *N*-glycans (20 mL) was transferred from the well to 1.5 mL polypropylene tube. The sample well was washed with 50 mL of water twice, and the washed water (100 mL) was combined with the solution including the released *N*-glycans. To form the reducing type of *N*-glycans completely, ammonium acetate buffer (100 mM, pH 5.0, 20 mL) was added to the combined solution followed by incubation for 1 h at room temperature. After the sample was dried by evaporation, 20 mL of 1 M NaBH4 in 50 mM KOH was added to the dried sample and then the mixture was incubated at 50°C for 3 h in order to form alditol type *N*-glycans. After 1 mL of acetic acid was added to stop the reaction, the mixture was passed through a cation-exchange column which already activated according to the procedure described above in order to remove salts. The column was washed with water (50 mL) twice, and the all effluents were combined together and then were evaporated to dryness. The remaining borate was removed by the addition of 100 μL of methanol and drying under vacuum by repeating three times. The *N*-glycan alditols were re-suspended in 20 μL of water immediately before injection to LC-ESI/MS. An 8 mL aliquot of the sample re-suspended with water was used for analysis of *N*-glycan profiling.

β-Elimination release of O-glycans from cell membrane

After the removal of the *N*-glycans, each protein spot remaining on the membrane was re-wet with 2.5 μL of methanol. A solution of 0.5 M NaBH4 in 50 mM KOH (20 μL) was applied, and the spots were incubated for 16

h at 50°C in order to release *O*-glycans as alditol form. After adding 4 mL of acetic acid to stop the reaction, the mixture was passed through a cation-exchange column to remove salts. The following procedures are same as that for *N*-glycans. Finally 8 mL of the sample re-suspended with water was injected to LC-ESI/MS for analysis of *O*-glycan profiling.

LC-ESI/MS for analysis of N- and O-glycan alditols

Liquid chromatography coupled to negative electrospray tandem mass spectrometry (LC/MSD Trap XCT Plus Series 1100, Agilent Technologies) was used for the analysis of both *N*- and *O*-glycan alditols. Samples were applied to a Hypercarb porous graphytized carbon capillary column (5 mm Hypercarb KAPPA, 180 mm × 100 mm, Thermo Hypersil) using a Surveyor autosampler. The separation of glycan alditols was carried out with a linear gradient of 0-45% (v/v) acetonitrile/10 mM ammonium bicarbonate for 83 min (*N*-glycan alditols) and 0-90% (v/v) acetonitrile/10 mM ammonium bicarbonate for 45 min (*O*-glycan alditols), followed by a 10 min wash using 90% (v/v) acetonitrile/10 mM ammonium bicarbonate at a flow rate of 5 mL/min. The sample injection volume was 8 mL and ESI-MS/MS was performed in a negative ion mode with three scan events: a full scan with the mass range of m/z 100-2500 amu, a dependent zoon scan, and a dependent MS/MS scan after collision induced fragmentation.

Relative abundance of N- and O-glycan alditol structures

The relative abundance of each glycan structure on cell membrane glycoproteins was calculated based on the peak area on EIC of the corresponding glycan structure after processing smooth of peaks (smoothing algorithm; Gauss, smoothing widths; 3 pnts (for *N*-glycan) or 1 pnts (for *O*-glycan) using Bruker Daltonics DataAnalysis software). Total peak area of glycans from each cell was set to 100%, and the relative abundance (%) was calculated for each glycan structure for each cell.

Reference

1. Takahashi, N., Nakagawa, H., Fujikawa, K., Kawamura, Y., Tomiya, N.: Three-dimensional elution mapping of pyridylaminated N-linked neutral and sialyl oligosaccharides. Anal. Biochem. 226, 139–146 (1995).

2. Takahashi, N., Kato, K.: GALAXY (Glycoanalysis by the Three Axes of MS and Chromatography): a Web Application that Assists Structural Analyses of *N*-Glycans. Trends Glycosci. Glycotechnol. 15, 235–251 (2003).

3. Yagi, H., Takahashi, N., Yamaguchi, Y., Kimura, N., Uchimura, K., Kannagi, R., Kato, K.: Development of structural analysis of sulfated N-glycans by multidimensional high performance liquid chromatography mapping methods. Glycobiology. 15, 1051–1060 (2005).

4. Yagi, H., Saito, T., Yanagisawa, M., Yu, R.K., Kato, K.: Lewis X-carrying N-glycans regulate the proliferation of mouse embryonic neural stem cells via the Notch signaling pathway. J. Biol. Chem. 287, 24356–24364 (2012).

5. Yagi, H., Ohno, E., Kondo, S., Yoshida, A., Kato, K.: Development and Application of Multidimensional HPLC Mapping Method for O-linked Oligosaccharides. Biomolecules. 1, 48–62 (2011).

6. North, S.J., Huang, H.-H., Sundaram, S., Jang-Lee, J., Etienne, A.T., Trollope, A., Chalabi, S., Dell, A., Stanley, P., Haslam, S.M.: Glycomics profiling of Chinese hamster ovary cell glycosylation mutants reveals N-glycans of a novel size and complexity. J. Biol. Chem. 285, 5759–5775 (2010).

7. Wada, Y., Tajiri, M., Yoshida, S.: Hydrophilic affinity isolation and MALDI multiple-stage tandem mass spectrometry of glycopeptides for glycoproteomics. Anal. Chem. 76, 6560–6565 (2004).

8. Kang, P., Mechref, Y., Novotny, M. V: High-throughput solid-phase permethylation of glycans prior to mass spectrometry. Rapid Commun. Mass Spectrom. 22, 721–734 (2008).

9. Lee, A., Kolarich, D., Haynes, P.A., Jensen, P.H., Baker, M.S., Packer, N.H.: Rat liver membrane glycoproteome: enrichment by phase partitioning and glycoprotein capture. J. Proteome Res. 8, 770–781 (2009).

10. Wilson, N.L., Schulz, B.L., Karlsson, N.G., Packer, N.H.: Sequential analysis of N- and O-linked glycosylation of 2D-PAGE separated glycoproteins. J. Proteome Res. 1, 521–529 (2002).

Supplementary Method 2

Preparation of membrane and cytosolic fractions

MATERIALS:

L428 cells (total in ~1.9×10^9 cells)

U937 cells (total in ~1.6×10^9 cells)

PROTOCOL

[Modify and refer from Cox, B. and Emili, A. (2006) *Nat Protocols.* 1:1872-1878.]

Preparation of cytosolic and membrane fraction was performed based on a published method [1].

Each of L428 and U937 cell pellets (each 4×10^8 cells) was resuspended in 10 mL ice-cold 250-STMDPS buffer (50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 5 mM MgCl2, spermine (25 μg/mL), spermidine (25 μg/mL), 2 mM DTT and 2 mM PMSF) on ice. The cell suspensions were homogenized by the Teflon-glass Dounce homogenizer. The cell homogenate was centrifuged at 800*g* at 4°C for 15 min. The supernatant was moved to a new centrifuge tube and the remaining cell pellets were rehomogenized with the Teflon-glass Dounce homogenizer in 5 mL ice-cold 250-STMDPS buffer on ice. The cell homogenate was centrifuged at 800*g* at 4°C for 15 min. Combined supernatants were centrifuged at 6000*g* at 4°C for 15 min to isolate the mitochondria fraction. The supernatant was ultracentrifuged at 100000*g* at 4°C for 70 min (BECKMAN: Optima™ Ultracentrifuge). The supernatant was served as a pure cytosolic fraction. The pellet was resuspended in 0.6 mL ME buffer (20 mM Tris-HCl, pH 7.8, 0.4 M NaCl, 15% glycerol, 2 mM DTT, 2 mM PMSF and 1.5% Triton X-100) and incubated at 4°C for 60 min with gentle agitation. The suspension was centrifuged at 9000*g* at 4°C for 30 min and the supernatant was served as the final membrane fraction. Each of the cytosolic and membrane fractions was precipitated with ice-cold acetone followed by ethanol, and precipitated proteins were lyophilized to dryness.

Reference

1. Cox, B., Emili, A.: Tissue subcellular fractionation and protein extraction for use in mass-spectrometry-based proteomics. Nat. Protoc. 1, 1872–1878 (2006).

Supplementary Method 3

***N*- and *O*-glycan analyses in membrane and cytosolic fractions by each laboratory**

Laboratory A

Technology from Lab A is based on the GALAXY-based HPLC profiling using 2-aminopyridine (PA)-derivatized glycans (http://www.glycoanalysis.info/galaxy2/index.jsp). This enables quantitative evaluation of the incidence of each oligosaccharide on the basis of the fluorescence intensity of PA-glycans. The elution time expressed in real time or volume can vary depending on the individual column, its age, or the batches of buffers used. In our method, elution time is normalized as the glucose unit (GU), which is daily calibrated with a PA-isomalto-oligosaccharides mixture in order to reduce any variations. Moreover, we use an extensive array of PA-standard oligosaccharides for co-injection into the column with the sample oligosaccharide in order to confirm the identification by observing a single peak. All these controls ensure the robustness and reproducibility of our technologies.

N-glycans

Experimental procedures, including chromatographic conditions and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), were as described previously [1–4].

Cell lysates were proteolyzed with chymotrypsin-trypsin mixture and further digested with glycoamidase A to release *N*-glycans. After removal of the peptides, the reducing ends of the *N*-glycans were derivatized with 2-aminopyridine (PA). The PA derivatives of the *N*-linked oligosaccharides were separated by HPLC on a TSK-gel diethylaminoethyl (DEAE)-5PW column (Tosho) according to their sialic acid content. Each fraction separated from the DEAE column was collected, evaporated, and then applied to a Shim-pack HRC-octadecyl silica (ODS) column. The fractions separated on the ODS column were subjected to MALDI-TOF-MS analysis and the fractions possibly including two or more *N*-glycans were further separated using an amide column (Tosho). Identification of *N*-glycan structures was based on their elution positions on the column and their molecular mass values compared with those of PA-glycans in the GALAXY database (http://www.glycoanalysis.info/galaxy2/ENG/index.jsp)[2].

O-glycans.

The experimental procedure used for structural analysis including hydrazinolysis, fluorescence-tagging, and chromatographic conditions have been described previously[5], with slight modification of the separation condition of the anion exchange column.

O-glycans were released from delipidated cell lysates by hydrazinolysis and then labeled with

2-aminopyridine (PA). The PA-glycan mixture was firstly separated on a TSKgel diethylamino ethanol (DEAE)-5PW column (7.5 mm i.d.×75 mm; Tosoh, Tokyo, Japan) at 30°C with a flow rate of 1.0 mL/min using two solvents, A and B: Solvent A was aqueous ammonia (pH 9.0) and solvent B was a 50 mM ammonium acetate solution (pH 9.0). The column was equilibrated with solvent A. The gradient elution parameters were 0–3 min, linear gradient 0%–12% B; 3–17 min, linear gradient 12%–40% B; 17–22 min, linear gradient 40%–100% B. Each oligosaccharide was separated according to its anionic charges.

Next, each fraction separated from the DEAE column was collected, evaporated, and then applied to a Decelosil C30-HG-5 (C30) column (Nomura Chemical Co., Ltd.). The HPLC elution times were represented by glucose units (GUs) on the columns calibrated with a PA-derivatized isomalto-oligosaccharides mixture. The structures of the PA-saccharides were characterized by HPLC mapping[5] and MALDI-TOF/MS analysis using a MALDI-TOF/MS spectrometer (AXIMA-CFR; Shimadzu or ABI 4700 Proteomics analyzer; ABI).

Laboratory B

N-glycans

Lyophilized material was suspended in 2 M thiourea, 5 M urea (267 mL), 1 M DTT (16.7 mL), benzonase (125 units), and then incubate at room temperature for 30 min.

After centrifugation at 15000 rpm for 10 min, the supernatant was mixed with cold acetone (1.5 mL), and then keep at −20°C for 30 min. After centrifugation at 15000 rpm for 10 min, the pellet was washed with 75% ethanol (1 mL), and then lyophilized to dryness. The lyophilized material was supended in water-10% SDS-2-ME (210:24:2.4, 250 mL), and then the mixture was kept in the boiling water bath for 5 min. After cooling, 10% NP-40 (25 mL) and 1 M phosphate buffer pH 7.5 (25 mL) were added, after addition of *N*-Glycanase F (2 unit, 4 mL), the mixture was kept at 37°C overnight. After keeping the mixture in a boiling water bath for 5 min, the mixture was centrifuged at 12000*g* for 15 min. The supernatant was collected and lyophilized to dryness.

The mixture of the released glycans was dissolved in 2-AA solution (200 mL) which was prepared by dissolution of 2-AA (30 mg) and sodium cyanoborohydride (30 mg) in methanol (1 mL) containing 4% sodium acetate and 2% boric acid. The mixture was kept at 80°C for 60 min. After addition of distilled water (200 mL), the mixture was applied to a column of Sephadex LH-20 (1.0 cm i.d., 30 cm length) equilibrated with 50% aqueous MeOH. The earlier eluted fluorescent fractions were collected and evaporated to dryness.

O-glycans

Lyophilized material was digested with pronase (50 mg) in 50 mM Tris-HCl (pH8.0, 1 mL) at 37°C for 24 h. The

reaction mixture was kept in the boiling water bath for 10 min, the supernatant was collected after centrifugation. The supernatant was passed through an ultrafiltration membrane (MW 5000 cutoff, Ultrafree-MC, Millipore) at 10000*g*. The mixture of glycopeptides on the membrane was recovered by dissolving in distilled water. The releasing reaction of *O*-glycans was performed with the automatic glycan releasing system according to the method reported previously. An aqueous solution of 0.5 M LiOH was used as the releasing reagent. To the flow of the eluent at 1.0 mL/min, an aqueous solution of the glycopeptides obtained as described above was injected. After the sample solution was mixed with the 0.5 M LiOH in the device. The mixture was moved to the reactor maintained at 60°C, in which a reaction tube (0.25 mm i.d., 10 m length: 700 mL volume) was set. The eluate containing the reaction mixture from the reactor was immediately passed through a cation exchange cartridge (0.7 mL volume), and collected to a fraction collector. The collected solution containing the released *O*-glycans was evaporated to dryness by a centrifugal evaporator.

Fluorescent labeling of N- and O-glycans

The mixture of the released glycans was dissolved in 2-AA (200 mL) solution which was prepared by dissolution of 2-AA (30 mg) and sodium cyanoborohydride (30 mg) in methanol (1 mL) containing 4% sodium acetate and 2% boric acid. The mixture was kept at 80°C for 60 min. After addition of distilled water (200 mL), the mixture was applied to a Sephadex LH-20 column (1.0 cm i.d., 30 cm length) equilibrated with 50% aqueous MeOH. The earlier eluted fluorescent fractions were collected and evaporated to dryness.

Serotonin-affinity chromatography for group separation of N-glycans based on the number of sialic acid residues.

HPLC was performed with a Jasco apparatus equipped with two PU-980 pumps and a Jasco FP-920 fluorescence detector. The *N*-glycan pool obtained from cancer cells as described above was separated based on the number of sialic acid residues using a serotonin-immobilized column (4.6 × 150 mm) with a linear gradient from water (solvent A) to 40 mM ammonium acetate (solvent B) at a flow rate of 0.5 mL/min. Initially, solvent B was used at 5% concentration as eluent for 2 min, and then linear gradient elution was performed to 30 mM ammonium acetate for 35 min, and finally the eluent was changed to solvent B (40 mM ammonium acetate) during the following 10 min. Peaks were collected and lyophilized to dryness.

After separation of *N*-glycan pool based on the number of sialic acid residues as described above, oligosaccharide fractions containing sialic acids were digested with neuraminidase. Neuraminidase (2 units, 4 mL) was added to the mixture of sialo-*N*-glycans in 20 mM acetate buffer (pH 5.0, 20 mL), and the mixture was incubated at 37°C overnight. After keeping the mixture in the boiling water bath for 3 min followed by centrifugation, a portion of the supernatant was used for MALDI-QIT-TOF/MS analysis.

Analysis of O-glycans on a polymer-based amino column

The apparatus was the same as described for the analysis of 2-AA labeled *O*-glycans by serotonin affinity chromatography. Separation was done with a polymer-based Asahi Shodex NH2P-50 4E column (Showa Denko, Tokyo, Japan: 4.6 mm i.d., 250 mm length) using a linear gradient formed by 2% acetic acid in acetonitrile (ACN) (solvent A) and 5% acetic acid in water containing 3% triethylamine (solvent B). The column was initially equilibrated and eluted with 70% solvent A for 2 min, then solvent B was increased to 95% over 80 min and kept at this composition for further 100 min.

MS analysis of O-glycans

The analyses of the *N*- and/or *O*-glycans were performed by a MALDI-QIT TOF mass spectrometer (AXIMA-QIT, Shimadzu, Kyoto, Japan) in negative or positive ion linear mode. Acquisition and data processing were controlled by Launchpad software (Kratos Analytical, Manchester, U.K.). For sample preparation, a 0.5-µL volume of the matrix solution (DHB; 10 mg/mL in 30% ethanol/0.1% trifluoroacetic acid (TFA)) was deposited on the stainless steel target plate and allowed to dry. Then, a portion (0.5 mL) of the appropriately diluted analyte solution (typically ca. 1 pmol/mL) was used to cover the matrix on the target plate and allowed to dry.

Laboratory C

Reduction and carboxymethylation

Proteins were reduced by dithiothreitol in 6M guanidine, 0.25 M Tris, pH 8.5, and carbamidomethylated with iodoacetamide. After alkylation, the samples were dialysed against 2 L of 50mM ammonium bicarbonate, pH 8.5, at 4°C for 24 h.

Tryptic digest

The carbamidomethylated proteins were digested with trypsin (Promega) and lysyl endopeptidase (Wako) in 50 mM ammonium bicarbonate, pH 8.4, at 37°C for 16 h.

Peptide N-Glycosidase F digestion of glycopeptides

Peptide *N*-glycosidase F (PNGase F, EC 3.5.1.52; Roche Applied Science) digestion was carried out in 100 µL of ammonium bicarbonate (50 mM, pH 8.5) at 37°C for 16 h using 3 units of enzyme. The released *N*-glycans were separated from peptides and *O*-glycopeptides by C18 Sep-Pak (Waters) as described previously [6]

Reductive elimination

O-Glycans were released by reductive elimination in 100 μL of 0.1 M sodium borohydride hydroxide 2M sodium borohydride solution at 45°C for 16 h. The reaction was terminated by dropwise addition of glacial acetic acid, followed by Dowex 50W-X8 (H) 50–100 mesh (Sigma) chromatography for borate removal.

Solid-phase permethylation

A microspin column was placed in a 2-mL microcentrifuge vial and filled with ACN. Sodium hydroxide beads were prepared in ACN to prevent the absorption of moisture from the atmosphere. Next, the spin columns were packed with sodium hydroxide beads using a 1-mL syringe. A spin column was filled with sodium hydroxide beads to approximately 1 cm below the top. The spin column was then centrifuged at 1,500*g* for a few seconds to remove ACN, and 200 μL DMSO was added to the column and centrifuged to wash the content. Then, 200-μL DMSO was added again to keep wet the column bed with DMSO. Optimum permethylation was achieved for the dried *N*-glycan samples dissolved in 90 μL DMSO and 40 μL iodomethane (TCI).

Sodium hydroxide-packed spin columns were centrifuged to remove DMSO prior to sample application. The glycan sample dissolved in DMSO/TCI was passed through the column by centrifugation at 40*g* for 30 s. The sample was then recycled through the column at least eight times. The packed spin column was then washed with 100-μL ACN. Finally, the column was centrifuged at 7,000*g* for 30 sec to ensure complete collection of all liquid in the column. The reaction mixture was then subjected to liquid-liquid extraction as follows. A solution of 200-μL chloroform and 2 mL water was added to the sample and vortexed. Then, the chloroform layer was collected and washed by water repeatedly. Finally, the permethylated glycans in the chloroform layer were dried by SpeedVac.[7, 8]

Laboratory E

Reduction and carboxamidomethylation

Each lyophilized cell fraction was resuspended in 7 M guanidine hydrochloride, 0.5 M Tris-HCl (pH 8.6), 10 mM EDTA-Na and was reduced with 10 mM dithiothreitol followed by alkylation with 20 mM iodoacetamide. Samples were dialyzed against 10 mM NH_4HCO_3 using a Mini Dialysis Kit with a molecular mass cut-off of 8 kDa (GE Healthcare).

Release and reduction of N-glycans from reduced carboxymethylated proteins

The dialyzed samples were digested with 50 μg of trypsin (Wako) at 37ºC for 12 h, followed by heat inactivation of the trypsin. Subsequently, 8 mU PNGaseF (Takara Bio Inc.) were added to the digested solutions, followed by

incubation at 37°C overnight to remove *N*-glycans. The released *N*-glycans were purified using a reverse-phase column Oasis HLB (30 mg/mL; Waters) and PGC column HyperCarb (25 mg/mL; Thermo). The desalted *N*-glycans were subjected to reduction. 50 µL of 500 mM NaBH4 in 50 mM NaOH was applied to the purified *N*-glycans and incubated at 45°C overnight. Each reaction solution was neutralized by adding 5 µL of 50% aqueous acetic acid. The reduced *N*-glycans were desalted by a cation-exchange column AG50W-X (H+) (Bio-Rad). Alditols were eluted with 750 µL of distilled water and dried with a Speed-Vac. The remaining borate was removed by the addition of 100 µL of 1% acetic acid in methanol and dried under vacuum three times.

Permethylation of released N-glycans

Permethylation was performed using the solid NaOH technique. Small NaOH pellets (approximately 50 mg; Fluka) were first mixed with 250 µL of DMSO (∞Pure; Wako). The released *N*-glycans (alditols) were dried in a glass tube and approximately 50 µL of the NaOH/DMSO slurry was added to the sample followed by 50 µL of TCI. The reaction mixture was agitated at room temperature for 30 min. The reaction was terminated by addition of 150 µL of ice-cold 50% aqueous acetic acid and diluted with ice-cold distilled water. The diluted reaction solution was applied to a reverse-phase column Oasis HLB (10 mg/mL; Waters), followed by elution of permethylated alditols with 500 µL of 95% ACN and dried with a Speed-Vac.

Release of O-glycans

O-glycans were directly subjected to reductive β-elimination on a reverse-phase column Oasis HLB (30 mg/mL; Waters). To each column, 150 µL of 500 mM NaBH4 in 50 mM NaOH was applied and incubated at 45°C overnight. The released *O*-glycans were passed through an Oasis HLB column and neutralized by adding 10 µL of 50% aqueous acetic acid. The released and reduced *O*-glycans were desalted by a cation-exchange column AG50W-X (H+) (Bio-Rad). Alditols were eluted with 750 µL of distilled water and dried with a Speed-Vac. The remaining borate was removed by the addition of 100 µL of 1% acetic acid in methanol and dried under vacuum three times.

Permethylation of O-glycans

Permethylation was performed using the solid NaOH technique. Small NaOH pellets (approximately 50 mg; Fluka) were first mixed with 250 µL of DMSO (∞Pure; Wako). The released *N*-glycans (alditols) were dried in a glass tube and approximately 50 µL of the NaOH/DMSO slurry was added to the sample followed by 50 µL of TCI. The reaction mixture was agitated at room temperature for 30 min. The reaction was terminated by addition of 150 µL of ice-cold 50% aqueous acetic acid and diluted with ice-cold distilled water. The diluted reaction

solution was applied to a reverse-phase column Oasis HLB (10 mg/mL; Waters), followed by elution of permethylated alditols with 500 µL of 95% ACN and dried with a Speed-Vac.

MALDI MS and MSn analysis of permethylated N- and O-glycans.

The MS spectra and MSn spectra were carried out in reflectron positive ion mode with a Reflex IV MALDI-TOF (Bruker-Daltonik GmbH, Bremen, Germany) and an AXIMA-QIT MALDI quadrupole ion trap TOF instruments (Shimadzu Corp., Kyoto, Japan), respectively. For sample preparation, the dried permethylated sample was resuspended in 50 µL of ACN. 0.5 µL of matrix solution (10 mg of 2,5-DHB dissolved in 1 mL of 50% ethanol) and 0.5 µL of the diluted analyte solution were spotted on the plate and mixed on the plate. Finally, the dried matrix-analyte mixture was recrystallized with 1 µL of ethanol. All MS and MSn spectra were obtained from Na+ adduct ions.

Laboratory F

Initial sample handling

The four provided samples (membrane and cytosolic fractions of Hodgkin lymphoma cells L428 and membrane and cytosolic fractions of lymphoma cells U937) were stored at 5°C until use. Initially they were dissolved to a concentration of 10 mg/ml in a denaturing buffer consisting of 0.5 M Tris-HCl, pH 8.5, 7 M Guanidine-HCl, 10 mM EDTA. Fractions of these samples were reduced by incubation with 10 mM DTT (final concentration) at 56°C for 30 min and subsequently alkylated by incubation with 20 mM iodoacetamide (final concentration) at room temperature for 45 min. The reaction was quenched by the addition of DTT to a final concentration of 20 mM. These samples were stored at 5°C until used.

N-glycans

Each sample (10–15 µg) was immobilized in discrete spots on a primed and blocked 0.45 µm Immobilon-PSQ PVDF membrane (Millipore) and processed essentially as previously described [9]. In brief, the spots were stained with Direct Blue (Sigma-Aldrich), excised and washed in separate wells in a flat bottom polypropylene 96 well plate (Corning Incorporated, Corning, NY). *N*-glycans were released from the membranes of all spots using 2.5 U PNGase F (*Flavobacterium meningosepticum*, Roche) in 10 µL water/well and incubated for 16 h at 37°C. To remove the glycosylamines from the reducing end of the released *N*-glycans, 10 µL 100 mM ammonium acetate, pH 5 was added and the mixtures incubated for 1 h at room temperature. Subsequently, the samples were dried and reduced to alditols by redissolving the *N*-glycans in 20 µl 1 M $NaBH_4$ in 50 mM KOH with incubation for 3 h at 50°C. After neutralisation with 2 µL glacial acetic acid, the *N*-glycans were desalted

prior to LC-MS/MS.

O-glycans

The same spots from where the *N*-glycans were released were subsequently used for *O*-glycan release. *O*-glycans were released from the membranes of all three spots using reductive β-elimination. This was performed in 20 µL 0.5 M $NaBH_4$ in 50mM KOH with incubation at 50°C for 16 h. After neutralisation with 2 µL glacial acetic acid, the *O*-glycans were desalted prior to LC-MS/MS.

Desalting

Desalting of the glycan samples were performed in two steps using two separate SPE columns: 1) cation exchange columns and 2) carbon columns. The cation exchange resin (25 µL column volume, AG 50W X8, Bio-Rad, Hercules, CA) was packed onto TopTip (Glygen), washed with 3 × 50 µL 1 M HCl, 3 × 50 µL methanol and then with 3 × 50 µL water before the sample was added. The flow-through from the sample load and two washing steps with 50 µL water were pooled. The samples were dried and then washed 3 times with 100 µL methanol to remove residual borate. Each sample was then redissolved in 10 µL water and cleaned-up with carbon SPE. For this, carbon SPE material was packed into empty TopTips. Prior to sample load, the columns were washed with 0.1% TFA in ACN and then with 0.1% TFA in water. After sample load, which was performed twice on the same column, the samples were washed with 0.1% TFA in water and oligosaccharides were eluted with 0.1% TFA in 50% ACN/50% water. The eluted samples were dried and taken up in 10 µL water and analysed with LC-MS/MS.

LC-MS/MS

Released *N*- and *O*-glycans (alditols) were separated on Hypercarb porous graphitised carbon column (PGC) (5 µm particle size, 180 µm (ID) × 10 cm, Thermo Scientific) on an Agilent 1100 capillary LC (Agilent Technologies) or a Dionex Ultimate 3000 LC (Dionex, Thermo) and analysed using an Agilent MSD 3D iontrap XCT Plus mass spectrometer or a Bruker HCT 3D ion trap coupled directly to the LC. Separation was carried out at a constant flow rate of 2 µL/min using aqueous 10 mM NH_4HCO_3 (A solvent) and linear gradients made with either of the two B solvents: 45% (v/v) ACN/10 mM NH_4HCO_3 (for *N*-glycans, total run time 100 min) and 90% (v/v) ACN/10 mM NH_4HCO_3 (for *O*-glycans, total run time 60 min). ESI–MS was performed in negative ion mode with the following scan events: MS full scan with mass range *m/z* 400–2,000 and data dependent MS/MS scan after CID of the top three most intense precursor ions. Compositions and glycan structures and substructures were verified using MS/MS (where available) and the quantitation was based on extracted ion chromatograms (EIC) for the individual *m/z* corresponding to the glycan compositions. If multiple isoforms for a

given composition were present these were pooled for the quantitation purposes. If multiple charge states were observed for a given glycan composition these were pooled as well. No significant adduct formation or molecular loss were observed.

Laboratory H

Chemicals and materials

Sequencing grade trypsin was obtained from Promega (Madison, WI). PNGase F (glycerol free) was obtained from New England Biolabs (Ipswich, MA). Complete protease inhibitor mixture tablets were purchased from Roche Applied Science (Basel, Switzerland). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The 10 kDa MWCO centrifugal filters were obtained from Millipore (Bedford, MA). Sep-Pak C18 1 cc Vac cartridges were obtained from Waters (Milford, MA). Water used in all experiments was produced by a Milli-Q Plus system from Millipore (Bedford, MA).

Sample preparation

The freeze-dried samples of membrane and cytosolic fraction from Hodgkin lymphoma cell (L428-Mem, L428-Cyto) and lymphoma cell (U937-Mem, U937-Cyto) were dissolved in lysis buffer (7 M urea, 2 M thiourea, 1 mM PMSF and cocktail) to a final concentration of 10μg/μL respectively. After being incubated in an ice-cold ultrasonic bath for 15 min, centrifuged for 20 min at 12000*g*, 4°C, the supernatant was collected.

Proteins digestion

The proteins were in-solution digested. The total proteins of 550 μg of each sample were diluted with 50 mM NH_4HCO_3 buffer to a final concentration of 2.5 μg/μL. Then, the proteins were reduced by 10 mM dithiothreitol at 37 °C for 1 h, and alkylated by 20 mM iodoacetamide in the dark, at room temperature for 30 min. Then the trypsin was added to the protein solution (1:50, w/w) and incubated at 37 ºC overnight. The tryptic peptides were desalted by passing through C18 columns. In detail, a C18 column was activated with 700 μL 100% ACN, then balanced with 700 μL 0.1% TFA. The peptides were loaded into the column. Then the column was washed with 500 μL 0.1% TFA. Finally the peptides were eluted with 700 μL eluting buffer 1 (50% ACN, 0.1% TFA) and 700 μL eluting buffer 2 (80% ACN, 0.1% TFA) sequentially. The peptide solution was dried in a vacuum centrifuge.

Release and purification of N-glycans

The glycans were released by PNGase F. The dried tryptic peptides were dissolved in 550μL50 mM NH_4HCO_3 buffer, then 1μL PNGase F was added to the peptide solution. The mixture was incubated at 37°Cfor 18 h.

Finally, the released N-glycans were purified from the mixture by passing through C18 columns. In detail, a C18 column was activated with 700 μL 100% ACN, then balanced with 700 μL0.1% TFA. The peptide and *N*-glycan mixture was loaded into the column. The unbounded faction was collected. Then the column was washed with 500 μL 0.1% TFA. The wash solution and unbounded fraction containing purified *N*-glycans were combined and dried in a vacuum centrifuge.

Mass spectrometry and data analysis

The purified glycans from four samples were analyzed using a LC/MS system consisting of Shimadzu Integrated System connected to a AB SCIEX Triple TOF^{TM} 5600 mass spectrometer. Each sample was suspended in 55 μL 95% ACN and the injection volume was 50 μL. A 2.0 mm id × 15 cm TSK gel Amide-80 column was used for the separation with a linear gradient of solvent A (95% ACN, 5% H_2O, 5mM ammonium formate) and solvent B (95% H_2O, 5% ACN, 5 mM ammonium formate). Gradient conditions: 20–50% solvent B over 10 min followed by 8 min at 50% solvent B and the gradient went back to 20% solvent B over 0.2 min (hold 9.8 min). The flow rate was 250 μL/min. The mass spectrometer was operated in the positive ion and information dependent acquisition (IDA) mode. A survey full-scan MS spectrum (m/z 500-5000) was followed by MS/MS of the six most intense ions. For MS/MS, the CE was set as 50±10.

The wiff files obtained from AB SCIEX Triple TOF^{TM} 5600 mass spectrometer were concerted to mgf format using AB SCIEX MS Data Converter. Then the peak lists of the precursor ion were extracted from mgf flies using the in-house made software. The Profiler option in Glyco Workbench software was used to search the database for structures with a given peak list. Glycome DB was chosen as the database and the tolerance was set as 20 ppm. The percentages of the glycans were calculated with the assistance of the progenesis LC-MS software.

Laboratory I

The purpose of this study was to characterize *O*-glycan and *N*-glycan on proteins isolated from membrane and cytoplasmic fractions of two lymphoma cell lines, L428 and U937. Solubilized samples were treated with trypsin. Polypeptides/glycopeptides were purified by SPE C18 cartridge. Dried (glycol)peptides were subjected to PNGase F digestion. Released *N*-glycans were separated from (*O*-glyco)peptides by SPE cartridge. *O*-glycans were released by β-elimination from (*O*-glyco)peptides. Partial *N*-glycans (native and desialylated) were reduced in mild base. All glycans were analyzed by capillary graphitized carbon LC-MS and LC-MS/MS in negative mode using a LTQ XL ion trap mass spectrometer.

Samples

All samples were dissolved in urea-thiourea buffer (7 M urea, 2 M thiourea, and 40 mM Tris) to reach a final concentration of 2 mg/mL in cold room overnight with rotation.

Enrichment of (glyco)peptides

Each sample (0.2 mg) was reduced and alkylated. Samples were then incubated with sequencing grade trypsin (1% w/w, Promega, Promega Biotech AB, Nacka, Sweden) at 37°C overnight.

Resultant (glyco)peptides were loaded to Sep-Pak C18 (Waters Corporation, Milford, MA), which was previously washed with 0.5 mL 65% ACN in 0.5%TFA, 0.5 mL 10% ACN in 0.5% TFA, and 3 × 0.5 mL 1% HOAc. After loading the sample, the cartridge was washed subsequently with 2 × 0.2 mL 1% HOAc. (Glyco)peptides were eluted with 3 × 0.2 mL 65% ACN with 0.5% TFA. All samples were dried in SpeedVac.

Purification of *N*-glycans

Dried (glycol)peptides were resuspended in 50 mM NH_4OAc (pH 8.4). Then additional 2 µL of PNGase F (ca. 2 U, Roche Diagnostics Deutschland GmbH, Mannheim, Germany) were added and incubated at 37°C overnight.

N-glycan was separated from (*O*-glyco)peptides by Sep-Pak C18, which was pre-washed with 5 mL of methanol. After loading the sample, cartridge was washed with 2 mL 0.1% of TFA. This washout and breakthrough contain released *N*-glycans. (*O*-glyco)peptides were further eluted with 80% ACN with 0.1% TFA. Both washout and elutant were dried in SpeedVac.

40% of released *N*-glycans treated with sialidase A (Prozyme, Hayward, CA) at 37°C overnight in 250 mM sodium phosphate, pH 6.0. Desialylated N-glycan and additional 40% released N-glycan were reduced by 0.5 M $NaBH_4$ in 10 mM NaOH at 50°C overnight. Both samples were desalted by using a C18 Zip tip fill with Dowex (see section of "purification of *O*-glycan").

Purification of O-glycans

Dried (*O*-glyco)peptides were suspended in 50 µL of 0.5 M $NaBH_4$ in 50 mM NaOH for 16 h at 50°C. The resultant sample was neutralized by adding 2.5 µL glacial acetic acid. Meantime, a micro-column in C18 Zip tip was packed. To do so, 25 µL Dowex 50W-X8 (H+, 200-400 mesh, BioRad, Bio-Rad Laboratories AB, Sundbyberg, Sweden) were packed to zip tip. Micro-column was washed with 2 × 60 µL of methanol, 2 × 60 µL of 1 M HCl, followed by 2 × 60 µL MQH_2O. Once the sample was applied, the microcolumn was further washed with 3 × 25 µL of MQH_2O. These washouts and breakthrough were combined and dried in SpeedVac. To remove boric acid, the dried samples were repeatedly dried in 5 × 50 µL of 1% HOAc in methanol.

LC-MS analysis

Dried *N*- and *O*-glycan samples were suspended in MQH_2O and analyzed with LC-MS/MS in negative mode. LC was carried out with a homemade capillary graphitized carbon (250 μmi.d. × 10 cm fused silica capillary). Oligosaccharides were identified from their MS/MS spectra manually and validated using UniCarb-DB (http://unicarb-db.biomedicine.gu.se/) hosted in this group.

Laboratory J

Release of N-glycans

The dried sample was dissolved in 0.1 M Tris-HCl buffer, pH 8.2 containing 0.01 M $CaCl_2$. The sample was denatured by heating for 5 min at 100°C. After cooling, the sample was digested with the trypsin (37°C, overnight). The sample was then heated at 100°C for 5 min to inactivate trypsin and spun at 3000 rpm in a refrigerated centrifuge for 15 min. The supernatant was collected and dried. The sample was then passed through a C18 sep-pak cartridge and washed with 5% acetic acid to remove contaminants (salts, free sugar, etc.). Peptides and glycopeptides were eluted in series with 20% isopropanol in 5% acetic acid, 40% isopropanol in 5% acetic acid and 100% isopropanol and dried in a speed vacuum concentrator. The dried samples were combined and incubated with PNGase F at 37°C overnight to release *N*-glycans. After digestion, the sample was passed through a C18 sep-pak cartridge and the released *N*-glycans was eluted with 5% acetic acid and dried by lyophilization.

Release of O-glycans

O-linked carbohydrate fractions were cleaved from *O*-glycopeptides, which were recovered after PNGase F digestion, by β-elimination procedures. Briefly, 1 M sodiumborohydride in 50 mM sodiumhydroxide (NaOH) were added to the samples and incubated overnight at 45°C. The incubated samples were neutralized with 10% acetic acid and desalted by passing through a packed column of Dowex™ resins (50 W × 8–100, Sigma Aldrich, St. Louis, MO) and lyophilized. The borate was removed with methanol/acetic acid (9:1) under a stream of nitrogen gas, and the samples were passed through a C18 reversed phase cartridge. The carbohydrate fractions (*O*-linked glycans) were eluted with 5% acetic acid. The carbohydrate fractions were dried by lyophilization.

Preparation of the per-O-methylated carbohydrates

The released *N*-linked glycan fraction was dissolved in dimethylsulfoxide and then permethylated based on the method of Anumula and Taylor (Anumula and Taylor, 1992). The reaction was quenched by addition of water and per-O-methylated carbohydrates were extracted with dichloromethane. Per- *O*-methylated glycans were

further cleaned of contaminants by passing the sample to C18 cartridges. Purified glycans were dried under a stream of nitrogen gas and were dissolved with methanol and profiled by mass spectrometry.

Mass spectrometry

MALDI-TOF/MS was performed in the reflector positive ion mode using α-dihydroxybenzoic acid (DHBA, 20 mg/mL solution in 50% methanol:water) as a matrix. The spectrum was obtained by using AB SCIEX TOF/TOF™ 5800 (AB SCIEX).

NSI-MSn analysis was determined by using on a LTQ Orbitrap XL mass spectrometer (ThermoFisher) equipped with a nanospray ion source. Permethylated glycans were dissolved in 1 mM NaOH in 50% methanol and infused directly into the instrument at a constant flow rate of 0.5 μL/ min. A full FTMS spectrum was collected at 30 000 resolution. The capillary temperature was set at 210°C and MS analysis was performed in the positive ion mode.

For total ion mapping (automated MS/MS analysis), m/z range, 800 to 2000 was scanned with ITMS mode in successive 2.8 mass unit windows that overlapped the preceding window by 2 mass units.

For calculating the relative intensity percentage of each *N*-glycan signal, the highest isotopic peak from peak cluster of isotopic ions was chosen.

Laboratory K

Preparation of N-glycans with PNGase F

Cellular *N*-glycans were enzymatically released with PNGaseF from the glycoproteins blotted onto PVDF membrane. The membrane or cytosolic proteins were re-dissolved in 1% SDS (100 ml). The portions of soluble sample solutions were dot-blotted (2.5 mL × 3) on the PVDF membranes and left to dry overnight. We performed the following preparation according to the procedure reported by Wilson et al [10] and Nakano et al [11] . Protein spots were cut from PVDF membranes and placed in separate wells of a 96-well microtiter plate. The spots were covered with 100 mL of 1% (w/v) poly(vinylpyrrolidone) 40000 in 50% (w/v) methanol for 20 min, and washed with water (100 mL × 5 times). To release N-glycans, PNGase F (3 U, Roche Diagnostics) in 10 mL in 20 mM phosphate buffer (pH 7.3) was added to each sample well and the 96-well plate was incubated at 37^{o}C for 15 min. After additionally adding 10 mL of water to the well, the 96-well plate was incubated at 37^{o}C overnight. After incubation, the plate was sonicated. Released *N*-glycans (20 mL) were collected, and each sample well was washed with water (40 mL × 3), and the washing solution was combined. Ammonium acetate buffer (100 mM, pH 5.0, 24 mL) was added to the combined solution followed by incubation for 1 h at room temperature. After evaporating to dryness of sample, the *N*-glycan samples were reduced with 1 M $NaBH_4$/50

mM KOH (20 mL) at 50°C for 3 h. The samples were neutralized with 1 mL glacial acetic acid, and purified with carbon microcolumns. The *N*-glycan alditols were collected in 0.1% formic acid containing 25% ACN, and dried under vacuum.

Preparation of O-glycans with β-Elimination

After the removal of the *N*-glycans, each protein spot remaining on the membrane was re-wet with 2 μL of methanol. A solution of 0.5 M $NaBH_4$ in 50 mM KOH (20 μL) was applied, and the spots were incubated for 16 h at 50°C. After adding 2 μL of acetic acid to stop the reaction, the mixture was passed through a cation-exchange column to remove salts. After dryness, finally the sample re-suspended with water was injected to LC-ESI MS.

LC-ESI/MS for analysis of N- and O-glycan alditols

Capillary LC-ESI/MS and MS/MS were performed using Agilent 1100 series HPLC Cap LC system coupled to an Esquire HCT (Bruker Daltonics). *N*-glycan alditols were applied to a HyperCarb column (5 mm HyperCarb, 0.32 mm I.D. × 100 mm, Thermo Hypersil, Runcorn, UK) in combination with a guard column (5 mm HyperCarb, 0.32 mm I.D. × 20 mm). Mobile phases were: (A) 10 mM NH_4HCO_3 and (B) ACN. The elution gradient for *N*-glycan analysis was as follows: 2–16%(v/v) linear gradient of B over 45 min; 16–25% linear gradient of B over 20 min, 25–45% linear gradient of B over 15 min, 45% of B for 10 min, and re-equilibrated with A for 20 min. 0–25% (v/v) linear gradient of B over 25 min, 25–80% (v/v) linear gradient of B for 15 min over 15 min. Flow rate was 5 μL/ min. The sample injection volume was 10 μL and ESI-MS/MS was performed in a negative ion mode with three scan events: a full scan with the mass range of m/z 200–2500. The scan rates were 8100 amu/s for the MS mode and, for MS/MS mode, the scan rates were 26000 amu/s. Monoisotoic masses were assigned with possible monosaccharide compositions using the GlycoMod tool available on the ExPASy server (mass tolerance for precursor ions is +/− 0.2 Da) and proposed oligosaccharide structures were further verified through annotation using a fragmentation mass matching approach based on the MS/MS data.

The relative abundance of N- and O-glycan alditols

The relative abundance of each glycan structure was calculated based on the peak area on EIC of the corresponding glycans. Total peak area of glycans from each cell was set to 100%, and the relative abundance (%) was calculated for each glycan structure for each cell.

Laboratory L

Sample preparation

Depending on their solubility the obtained samples were taken up in 200–2000 µL buffer (10% glycerin, 1% SDS, 50 mM DTT, 0.0625 M Tris-HCl pH 6.8). Per sample a volume corresponding to 75 µg of protein was dot-blotted onto PVDF membranes as described by Jensen *et al*. and the respective spots were subsequently used for sequential *N*- and *O*-glycan release as described in detail by Jensen *et al*.[9].

MS analysis

Porous graphitized carbon (PGC) LC-ESI-MS/MS analysis was performed on an AmaZon speed ETD ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) coupled to an Ultimate 3000 UHPLC system (Dionex, Part of Thermo Fisher, Germany).

The LC-ESI/MS instrument was controlled by Hystar 3.2 – SR2 software (Bruker Daltonics); this software allows combined control of the LC-software [Chromeleon Xpress (Dionex)] and the MS instrument specific control software [trapControl 7.1. (Bruker Daltonics)].

The *N*- and *O*-glycans were dissolved in 12 µL water prior PGC-LC-ESI MS/MS analysis (5 µm Hypercarb material, Thermo Hypersil, Runcorn UK [precolumn: 30 mm × 0.32 mm I.D., analytical column: 100 mm × 0.32 mm I.D.]). The column temperature was held at 40°C throughout the whole analysis. PGC-LC-ESI MS/MS analysis was performed as described in detail previously [9] using a linear separation gradient from 5% up to 20% solvent B (90% ACN in 10 mM ammoniumbicarbonate) within 54.8 min, followed by 5 min increase of 15%/min and a final 5 min column washing step of 95% B before equilibrating the column for 12 min in starting conditions (2% solvent B). Meanwhile the precolumn was equilibrated in 100% 10 mM ammoniumbicarbonate (solvent A, sample loading conditions).

The LC system was coupled to an AmaZon ETD speed ESI ion trap MS using the capillary standard sprayer (Bruker Daltonics). In the MS device, the voltage of the capillary outlet was set to 3500 V and the end plate to 500 V. Transition parameters were optimized to exclude any insource decay during ionisation and ion transport within the mass spectrometer. The solvents were evaporated at a temperature of 220°C with a nitrogen gas flow rate of 5 L/min and 8 psi. Negative ion mode MS spectra were obtained within a mass range from *m/z* 350 to *m/z* 1800. For MS/MS precursor selection the three most intense ions above the absolute intensity of 3000 and 20% relative intensity threshold were isolated with an isolation width of m/z 3. CID was performed using helium as a collision gas with a pressure of 4.89×10^{-6} mbar inside the 3D ion trap. "Smart fragmentation" setting of the instrument was used with a start amplitude of 30% and 120% end amplitude over 32 ms for performing CID

within the trap.

Structural assignment and relative quantification

The identified signal masses were obtained by manual evaluation of the LC-ESI MS/MS spectra and the detected/calculated $[M-H]^-$ masses were submitted to a Glycomod (http://web.expasy.org/glycomod/) search to obtain theoretical possible glycan compositions. The applied settings were "reduced glycans", "$[M-H]^-$" and a mass tolerance of 0.5 Da was set. Structural assignment was performed based on the detected masses, retention time and MS/MS fragmentation spectra [9, 12, 13]. Furthermore the well-known biosynthetic glycan pathways were applied in interpretation of the obtained results for glycan structure determination. Presented glycan structures were drawn using ProteinScape 3.0.3.346 (Bruker Daltonics).

For quantification of the data set the peak areas of the EIC were integrated and the relative glycan intensities were calculated based on the total area of each sample.

Reference

1. Takahashi, N., Nakagawa, H., Fujikawa, K., Kawamura, Y., Tomiya, N.: Three-dimensional elution mapping of pyridylaminated N-linked neutral and sialyl oligosaccharides. Anal. Biochem. 226, 139–146 (1995).

2. Takahashi, N., Kato, K.: GALAXY (Glycoanalysis by the Three Axes of MS and Chromatography): a Web Application that Assists Structural Analyses of *N*-Glycans. Trends Glycosci. Glycotechnol. 15, 235–251 (2003).

3. Yagi, H., Takahashi, N., Yamaguchi, Y., Kimura, N., Uchimura, K., Kannagi, R., Kato, K.: Development of structural analysis of sulfated N-glycans by multidimensional high performance liquid chromatography mapping methods. Glycobiology. 15, 1051–1060 (2005).

4. Yagi, H., Saito, T., Yanagisawa, M., Yu, R.K., Kato, K.: Lewis X-carrying N-glycans regulate the proliferation of mouse embryonic neural stem cells via the Notch signaling pathway. J. Biol. Chem. 287, 24356–24364 (2012).

5. Yagi, H., Ohno, E., Kondo, S., Yoshida, A., Kato, K.: Development and Application of Multidimensional HPLC Mapping Method for O-linked Oligosaccharides. Biomolecules. 1, 48–62 (2011).

6. North, S.J., Huang, H.-H., Sundaram, S., Jang-Lee, J., Etienne, A.T., Trollope, A., Chalabi, S., Dell, A., Stanley, P., Haslam, S.M.: Glycomics profiling of Chinese hamster ovary cell glycosylation mutants reveals N-glycans of a novel size and complexity. J. Biol. Chem. 285, 5759–5775 (2010).

7. Lei, M., Novotny, M. V, Mechref, Y.: Sequential enrichment of sulfated glycans by strong anion-exchange chromatography prior to mass spectrometric measurements. J. Am. Soc. Mass Spectrom. 21, 348–357 (2010).

8. Kang, P., Mechref, Y., Novotny, M. V: High-throughput solid-phase permethylation of glycans prior to mass spectrometry. Rapid Commun. Mass Spectrom. 22, 721–734 (2008).

9. Jensen, P.H., Karlsson, N.G., Kolarich, D., Packer, N.H.: Structural analysis of N- and O-glycans released from glycoproteins. Nat. Protoc. 7, 1299–1310 (2012).

10. Wilson, N.L., Schulz, B.L., Karlsson, N.G., Packer, N.H.: Sequential analysis of N- and O-linked glycosylation of 2D-PAGE separated glycoproteins. J. Proteome Res. 1, 521–529 (2002).

11. Nakano, M., Saldanha, R., Gobel, A., Kavallaris, M., Packer, N.H.: Identification of glycan structure alterations on cell membrane proteins in desoxyepothilone B resistant leukemia cells. Mol. Cell. Proteomics. 10, M111.009001 (2011).

12. Pabst, M., Bondili, J.S., Stadlmann, J., Mach, L., Altmann, F.: Mass + retention time = structure: a strategy for the analysis of N-glycans by carbon LC-ESI-MS and its application to fibrin N-glycans. Anal. Chem. 79, 5051–5057 (2007).

13. Everest-Dass, A. V, Jin, D., Thaysen-Andersen, M., Nevalainen, H., Kolarich, D., Packer, N.H.: Comparative structural analysis of the glycosylation of salivary and buccal cell proteins: innate protection against infection by Candida albicans. Glycobiology. 22, 1465–1479 (2012).

Glycoconjugate Journal
Supplementary material for

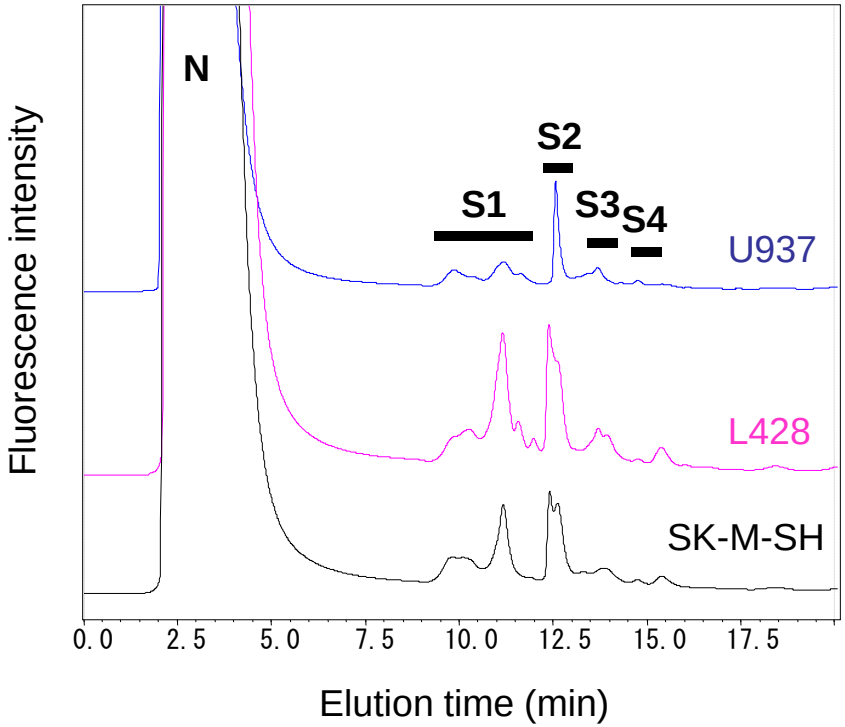
Comparison of analytical methods for profiling *N*- and *O*-linked glycans from cultured cell lines **—HUPO Human Disease Glycomics/Proteome Initiative Multi-institutional Study—**

Hiromi Ito, Hiroyuki Kaji, Akira Togayachi, Parastoo Azadi, Mayumi Ishihara, Rudolf Geyer, Christina Galuska, Hildegard Geyer, Kazuaki Kakehi, Mitsuhiro Kinoshita, Niclas G. Karlsson, Chunsheng Jin, Koichi Kato, Hirokazu Yagi, Sachiko Kondo, Nana Kawasaki, Noritaka Hashii, Daniel Kolarich, Kathrin Stavenhagen, Nicolle H. Packer, Morten Thaysen-Andersen, Miyako Nakano, Naoyuki Taniguchi, Ayako Kurimoto, Yoshinao Wada, Michiko Tajiri, Pengyuan Yang, Weiqian Cao, Hong Li, Pauline M. Rudd*, Hisashi Narimatsu

* to whom correspondence should be addressed:
Pauline M. Rudd (pauline.rudd@nibrt.ie.)
National Institute for Bioprocessing Research and Training (NIBRT) Dublin Ireland

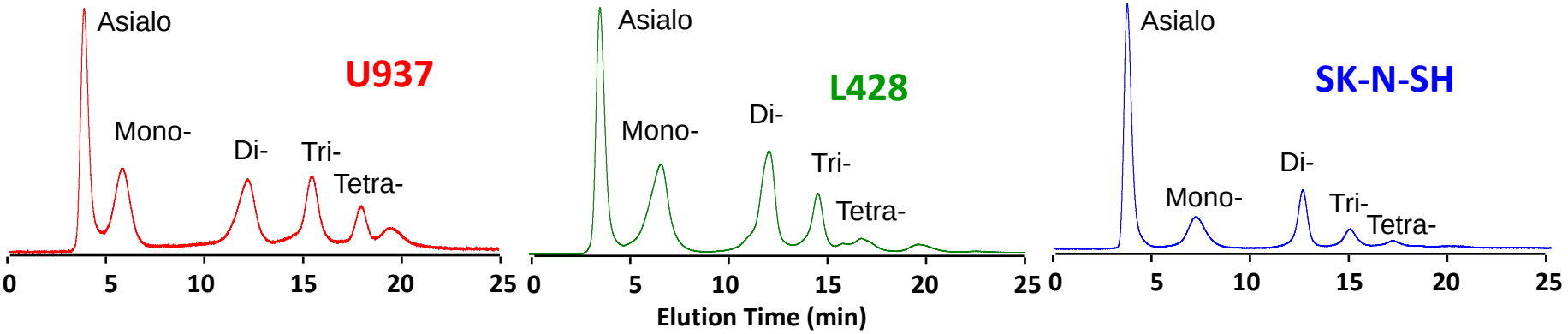
Supplementary Figure 1

N-glycosylation profiles classified according to charge on an DEAE (weak anion exchange) column by Lab A . Glycan structures were determined by the HPLC mapping using GALAXY. The N and S1-S4 fractions denote the neural and sialylated fractions, respectively. S1, S2, S3, and S4 denote the fractions of PA-N-glycans containing 1, 2, 3, and 4 sialic acid residues, respectively.



Supplementary Figure 2

HPLC profiling of *N*-glycans using serotonin affinity chromatography by Lab B. Glycan structures were determined by MSn after sialidase treatment. Mono-, mono-sialo; Di-, di-sialo; Tri-, tri-sialo; Tetra-, tetra-sialo



Supplementary Figure 3 **3rd-1_O**

Comparison of *O*-glycan profiles obtained from lyophilized cell pellets (a: L428, b: U937, c: SK-N-SH) by each laboratory used HPLC techniques. Percentages of the identified structures to the total glycans are indicated by the compositions as H, Hex; N, HexNAc; F, deoxyHex ; NA, NeuAc.