Deep Profiling Reveals Coordinated Remodeling of Ganglioside Metabolism in MCF-7 Breast Cancer Cell Line

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SUPPORTING INFORMATION

Methods

Materials and chemicals

HPLC grade acetonitrile (ACN), methanol (MeOH), isopropanol (IPA) and deionized water (H_2O) were purchased from Fisher Scientific Company (Ottawa, ON, Canada). Ethanol (EtOH) anhydrous, ethylene glycol, chloroform ($CHCl_3$) and ammonium hydroxide (NH_4OH) were purchased from Guoyao Chemical Reagent Company. Iron chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) was purchased from 3A materials. Titanium (IV) butoxide, Ammonium acetate (NH_4OAc), acetic acid (HOAc), and formic acid (FA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). N,N-Dimethylformamide (DMF) was obtained from Aladdin Industrial Corporation (Shanghai, China). The filter (Hydrophobic PTFE membrane, pore size 0.22 μm, diam. 13 mm) was purchased from Welch Materials, Inc. (Jinhua, Zhejiang, China).

Nomenclature of gangliosides

The abbreviation of the glycan portion of gangliosides follows Svennerholm's nomenclature[1], later adopted by IUPAC[2]. Using GT1b as an example: the first letter G indicates the ganglio-series; the second letter denotes the number of sialic acids (M = mono, D = di, T = tri, Q = tetra); the third character is an Arabic numeral indicating the number of neutral sugars (1 = 4 sugars, 2 = 3 sugars, 3 = 2 sugars, 4 = 1 sugar); the final lowercase letter indicates the position of the sialic acid(s) (a = one on the innermost Gal, b = two, c = three). Thus, GT1b refers to a ganglioside with three sialic acids (T), four neutral sugars (1), and two of the sialic acids located on the innermost galactose (b).

The annotation of the ceramide portion follows the Lipid MAPS nomenclature[3]. At the species level, ceramides are described by their total carbon number (C32–C46), number of double bonds (0–4), and number of hydroxyl groups (2–4), e.g., GT1b 36:1;O2.

At the chain compositional level, the long-chain base (LCB) is denoted by the carbon number (C16–C20), degree of unsaturation (0–2), and the number of hydroxyl groups (O2/O3), e.g., 18:1;O2. The N-acyl chain is similarly represented by its carbon number (C12–C26), number of double bonds (0–1), and hydroxyl groups (0–1), e.g., 18:0. The LCB and N-acyl chains are listed in order after the headgroup and separated by a slash, as in GT1b 18:1;O2/18:0.

At the C=C positional level, the C=C location is indicated using the "n-" notation, which counts from the methyl end, e.g., GT1b 18:1(n-14);O2/24:1(n-9).

Cell culture

The MCF-10A (CL-0525) and MCF-7 (CL-0149) cells were obtained from Procell (Wuhan, China). The MCF-10A cells were cultured in MCF-10A Cell Complete Medium (Procell, Wuhan, China), containing Dulbecco's Modified Eagle's Medium/ Nutrient Mixture F-12 (DMEM/F12), 5% horse serum, 20 ng/mL Epidermal Growth Factor (EGF), 0.5 μg/mL hydrocortisone, 10 μg/mL insulin, 1% non-essential amino

acids (NEAA) and 1% penicillin–streptomycin. The MCF-7 cells were cultured in DMEM (Analysis quiz, Beijing, China) supplemented with 10% fetal bovine serum (FBS) (Coning, Hartford, USA), 1% penicillin–streptomycin, 1× NEAA (Yeasen, Shanghai, China) and 2 mM L-Alanyl-L-Glutamine (Gibco, Waltham, USA). All cells were cultured under humidified atmosphere of 5% CO_2 at 37 °C.

Synthesis of TiO_2 MNPs

The TiO_2 MNPs were synthesized according to the protocol previously reported[4, 5]. To prepare the magnetic Fe_3O_4 core nanoparticles, 7.2 g of anhydrous sodium acetate and 2.7 g of $FeCl_3 \cdot 6H_2O$ were dissolved in 150 mL of ethylene glycol with vigorous stirring until a clear solution was obtained. The solution was then transferred to a Teflon-lined stainless-steel autoclave, sealed, and heated at 200 °C for 16 hours to facilitate the formation of Fe_3O_4 nanoparticles. The resulting magnetic Fe_3O_4 nanoparticles were collected from the bottom of the autoclave, washed three times with water and ethanol under ultrasonic treatment, and dried at 50 °C. Then, a total of 160 mg of magnetic Fe_3O_4 nanoparticles was added to 395 mL of EtOH. After a 60-minute ultrasound treatment, 1.44 mL of ammonia solution (28 wt%) was added to the mixture. Subsequently, a dropwise addition of 3.2 mL of titanium (IV) butoxide and 5 mL of EtOH was made to the reaction system, which was maintained at 45 ℃ for 24 hours. The resulting TiO_2 MNPs were washed three times with H_2O and three times with EtOH, then dehydrated in a vacuum drying oven. Finally, the TiO_2 MNPs were calcined at 400 ℃ for 2 hours and stored at room temperature.

Offline PB derivatization

PB derivatization was carried out using a homemade flow microreactor. A GM3 standard mixture (from bovine milk) was prepared at a concentration of 5 μM (based on the most abundant GM3 species, GM3 40:1;O2) and dissolved in 100 μL of ACN/H_2O (50:50, v/v) containing 10 mM 2-acetylpyridine (2-AcPy). The solution was purged with nitrogen gas for 10 minutes to remove residual oxygen prior to the PB reaction. It was then injected into the flow microreactor and exposed to UV irradiation at 254 nm for 2–25 seconds. Based on optimization, a 20-second irradiation time was selected as optimal. For biological samples, gangliosides enriched from 5.5×10^6 cells were dissolved in 100 μL of ACN/H_2O (50:50, v/v) containing 10 mM 2-AcPy and subjected to the PB reaction under the same 20-second UV irradiation conditions.

Amide HILIC-TIMS-MS/MS

Amide HILIC

LC separations were performed on a Waters ACQUITY UPLC I-Class system (Waters, Milford, MA, USA) hyphenated with a hybrid trapped ion mobility-quadrupole time-of-flight mass spectrometer (timsTOF, Bruker Daltonics, Bremen, Germany). An ACQUITY Premier BEH Amide column (150 mm × 2.1 mm, 1.7 μm; Waters, Milford, MA, USA) was used for separation. The mobile phase A contained H_2O with 10 mM aqueous ammonium acetate and 0.1% acetic acid. mobile phase B contained ACN with 0.1% acetic acid. The flow rate was set at 0.3 mL/min. Oven temperature was set at

25 °C. The chromatographic gradient was as follows: 0-15 min: 87.7%-60% B; 15 - 15.1 min: 60% B-87.7% B; 15.1-18 min: 87.7% B.

MS and TIMS

The MS parameters were optimized as follows: capillary voltage, 3500 V; end plate offset, 500 V; nebulizer pressure, 2 bar; dry gas flow rate, 8 L/min; dry temperature, 200 °C. The MS acquisition range was set to m/z 500–2500 in both positive and negative ion modes.

The TIMS parameters were optimized in "detect" mode with a $1/K_0$ range of 0.87–2.24 $V \cdot s/cm^2$. The ramp time was approximately 370 ms, and the spectra rate was 2.68 Hz.

For MS/MS analysis, CID was performed in MRM mode with a precursor isolation window of 2 Da. The CID energy was set to 45–80 eV for negative ion mode and 30–45 eV for positive ion mode.

Tuning Mix (ESI-L, Agilent, Santa Clara, CA, USA) was used for calibration of both m/z and ion mobility values.

RNA extraction and qRT-PCR

MCF-10A and MCF-7 cells (5.5×10^6 each) were cultured in 10 cm dishes and detached using 0.5% Trypsin-EDTA (Gibco, Waltham, USA). Trypsinization was quenched by adding fresh medium, and cells were collected by centrifugation at 800 rpm for 3 minutes. All consumables, including tips and tubes, were RNase-free to prevent RNA degradation. Total RNA was extracted using TRIzol® Reagent (Invitrogen, Carlsbad, USA), following the manufacturer's protocol. RNA quantity and purity were determined with a NanoDrop spectrophotometer (Thermo Scientific, Waltham, USA). Subsequently, 1 μg of total RNA was reverse-transcribed into cDNA using the NovoScript® Plus All-in-one 1st Strand cDNA Synthesis SuperMix (Novoprotein, Millburn, USA). Quantitative real-time PCR (qRT-PCR) was carried out on a CFX96 Real-Time PCR System (Bio-Rad, California, USA) with NovoStart® SYBR qPCR SuperMix Plus (Novoprotein, Millburn, USA). Gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method, with *GAPDH* serving as the internal reference. Primer sequences are listed in Table S7.

Label-free quantitative proteomics

Proteins were reduced with 100 mM tris(2-carboxyethyl)phosphine (TCEP) and alkylated with 400 mM 2-chloroacetamide, followed by overnight digestion at 37 °C using sequencing-grade modified trypsin. Resulting peptides were desalted using Styrenedivinylbenzene-Reversed Phase Sulfonate (SDB-RPS) and dried under vacuum in a SpeedVac concentrator.

LC-MS/MS analysis were performed using a Thermo Vanquish Neo UHPLC system coupled to a Thermo Scientific Orbitrap Astral mass spectrometer. A 25 min linear gradient was applied at a flow rate of 0.3 μL/min. The mobile phase consisted of 0.1%

formic acid in water (A) and 80% acetonitrile with 0.1% formic acid (B). Mass spectrometric analysis was operated in data-independent acquisition (DIA) mode. Full MS scans were acquired at a resolution of 240,000 (*m/z* 200), with the automatic gain control (AGC) target set to 500%. Fragment ions were detected by the Astral analyzer at a resolution of 80,000, with a maximum injection time of 3 ms. DIA windows were automatically distributed across the *m/z* range of 380–980. Fragmentation was achieved using a normalized collision energy (NCE) of 25%.

Raw MS data were analyzed using Spectronaut software (version 18). Peptide spectral matching was performed against a UniProt human FASTA database. Search parameters included: trypsin as the digestion enzyme with allowance for up to two missed cleavages; carbamidomethylation of cysteine residues as a fixed modification; and methionine oxidation as a variable modification. Precursor ion mass tolerance was set to 20 ppm, and fragment ion mass tolerance to 0.02 Da. Quantification was based on MS^2 ion intensity. Peptide and protein identifications were filtered at a false discovery rate (FDR) threshold of 1%.

Supplementary Figures

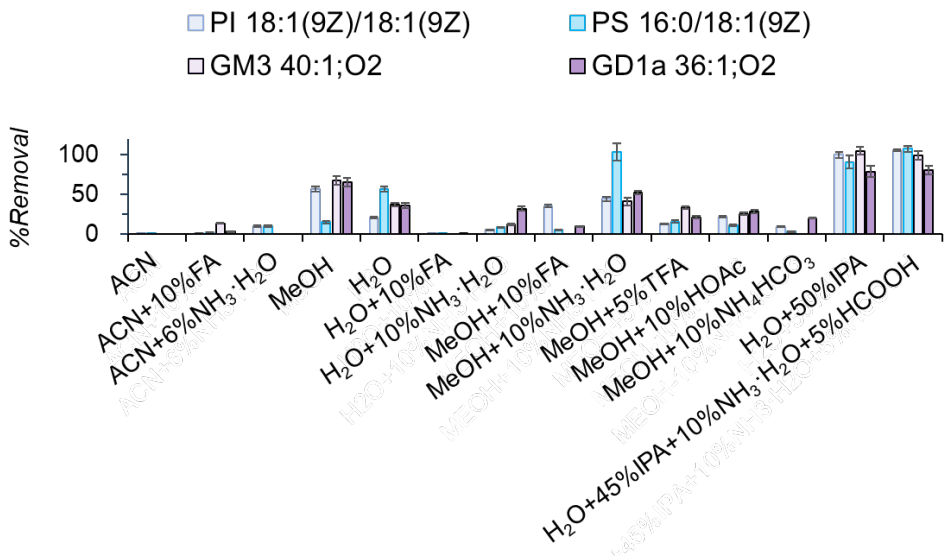


Fig. S1. Evaluation of washing buffer conditions for the removal of PI 18:1(9Z)/18:1(9Z), PS 16:0/18:1(9Z), GM3 40:1;O2, and GD1a 36:1;O2 from TiO_2 MNPs. Lipids were loaded onto TiO_2 MNPs using 6% aqueous NH_3/94% ACN as the loading buffer and then washed by each washing buffer solution. The lipids eluted by the washing buffer were quantified for calculating %removal.

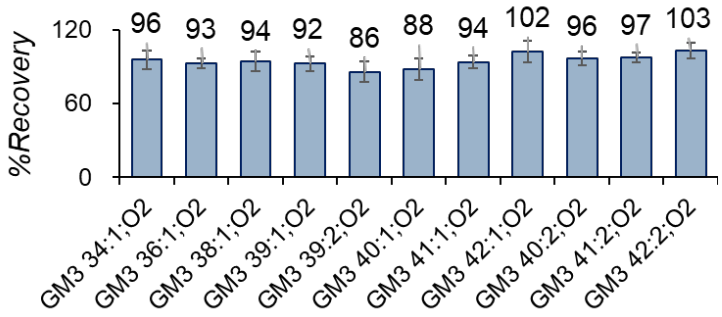


Fig. S2. %Recovery of GM3 standards with varying ceramide chain lengths (C34–C42) and degrees of unsaturation (1–2). The initial concentrations of the standards range from approximately 10 to 25 nM.

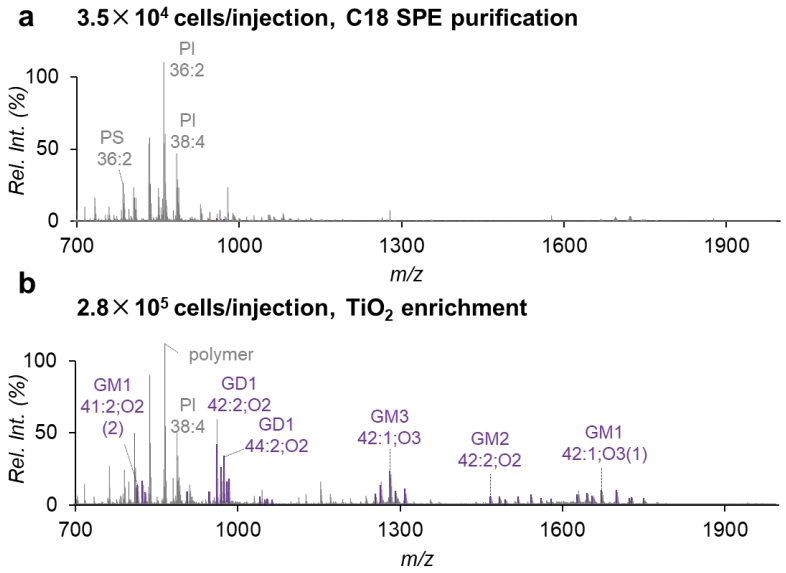


Fig. S3. MS^1 spectra obtained from HILIC-MS analysis of MCF-7 cells. (a) MS^1 spectra acquired from 3.5 × 10^4 cells per injection following C18 SPE purification, recorded over retention time of 0–12 min. (b) MS^1 spectra acquired from 2.8 × 10^5 cells per injection following TiO_2 enrichment, recorded over retention time of 0–12 min.

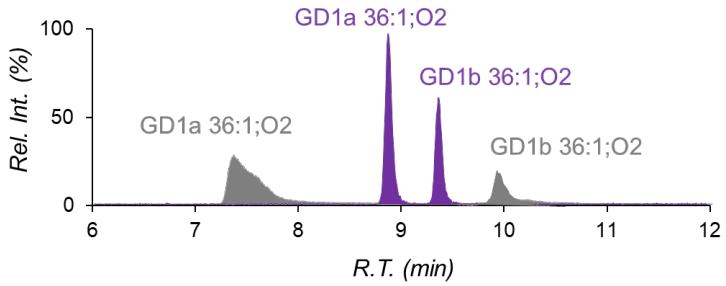


Fig. S4. Comparison of amide HILIC (purple) and conventional HILIC (gray) for the separation of gangliosides. Equal amounts (1 pmol each) of GD1a 36:1;O2 and GD1b 36:1;O2 were injected to evaluate chromatographic performance.

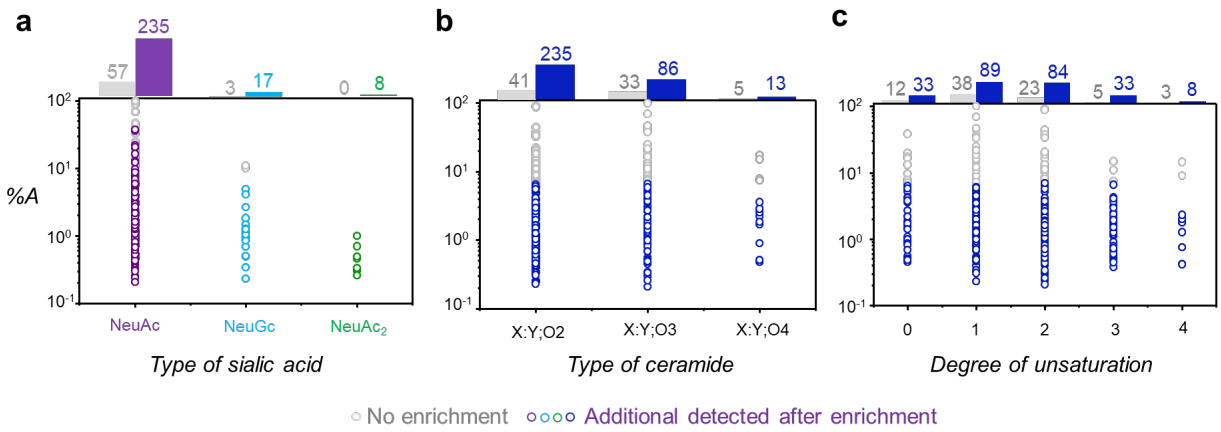


Fig. S5. Relative quantitation of ganglioside species in MCF-7 cells with (2.6×10^5 cells/injection) or without (3.3×10^4 cells/injection) selective enrichment. Species are plotted according to their (a) sialic acid type (NeuAc, NeuGc, $NeuAc_2$), (b) ceramide type (X:Y;O2, X:Y;O3, X:Y;O4), and (c) degrees of unsaturation (0-4).

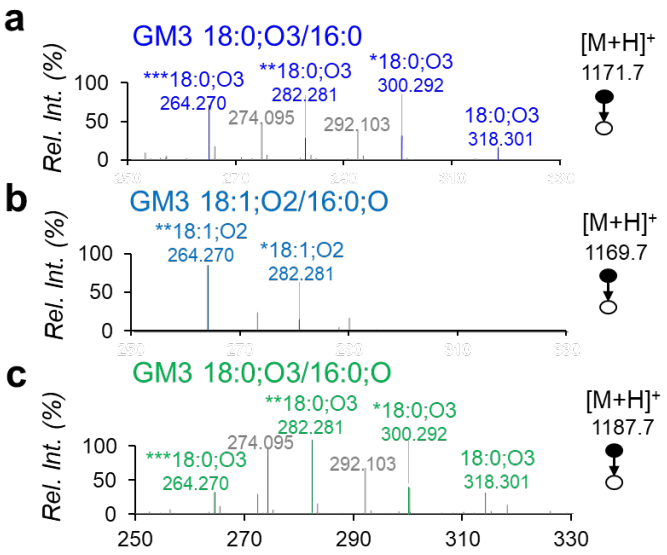


Fig. S6. Characteristic fragment ions in positive ion mode MS^2 CID spectra to determine the LCB and N-acyl chain compositions of ceramides with X:Y;O2, X:Y;O3, and X:Y;O4 structures. Positive ion mode MS^2 CID spectrum of (a) GM3 34:0;O3 ($[M+H]^+$, *m/z* 1171.7), (b) GM3 34:1;O3 ($[M+H]^+$, *m/z* 1169.7), (c) GM3 34:0;O4 ($[M+H]^+$, *m/z* 1187.7). The spectra demonstrate the characteristic fragments for ceramides containing a PHS-type LCB and a hFA.

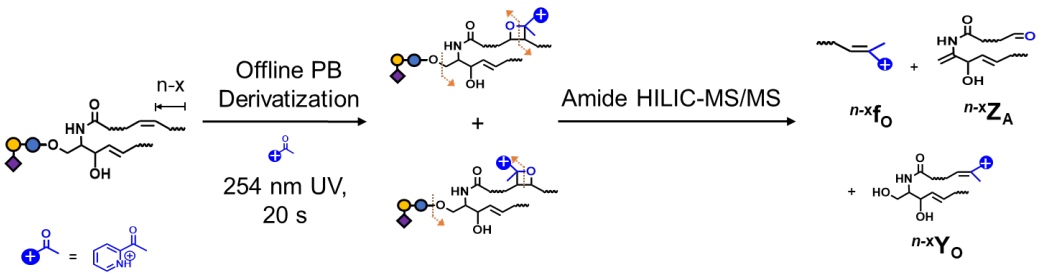
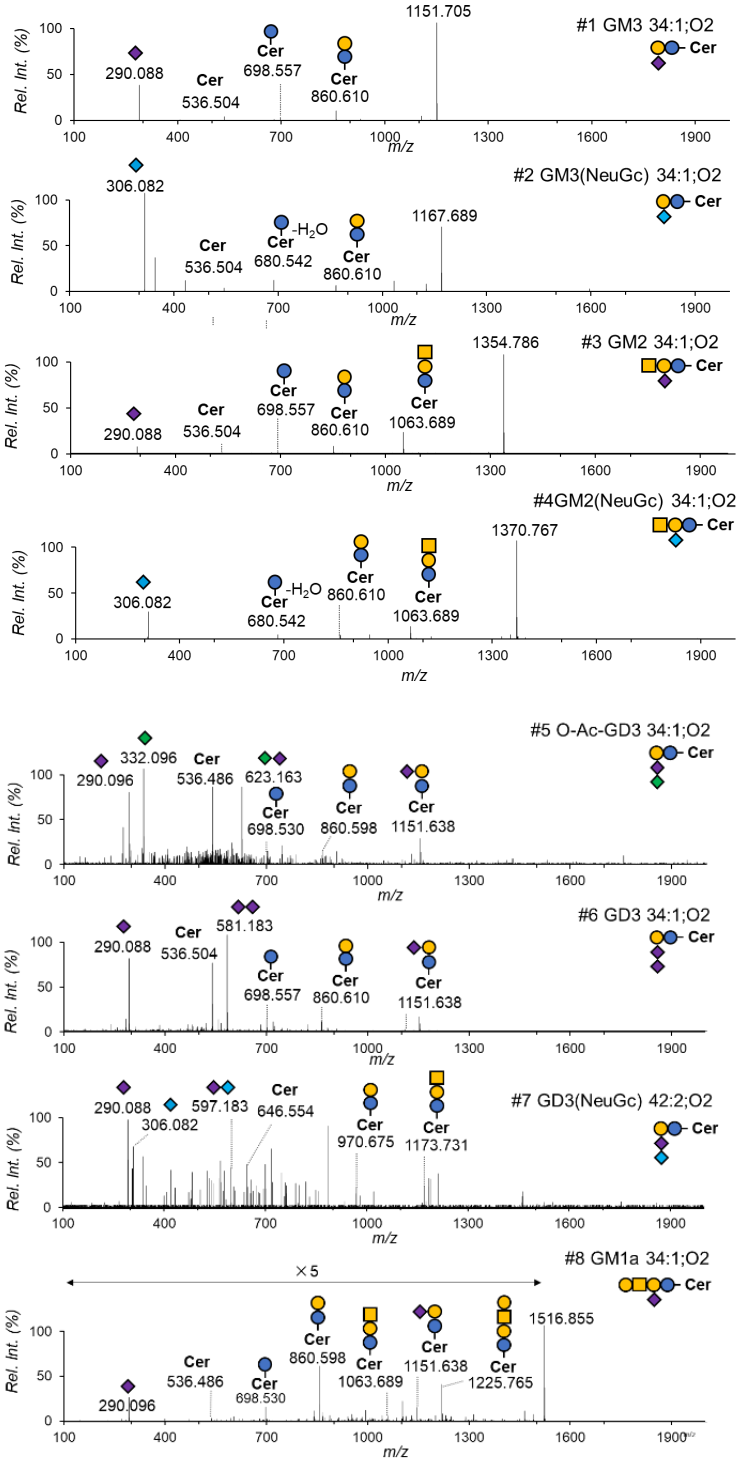
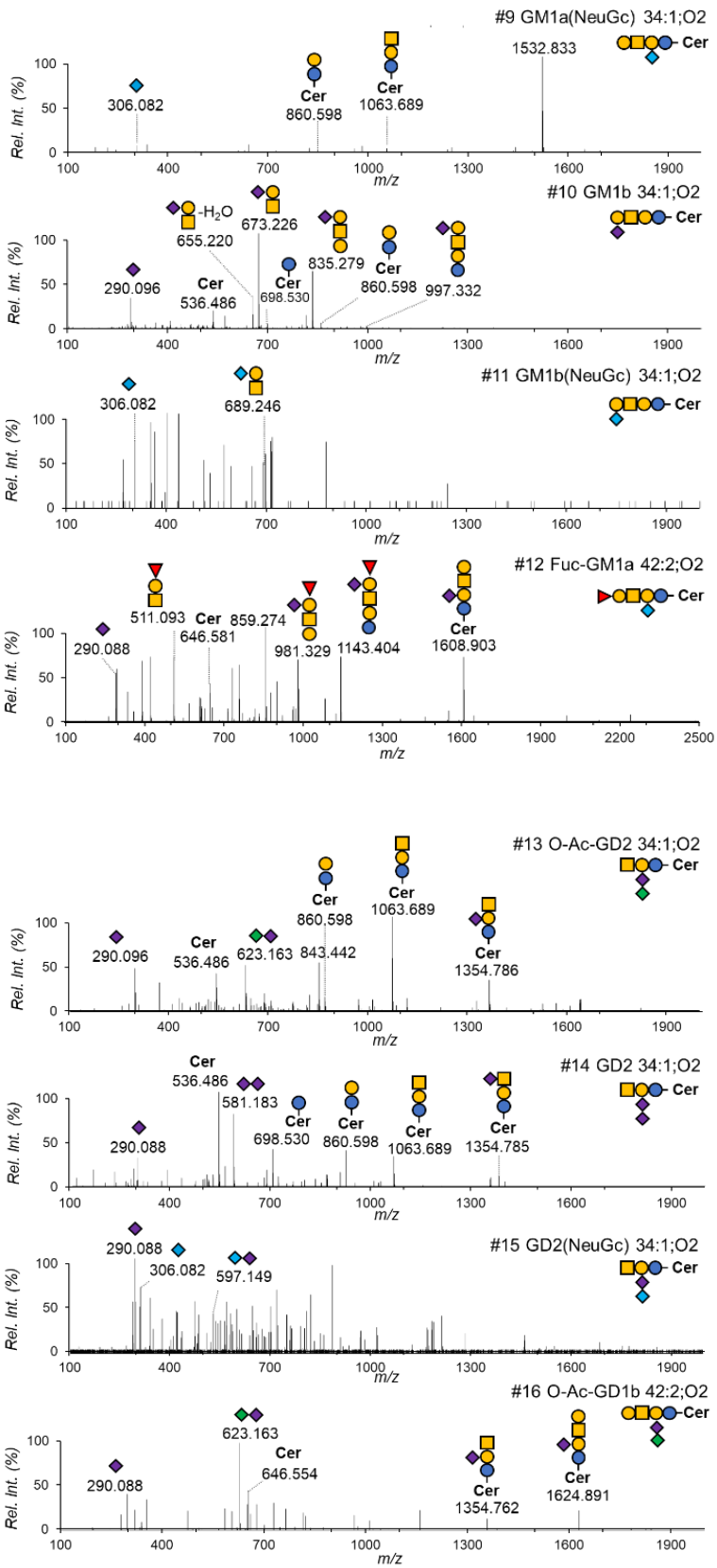
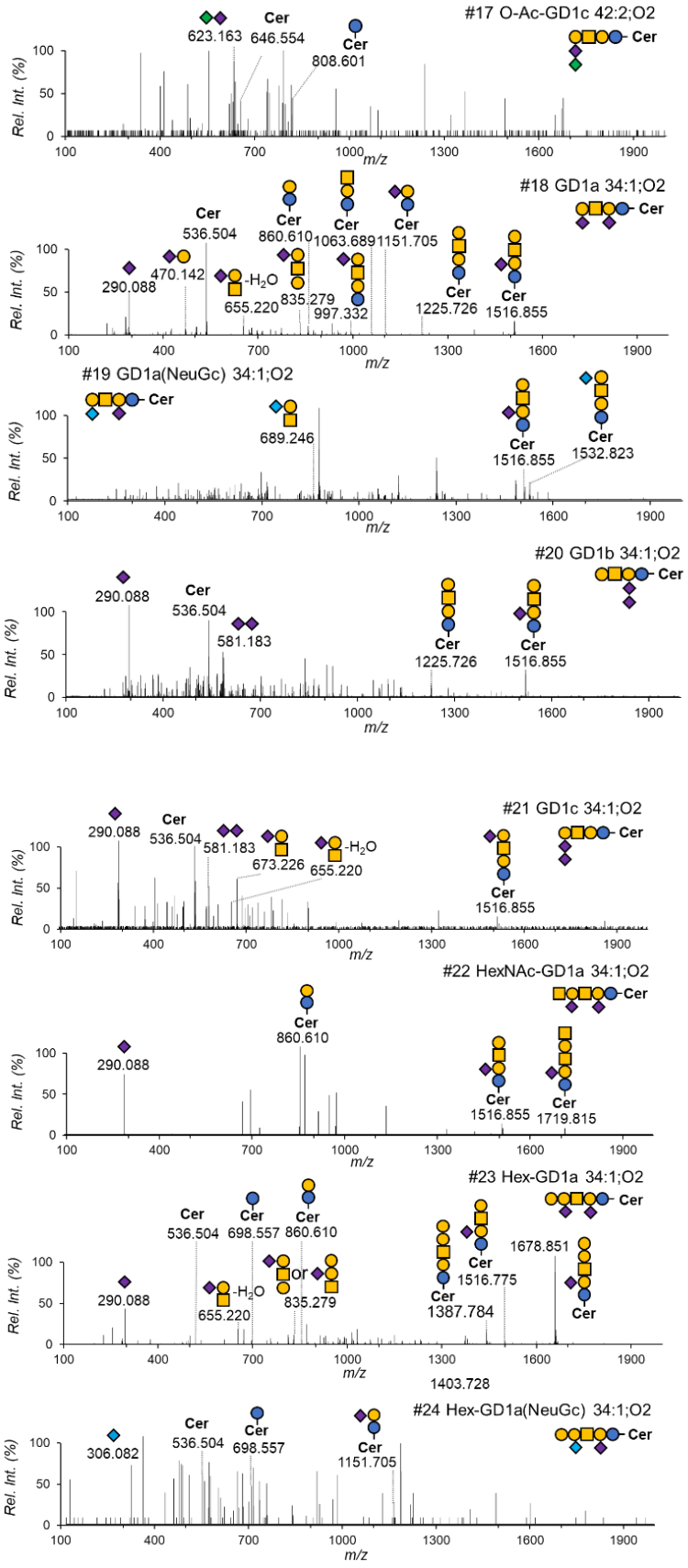
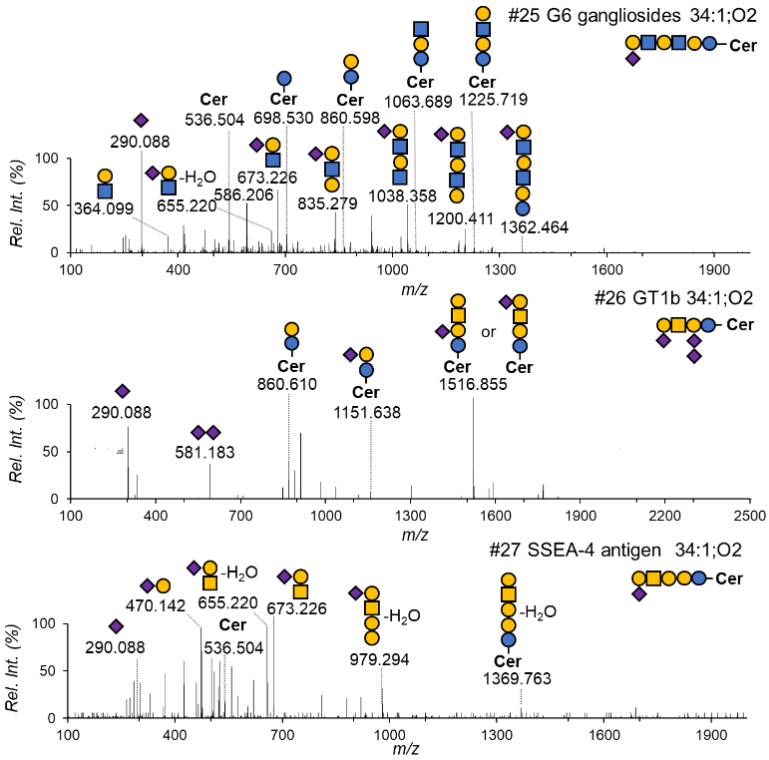


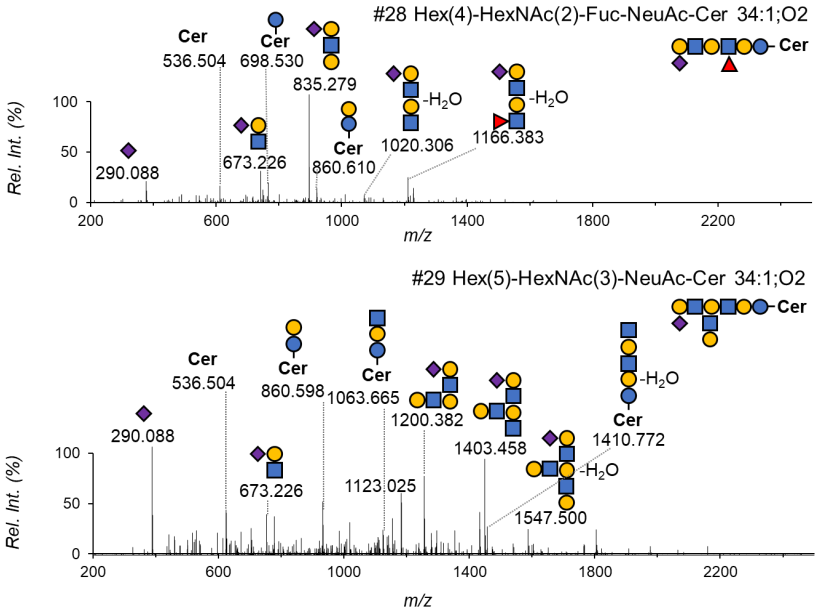
Fig. S7. Schematic illustration of the Paternò–Büchi (PB) derivatization of gangliosides using 2-acetylpyridine (2-AcPy) as the PB reagent.


#1 GM3 34:1;O2
1151.705
290.088
Cer 536.504
Cer 698.557
Cer 860.610
#2 GM3(NeuGc) 34:1;O2
306.082
Cer 536.504
Cer -H2O 680.542
Cer 860.610
1167.689
#3 GM2 34:1;O2
1354.786
290.088
Cer 536.504
Cer 698.557
Cer 860.610
Cer 1063.689
#4GM2(NeuGc) 34:1;O2
1370.767
306.082
Cer -H2O 680.542
Cer 860.610
Cer 1063.689
#5 O-Ac-GD3 34:1;O2
332.096
290.096
Cer 536.486
623.163
Cer 698.530
Cer 860.598
Cer 1151.638
#6 GD3 34:1;O2
581.183
290.088
Cer 536.504
Cer 698.557
Cer 860.610
Cer 1151.638
#7 GD3(NeuGc) 42:2;O2
290.088
306.082
597.183
Cer 646.554
Cer 970.675
Cer 1173.731
#8 GM1a 34:1;O2
×5
1516.855
290.096
Cer 536.486
Cer 698.530
Cer 860.598
Cer 1063.689
Cer 1151.638
Cer 1225.765
Rel. Int. (%)
m/z


#9 GM1a(NeuGc) 34:1;O2
1532.833
Cer
306.082
Cer
860.598
Cer
1063.689
#10 GM1b 34:1;O2
-H2O
655.220
673.226
835.279
290.096
Cer
536.486
Cer
698.530
Cer
860.598
997.332
#11 GM1b(NeuGc) 34:1;O2
306.082
689.246
#12 Fuc-GM1a 42:2;O2
511.093
Cer
646.581
859.274
290.088
981.329
1143.404
Cer
1608.903
#13 O-Ac-GD2 34:1;O2
Cer
860.598
Cer
1063.689
290.096
Cer
536.486
623.163
843.442
Cer
1354.786
#14 GD2 34:1;O2
Cer
536.486
581.183
Cer
698.530
Cer
860.598
Cer
1063.689
Cer
1354.785
290.088
#15 GD2(NeuGc) 34:1;O2
290.088
306.082
597.149
#16 O-Ac-GD1b 42:2;O2
623.163
Cer
646.554
290.088
Cer
1354.762
Cer
1624.891
Rel. Int. (%)
m/z


#17 O-Ac-GD1c 42:2;O2
Cer
623.163
646.554
808.601
#18 GD1a 34:1;O2
536.504
860.610
1063.689
1151.705
470.142
290.088
-H2O
655.220
835.279
997.332
1225.726
1516.855
#19 GD1a(NeuGc) 34:1;O2
689.246
1516.855
1532.823
#20 GD1b 34:1;O2
290.088
536.504
581.183
1225.726
1516.855
#21 GD1c 34:1;O2
290.088
536.504
581.183
673.226
-H2O
655.220
1516.855
#22 HexNAc-GD1a 34:1;O2
860.610
290.088
1516.855
1719.815
#23 Hex-GD1a 34:1;O2
536.504
698.557
860.610
290.088
-H2O
655.220
or
835.279
1516.775
1387.784
1678.851
1403.728
#24 Hex-GD1a(NeuGc) 34:1;O2
306.082
536.504
698.557
1151.705
Rel. Int. (%)
m/z


#25 G6 gangliosides 34:1;O2
Cer
Cer 536.504
Cer 698.530
Cer 860.598
Cer 1063.689
Cer 1225.719
290.088
364.099
-H2O
655.220
673.226
586.206
835.279
1038.358
1200.411
1362.464
Rel. Int. (%)
m/z
#26 GT1b 34:1;O2
Cer
Cer 860.610
Cer 1151.638
or
Cer 1516.855
Cer
290.088
581.183
Rel. Int. (%)
m/z
#27 SSEA-4 antigen 34:1;O2
Cer
-H2O
470.142
655.220
673.226
Cer 536.504
290.088
-H2O
979.294
-H2O
Cer 1369.763
Rel. Int. (%)
m/z


#28 Hex(4)-HexNAc(2)-Fuc-NeuAc-Cer 34:1;O2
Cer
Cer 536.504
Cer 698.530
835.279
-H2O
-H2O
673.226
Cer 860.610
1020.306
1166.383
290.088
Rel. Int. (%)
m/z
#29 Hex(5)-HexNAc(3)-NeuAc-Cer 34:1;O2
Cer
Cer 536.504
Cer 860.598
Cer 1063.665
1200.382
1403.458
-H2O
Cer 1410.772
290.088
673.226
1123.025
-H2O
1547.500
Rel. Int. (%)
m/z

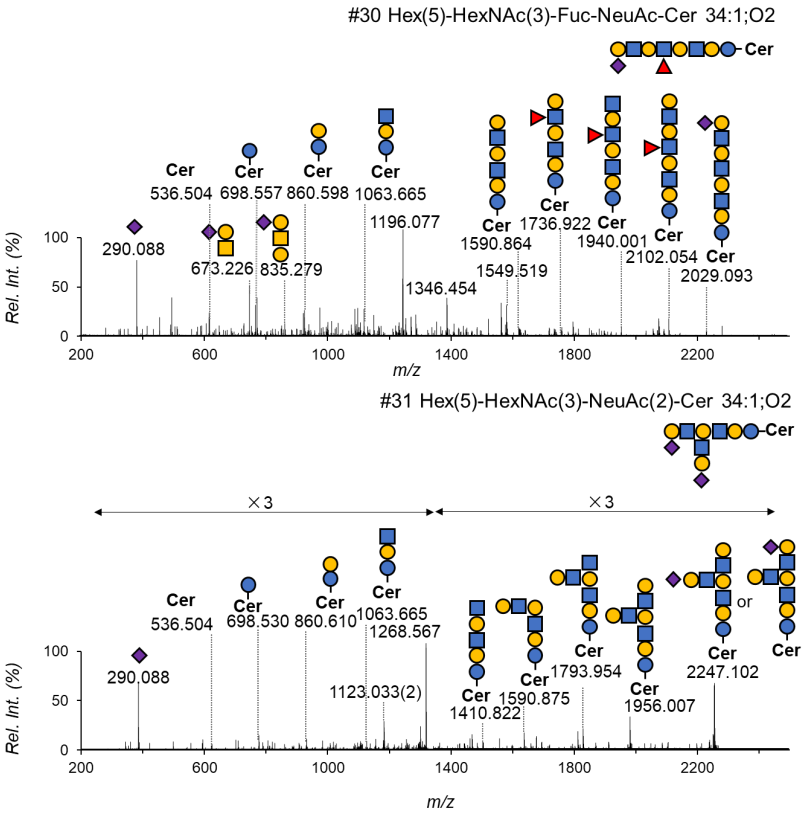


Fig. S8. Characteristic fragment ions observed in negative-ion mode MS^2 CID spectra used for the identification ganglioside subclasses (#1-#31). The identification was further supported by the diagnostic patterns of *m/z*, retention time (R.T.), and 1/K_0 values.

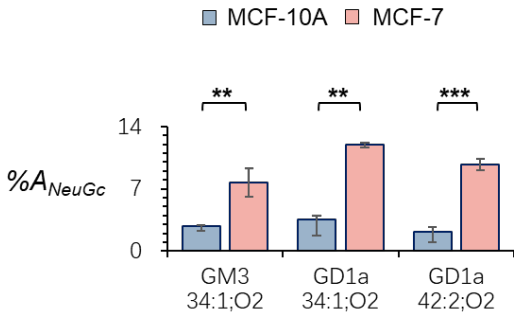


Fig. S9. Relative composition of NeuGc-containing species. $\%A_{NeuGc}$ was calculated as the proportion of NeuGc-containing species relative to the total pool of containing NeuAc and NeuGc-containing species. "%A" was calculated from EIC peak area.

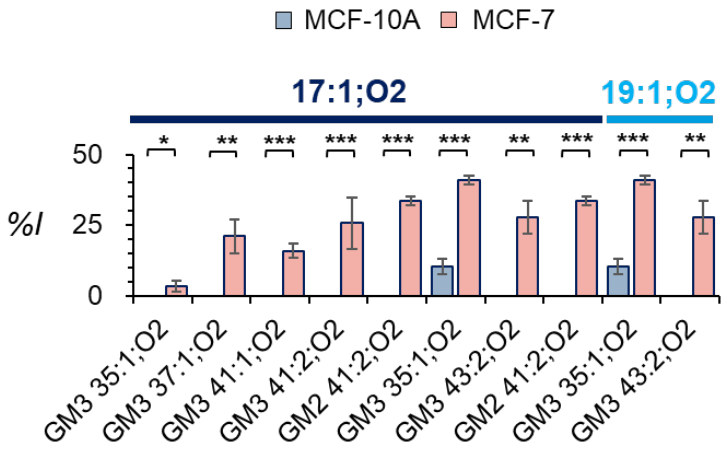


Fig. S10. Relative composition of 17:1;O2 across 8 pairs and 19:1;O2 across 2 pairs of ganglioside species between MCF-10A and MCF-7 cells. "%I" was calculated from peak intensity in MS^2 CID data.

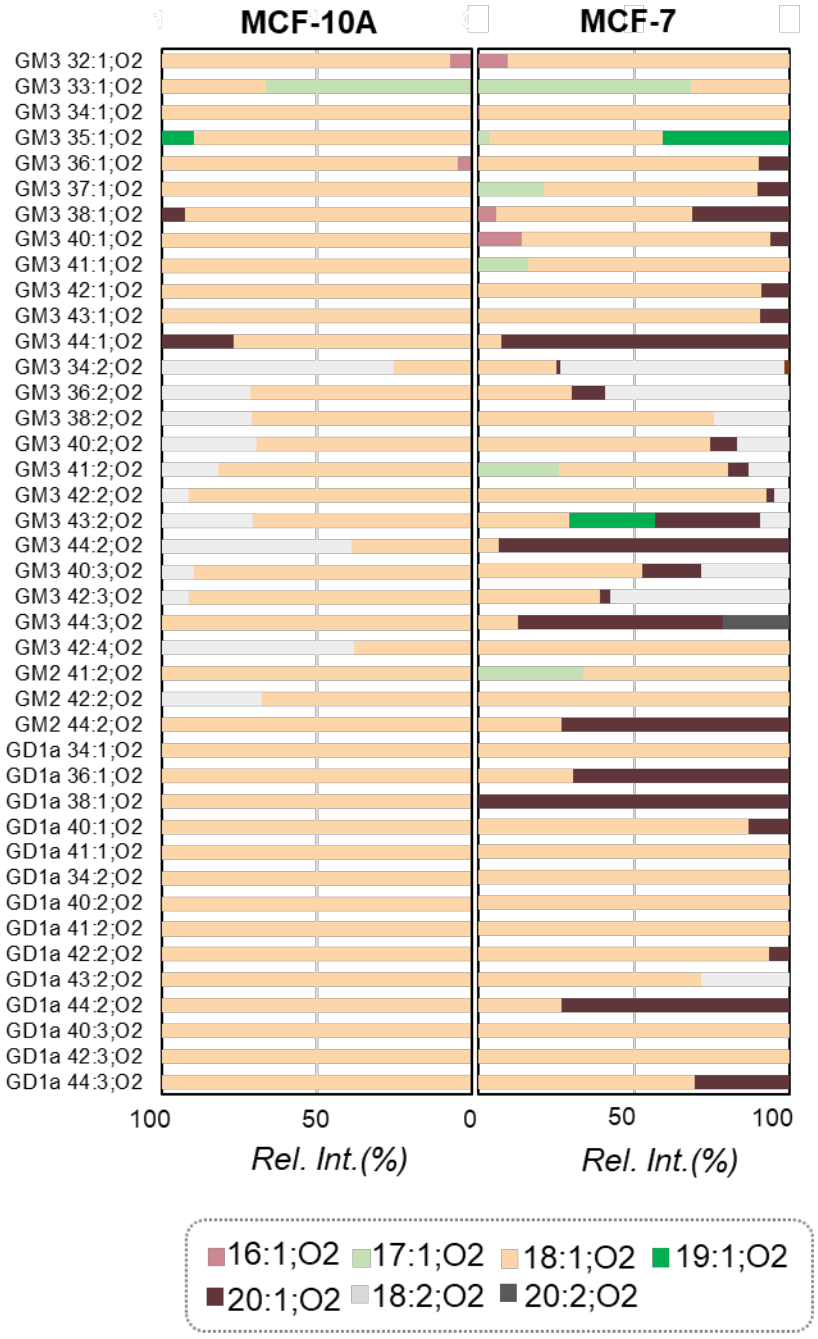


Fig. S11. Relative composition of chain composition isomers in MCF-10A and MCF-7 cells.

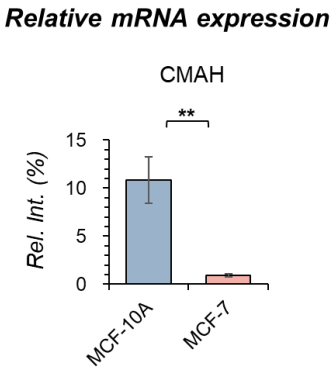


Fig. S12. Transcriptional levels of CMAH between MCF-10A and MCF-7 cells.

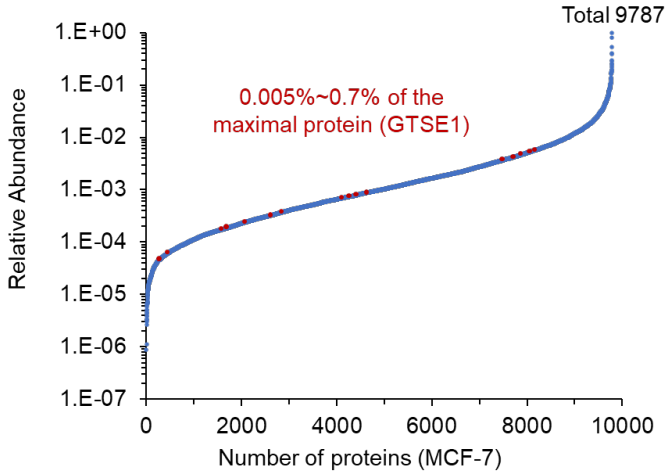


Fig. S13. Label-free quantitative proteomics of MCF-7 cells and MCF-10A cells. (a) Relative abundance of 9,789 proteins in MCF-7 cells, with red dots indicating the 22 identified enzymes involved in ganglioside biosynthesis.

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