PNAS
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Supplementary Information for

Signaling Pathway of Globo-series Glycosphingolipids and β1,3-galactosyltransferase V (β3GalT5) in Breast Cancer

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This PDF file includes:

www.pnas.org/cgi/doi/10.1073/pnas.1816946116

Supplementary Information Text

Materials and Methods

Cell culture. The human breast carcinoma cell lines MDA-MB-231 and MCF7 were acquired from American Type Culture Collection (ATCC). MDA-MB-231 cells were cultivated in high glucose DMEM (Invitrogen) supplemented with 10% (v/v) FBS and 10% (v/v) Antibiotic-Antimycotic. MCF7 cells were maintained in high glucose DMEM (Invitrogen) supplemented with 10% (v/v) FBS, 1 mM sodium pyruvate, 10% (v/v) non-essential amino acids, and 10% (v/v) Antibiotic-Antimycotic. All cell lines were cultivated in 5% (v/v) CO_2 damp atmosphere at 37°C. For Z-DEVD-FMK (caspase-3 inhibitor) (50 μM, R&D Systems) or FK506 (FK506 binding protein-4 inhibitor) (50 μM, Sigma) treatment, the cells were seeded overnight and treated with FK506, Z-DEVD-FMK, or DMSO containing medium .

Short hairpin RNA (shRNA) Infection and Knockdown Cells. To establish human β3GalT5 knockdown stable cell lines, the lentivirus-shRNA systems for human β3GalT5 were purchased from the National RNAi Core Facility in Academia Sinica. The β3GalT5-short hairpin sequence is 5'-CCGGGCAAGTGGTTTGTCAGTAAATCTCGAGATTTACTGACAAACCACTTGCTTTTTG-3'; the FAK-short hairpin sequence is 5' CCGGGCCCAGGTTTACTGAACTTAACTCGAGTTAAGTTCAGTAAACCTGGGCTTTTTG-3'; and the Galectin-8-short hairpin sequence is 5' CCGGCCTGGAACTTTGATTGTGATACTCGAGTATCACAATCAAAGTTCCAGGTTTTTG-3'. MCF7 and MDA-MB-231 cells were infected with shβ3GalT5 or shControl lentiviruses according to the manufacturer's protocols. Infected cells were harvested 48 h post-infection for further experiments and the knockdown efficiency was determined using Q-PCR and flow cytometry.

Q-PCR. The total mRNA from cell lines was extracted using GeneJET RNA Purification kit (Thermo Scientific) according to the manufacturer's guidelines: RNA (2 μg) was reverse-transcribed to cDNA using High Capacity cDNA Reverse Transcription kits (Invitrogen). The Q-PCR reactions were prepared in a total volume of 20 μL containing 2 μL of cDNA of the test sample or control sample with 2X Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific). cDNAs were examined for the expression of β3GalT5 (forward primer: 5'-AGCGGAAACGAAAGAGGTGGAC-3'; reverse primer: 5'-CCTGAGGACAAAAGCGATGGAC-3'), FAK (forward primer, 5'-CTTCGGACAGCGTGAGAGAGA-3'; reverse primer, 5'-GACGCATTGTTAAGGCTTCTTGA-3'), and GAPDH (forward primer, 5'-GCTGTTGTCATACTTCTCATG-3'; reverse primer, 5'-TCTTCCAGGAGCGAGATCCC-3') by Applied Biosystems 7300 Real-Time PCR system (Life Technologies). The relative gene expression was normalized against GAPDH gene expression based on the Ct values.

Flow Cytometry. For cellular surface staining, 10^5 cells were incubated with 0.5 μg Alexa Flour 488-conjugated anti-SSEA3 mAb (MC-631, eBioscience), anti-SSEA4 mAb (MC-813-70, eBioscience), and anti-Globo H mAb (VK9, a gift from Philip O. Livingston, Memorial Sloan–Kettering Cancer Center, New York) in 50 μL FACS buffer (PBS solution

with 1% FBS) at 4°C for 30 min in the dark. Corresponding isotype antibodies were used as staining controls. After washing with 200 μL FACS buffer for three times, cells were resuspended in 200 μL FACS buffer and analyzed by FACSCanto (BD Biosciences) with FACSDiva software (BD Biosciences). All data were analyzed by FlowJo software (Tree Star). For triton X-100 treatment, cells were fixed with 4% (wt/v) paraformaldehyde in PBS for 15 min at room temperature, followed by incubation in 0.1% (v/v) triton X-100 for 10 min before staining with appropriate antibodies.

Immunohistochemical Staining. Tissue slides were dried at 65°C for 30 min, deparaffinized in xylene, and rehydrated in ethanol. The antigen retrieval was carried out by antigen retrieval buffer in pH 6.1 (DAKO) for 10 min at 121°C pressure cooker. The sections were processed with 3% (v/v) hydrogen peroxide in methanol for 30 min to quench endogenous peroxidase activity, following PBS washing, and were treated with blocking buffer [10% (v/v) goat serum blocking reagent (Sigma) in PBS] for 1 h at room temperature to block nonspecific background. Moreover, sections were incubated with rabbit anti-human β3GalT5 polyclonal antibody (Abcam) with 1:50 dilution or rabbit IgG isotype antibody (Cell signaling) in incubation buffer [10% (v/v) goat serum blocking reagent in PBS, pH 7.4] overnight at 4 °C. Followed by washing with PBS, three times for 5 min, sections were incubated with universal immuno-peroxidase secondary antibody for 1 h. After washing with PBST, sections were developed with DAB (Vector Laboratories) and counterstained with slight hematoxylin. The slides were mounted and observed by light microscopy. According to a four-point staining intensity scoring system approach, the staining intensity score ranged from no expression (0) to maximal expression (3+), all the specimens used for determining the relative staining intensity of β3GalT5 expression were reviewed and scored independently by two pathologists.

Apoptosis Assay. The percentage of apoptosis in MCF7 and MDA-MB-231 cells was determined after treatment with β3GalT5 shRNA lentivirus (MOI=5), 1 μg/mL (wt/v) Fas neutralizing antibody (Merck Millipore), or 1 μg/mL (wt/v) TNFRI neutralizing antibody (R&D Systems). After three days, cells were washed three times with PBS and stained with allophycocyanin (APC)-conjugated annexin V [1:40 dilution in binding buffer (0.01 M HEPES, 0.14 M NaCl, 2.5 mM $CaCl_2$); BD Biosciences] for 15 min at 4ºC. Cells were washed with PBS and then harvested for flow cytometry analysis.

Migration Wound Healing Assay. MDA-MB-231 cells with or without β3GalT5 knockdown were grown to reach 90% confluency on 6-well plates and the culture medium was replaced with serum-free medium for starvation for 12 h. Cells were then trypsinized and seeded at 5 x 10^4 cells/well into a 2-well Culture-Insert (iBidi, Germany) with a 500 μm thick cell free gap (wound). After incubation at 37ºC for 12 h, the Culture-Insert was removed and the cells were allowed to migrate into the wound area. The images were recorded at 0 h and 18 h time points under a light microscope using Olympus digital camera to assess the ability of cell migration from the degree of wound closure.

Cell Invasion Transwell Assay. The assay was performed in BD Falcon FluoroBlok 24-Multiwell cell culture insert with 8-μm pore-size PET membrane. The insert well was coated with 100 μL of 1 μg/μL Matrigel matrix (BD Biosciences) at 37°C, 5% CO_2 for 2

h. Cells were resuspended in serum-free medium and added into the insert well at 5 x 10^4 cells/500 µL, and 750 µL of chemoattractant [10% (v/v) FBS in DMEM] was added to the lower chamber. Following the incubation at 37°C, 5% CO_2 for 22 h, the medium was carefully removed from the insert well, the insert well was transferred to a new 24-well plate containing 500 µL/well of 4 µg/mL Calcein AM in cell dissociation buffer (Life Technologies) and incubated at 37°C for 1 h. The fluorescence of invaded cells was measured at 494 nm/517 nm (excitation/emission) by a SpectraMax M5 microplate reader (Molecular Devices).

Adhesion Assay. The cells were seeded into 6-well plates and grown to reach 90% confluency. Following starvation for 6 h in a serum-free medium, cells were collected after treatment with cell dissociation buffer (Invitrogen), and resuspended in a serum-free medium. The cells were then seeded in a 96-well plate at the density of 1 x 10^5 cells/200 µL of serum-free medium/well and cultivated at 37°C for 4 h. After the removal of the medium, the adherent cells were stained with 5 µM Calcein AM (BD Biosciences) at 37°C for 1 h. The fluorescence of stained adherent cells were measured by SpectraMax M5 microplate reader (Molecular Devices) at 494 nm/517 nm (excitation/emission).

Western Blotting. MCF7 and MDA-MB-231 cells were lysed by lysis buffer [150 mM NaCl, 100 mM phosphate buffer at pH 7.4, 1% Nonidet P-40, 1 X EDTA-free protease inhibitor cocktail (Roche) and 1 X phosphatase inhibitor (Roche)]. The protein concentration of lysates was measured by Pierce 660 nm Protein Assay Reagent (Thermo). Protein lysates were denatured in 1 X SDS sample buffer (Invitrogen) at 95°C for 10 min and separated in 4-12% NuPAGE (Invitrogen) protein gels. Proteins in the gels were transfer to PVDF membranes and blocked with 5% (wt/v) nonfat milk in TBST (0.05% Tween 20) for 30 min before immunoblotting with primary antibodies at 4 °C overnight. The antibodies for FAK, cleaved FAK, and CAV1 were purchased from Santa Cruz Biotechnology. The antibody for RIP (Clone 38/RIP) was purchased from BD Biosciences. The antibody for FADD (Clone 1F7) was purchased from Merck Millipore. The antibodies for caspase-3, cleaved caspase-3, E-cadherin, AKT, pAKT (T308), and N-cadherin were purchased from Cell Signaling Technology. The antibody for beta-actin (Clone AC-15) was purchased from Sigma-Aldrich. After washing with TBS with 0.05% Tween 20, the membranes were incubated with HRP-conjugated secondary antibodies and the signals were developed with ECL western blot detection reagents (Merck Millipore). The intensity of signals was detected by ImageQuant LAS-4000 imaging system (GE Healthcare Life).

Co-immunoprecipitation (co-IP). In the immunoprecipitation experiments, 600 µg of total protein lysates were incubated with 3 µg of biotinylated anti-SSEA3, anti-SSEA4, mIgG3 or IgM isotype antibody (eBioscience) for 3 h and then incubated with streptavidin or protein A/G magnetic beads (Thermo) at 4°C overnight before pulldown with a magnet. For the co-IP of FAK, CAV1, RIP and AKT, cell lysates were treated with specific antibodies or isotype control antibodies for 3 h followed by incubation with protein A/G-magnetic beads (Thermo) at 4°C overnight. The beads were washed three times with lysis buffer for proteomic analysis or MS grade water for glycomic analysis. In order to detect the immunoprecipitated proteins, samples were resuspended in 1 X SDS sample buffer and separated using protein gels, followed by western blot with appropriate antibodies. To

release glycolipids from the immunoprecipitates, chloroform/methanol [2:1; (v/v)] was used for elution, and the eluent was subjected to glycolipid profiling by mass spectrometry.

High Resolution Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) for Protein Identification. Samples in 0.1% formic acid were injected onto a self-packed precolumn (150 μm I.D. x 30 mm, 5 μm, 200 Å) and 75-μm x 20-cm fused silica capillary column packed with 2.5-μm C18 beads (Reprosil-Pur Basic, Dr. Maisch), in a 250 μL/min gradient of 5% acetonitrile/0.1% formic acid to 40% acetonitrile/0.1% formic acid over the course of 40 min, with a total run of 60 min and a flow rate of 300 nL/min. Full MS survey scans from m/z 200 to 1400 were carried out on an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, San Jose, CA) at a resolution of 120000 using EASY-IC as lock mass for internal calibration. The MS/MS was run in top speed mode with 3 s cycles; while the dynamic exclusion duration was set to 60 s with a 25 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor ion selection was enabled and 1+ charge states were rejected from MS/MS. The analysis was carried out with higher collision energy dissociation fragmentation modes. Automatic gain control was employed and set to 2 x 10^5 for MS. The maximal allowed ionization time was 200 ms. For protein identification, raw files were analyzed using MaxQuant (Version 1.5.2.8), which consists of an integrated Andromeda search engine. MS/MS spectra were searched against the IPI Human database (Version 3.87). The search parameters were set as: trypsin digest up to 2 missed cleavages, false discovery rate (FDR) of 0.01, variable modifications of methionine oxidation and N-terminal acetylation, and fixed modification of carbamidomethyl cysteine.

High Resolution LC-MS for Glycolipids. Samples were detected by LC-ESI-MS on a LTQ Orbitrap XL ETD mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with Waters Acquity UPLC (Waters, Milford, MA), using an ZIC-HILIC column (1.0 mm X 150 mm, 3 μm, Merck). The gradient employed was 98% buffer B at 3 min to 60% buffer B at 45 min with a flow rate of 50 μL/min, where buffer A was 0.1% formic acid/H_2O and buffer B was 0.1% formic acid/acetonitrile. For the MS condition, the mass range was m/z 320-2000, and the resolution was 30000 at m/z 400. Electrospray voltage was maintained at 4.0 kV and capillary temperature was set at 275ºC. For glycolipid profiling, raw files were deconvoluted and deisotoped by Xtract, and the list of all MS peaks was combined to search against the home built glycolipid database.

Lipid Raft Extraction and Dot Blotting. Lipid raft fraction from breast cancer cells were extracted by the Focus Signal Protein Isolation kit (G Biosciences, St Louis, MO). Cells were harvested with enzyme-free dissociation buffer (Invitrogen), washed three times in ice-cold PBS, resuspended in ice-cold signal protein extraction (SPE) buffer-I, and then lysed by sonication for 10 s. The lysates were treated with SPE buffer-II followed by incubation on ice for 15 min, with an 1-min-vortex every 5 min. The lysates were centrifuged at 20000 x g for 15 min at 4°C for raft isolation. Non-lipid raft fractions were collected from supernatant, and the pellets that contained lipid rafts were solubilized in the protein solubilization buffer supplemented with 1 x protease inhibitor cocktail (Roche) at room temperature for 15 min, with an 1-min-vortex every 5 min. After centrifugation at 18000 x g for 10 min at room temperature, the supernatants that contained the lipid rafts and the associated proteins were collected. To check the isolated lipid raft, 1 μL lipid raft

fraction were spotted on nitrocellulose membrane (Merck Millipore). After drying, the membrane was blocked with 2% (wt/v) BSA in TBS buffer, incubated with HRP-conjugated cholera toxin (1:1000, sigma) in TBS buffer containing 2% (wt/v) BSA and 0.05% (v/v) Tween-20 to detect lipid-raft-associated GM1, and the signal was visualized by development with DAB (Vector Laboratories).

Immunofluorescence Microscopy. Cells cultured on glass coverslips were fixed with 4% (wt/v) paraformaldehyde in PBS for 10 min at room temperature, washed three times with 0.1% (wt/v) BSA in PBS for 5 min, and permeabilized with 0.1 % (v/v) triton X-100/4% (wt/v) paraformaldehyde/PBS at room temperature for 10 min. After incubation with 0.1% BSA/PBS for 5 min, and 0.5% (v/v) Tween-20 in 0.1% BSA/PBS at room temperature for 1 h, cells were incubated with 10 μg/mL biotinylated anti-SSEA3 antibody (eBioscience), 5 μg/mL FAK polyclonal antibody (Santa Cruz), and/or 5 μg/mL CAV1 monoclonal antibody (Clone 2297/Caveolin1, BD Transduction Laboratories) for 2 h at room temperature. After washing with PBS, cells were stained with Alexa Fluor 488-conjugated streptavidin (1:200) or Alexa Fluor 488-, Alexa Fluor 555 or Alexa Fluor 546-conjugated secondary antibody (1:500) for 1 h at room temperature in the dark. Coverslips were washed with PBS and mounted with 5 μL glycerol:PBS (1:1). All images were acquired by a confocal microscope (Leica, TCS-SP5-MP).

Tumor Growth Inhibition in Xenograft Mouse Model. Six-seven weeks old female athymic (nu/nu) nude mice were injected with supplement Estol/Depot (100 μg/mouse) subcutaneously twice weekly starting one week before cell inoculation. Viable 2 x 10^7 MCF7, HCC1428, and HPAC cells in 1:1 matrigel/media mixture at 0.2 mL/mouse were inoculated into the right flank of mice. Tumor implanted mice were divided into groups of which each group contain eight animals. Antibody administrations were initiated one week after cell inoculation (denoted as Day 1). Vehicle (sodium citrate, NaCl, pH6.5) and anti-Globo-H antibody at 3 mg/kg and anti-SSEA4 antibody at 3 mg/kg were administered intravenously twice weekly. The anti-Globo-H and anti-SSEA4 monoclonal antibodies were made by OBI Pharma Inc. (Taipei, Taiwan) and were disclosed in details in US patent US9902779B2 and US patent US2017283488A1, respectively. All test substances were administered in a dose volume of 10 mL/kg. Tumor volume (mm^3) was estimated according to the ellipsoid formula as: length x $(width)^2$ x 0.5. Percent tumor growth inhibition (TGI) was calculated by the following formula:
%TGI= (1-T/C) x100%.
T: Tumor size measured in the treatment group.
C: Tumor size measured in the control group (vehicle).

Pulldown of Globo-Series Glycan Binding Protein
Cells were harvested by cell scraper and centrifuged at 500 x g for 3 min at 4°C. Cell pellets were lysed with RIPA lysis buffer [0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA, 1 X protease inhibitor (Roche)] for 20 min on ice. After centrifugation at 13000 x g for 20 min at 4°C, the cell lysate was collected and stored at -80°C before use. Fifty μM globo-series glycan-biotin and methyl-PEG4-biotin were incubated with 1 mg breast cancer cell lysate in RIPA buffer, then the mixture was stirred for 16 h at 4°C. Fifty μL neutravidin agarose beads (Thermo Scientific) were added and

incubated for 1 h at 4°C. The supernatant was removed and beads were washed with PBST (PBS with 0.05% tween 20) for five times. The captured proteins were eluted from beads by 1 x SDS sample buffer.

Glycan Array

Before protein binding, the glycan microarrays were blocked with Superblock blocking buffer (Pierce) at room temperature for one h, followed by washing twice with PBST. Recombinant human galectin-8 (2.5 μg/mL) was diluted with PBST, followed by incubating with microarray at room temperature for 1 h. Excess galectin-8 protein were washed out and each microarray grid was incubated with 2.5μ g/mL of anti-galectin-8 antibody for 1 h. After washing with PBST, DyLight 647-conjugated rabbit anti-goat IgG (2.5 μg/mL) was added and incubated for 1 h. The slide was then washed thoroughly and scanned at 635 nm with a microarray fluorescence chip reader (GenePix 4300A; Molecular Devices) and scanned images were analyzed with GenePix Pro-6.0 analysis software (Axon Instruments, Union City, CA, USA).

Protein Purification

The DNA fragments encoding N-terminal and C-terminal domains of galectin-8 were cloned into pET28a to in frame with the N-terminal His-tag sequence for making His-tagged proteins. In order to increase the gene expression level, the DNA sequences were synthesized by codon optimization in *E. coli*. The constructs were transformed into *BL21(DE3)* competent cells by chemical transformation method. Single colonies were picked and inoculated into TB medium with kanamycin antibiotics overnight, and the culture were expanded and subjected to IPTG (0.1 mM) induction at 16°C for 24 h when OD600 reached 0.5. The *E. coli* cells were harvested and lysed by microfluidizer with the buffer containing 50 mM sodium phosphate, pH 8.0, 300 mM NaCl, and 10 mM imidazole. The lysate were centrifuged at 10000 rpm for 30 min at 4°C. Then, the supernatant was mixed with Ni-NTA agarose (Qiagen, Santa Clarita, CA). The bound protein was eluted with the same buffer containing a higher concentration of imidazole (250 mM). The protein concentration was determined by Qubit Protein Quantitation (Invitrogen, CA), and the purity was confirmed by SDS-PAGE.

Statistical Analysis. All results were statistically analyzed by GraphPad Prism 6. The overall survival rate of clinical specimen was evaluated using the Kaplan-Meier method. Statistical comparisons of pathological features and B3GalT5 expression were determined by Fisher's exact test. All data were verified at least three times and differences between experimental conditions were presented as mean ± SD. Intergroup results were estimated using two-tailed Student's t test and ANOVA with Tukey's multiple comparisons test for comparison of significant differences in the results between the paired exam groups, respectively. $p < 0.05$ was considered statistically significant.

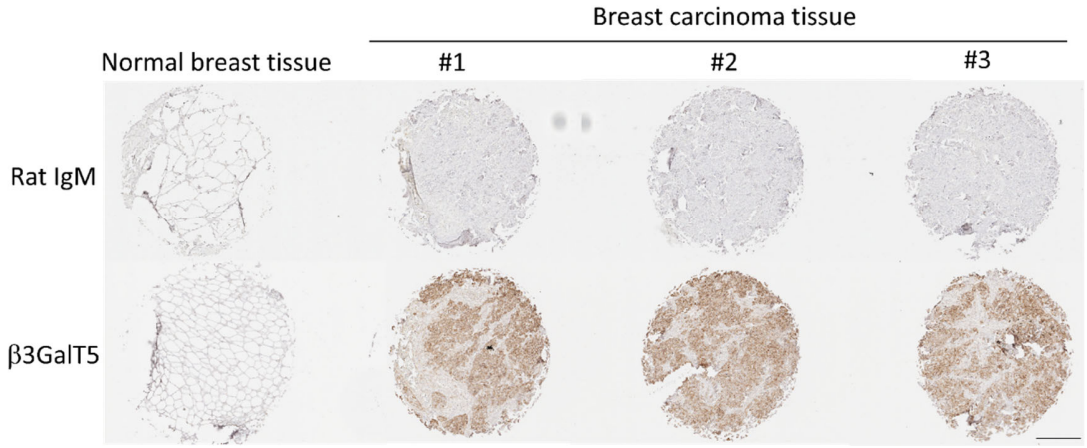

Fig. S1. Immunohistochemistry (IHC) of β3GalT5 in human specimens of normal breast tissues and caner tissues. Representative images of normal breast tissue isotype staining (**A**), β3GalT5 staining (**B**), and breast carcinoma tissue isotype staining (**C**), β3GalT5 staining (**D**) were shown. (Scale bars, 300 μm).

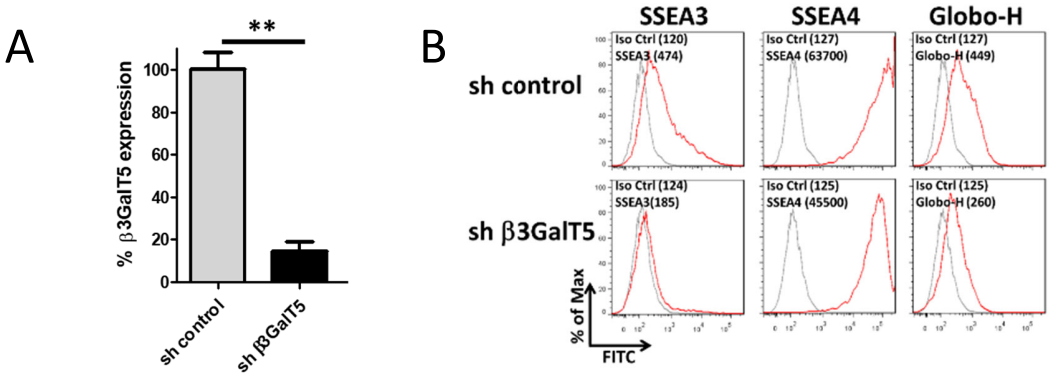

Fig. S2. Knockdown efficiency of β3GalT5 in MDA-MB-231 cells. (A) The mRNA expression of β3GalT5 in MDA-MB-231 cells with knockdown of β3GalT5. Results are shown as mean ± SD (n = 3). (**B**) The expressions of SSEA3, SSEA4, and Globo-H glycolipids were determined in MDA-MB-231 cells by flow cytometry. Representative data among triplicated experiments are shown.

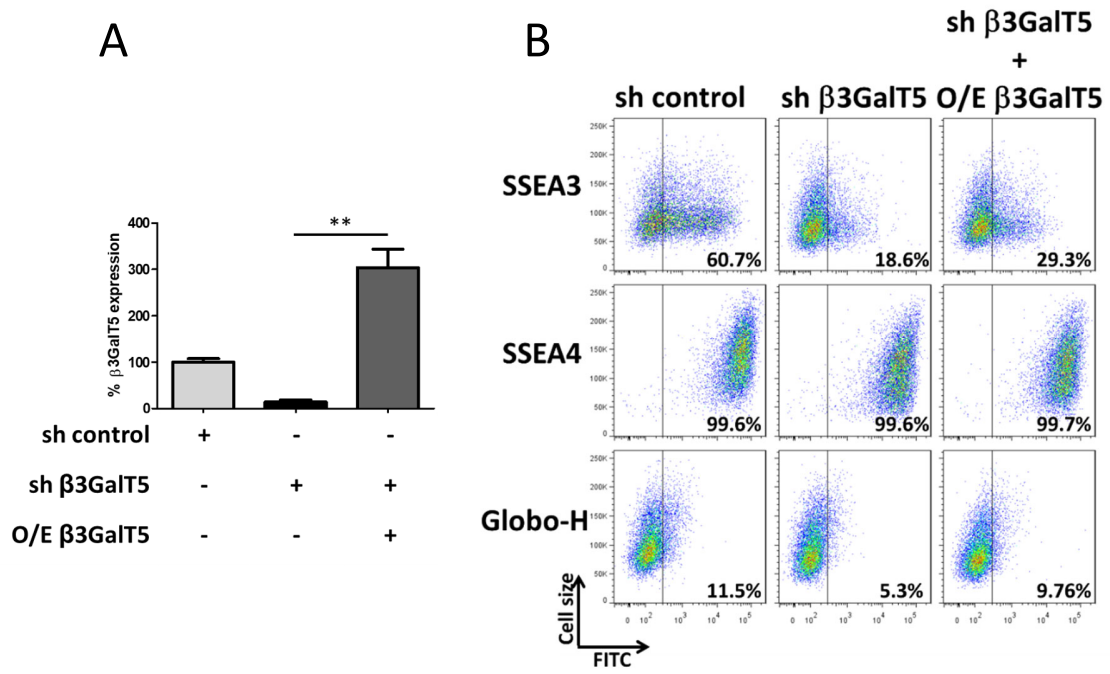

Fig. S3. Restoration of β3GalT5 expression in β3GalT5 knockdown MDA-MB-231 cells. (**A**) The mRNA expression of β3GalT5 in MDA-MB-231 cells with knockdown and/or overexpression of β3GalT5. Results are shown as mean ± SD (n = 3). (**B**) The expressions of SSEA3, SSEA4, and Globo-H were evaluated by flow cytometry. Representative data of triplicated experiments is shown.

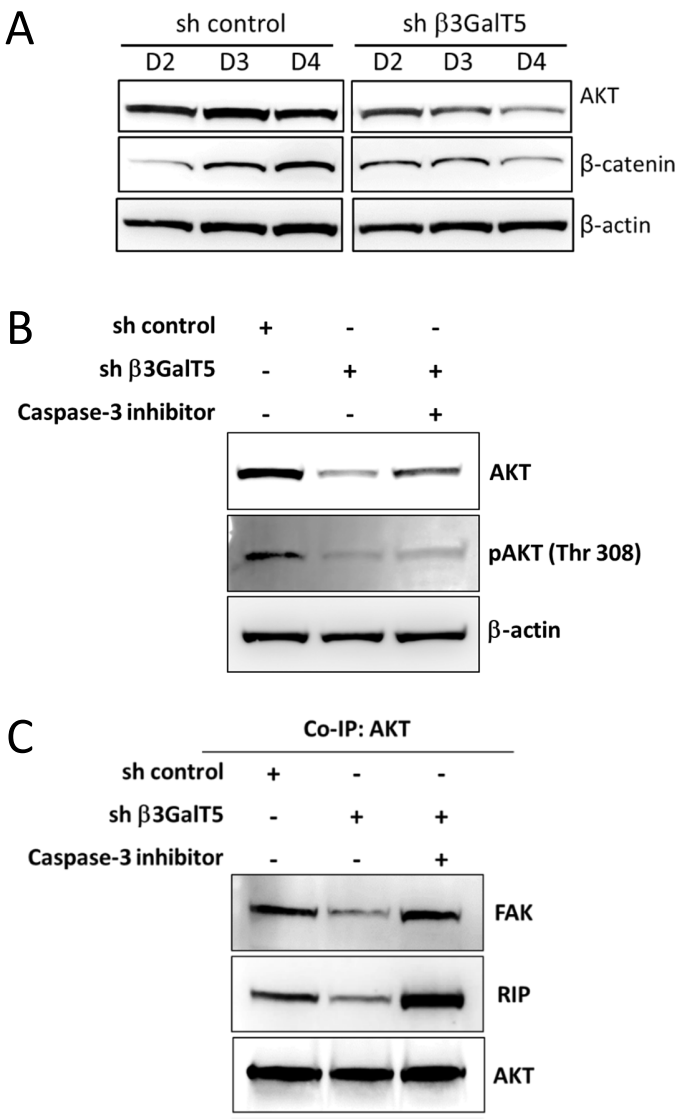

Fig. S4. MDA-MB-231 cells knocked down with β3GalT5 showed reduced expression of AKT and its association with FAK and RIP and inhibition of caspase-3 activation restored the phenomenon. (**A**) The expression levels of AKT and downstream β-catenin in β3GalT5 knockdown cells. (**B**) Caspase-3 inhibitor restored AKT expression in β3GalT5 knockdown cells. (**C**) Association of FAK, RIP and AKT. Total cell lysates were immunoprecipitated with anti-AKT antibody. Knockdown of β3GalT5 showed decreased association of AKT with FAK and RIP. Caspase-3 inhibitor restored the association.

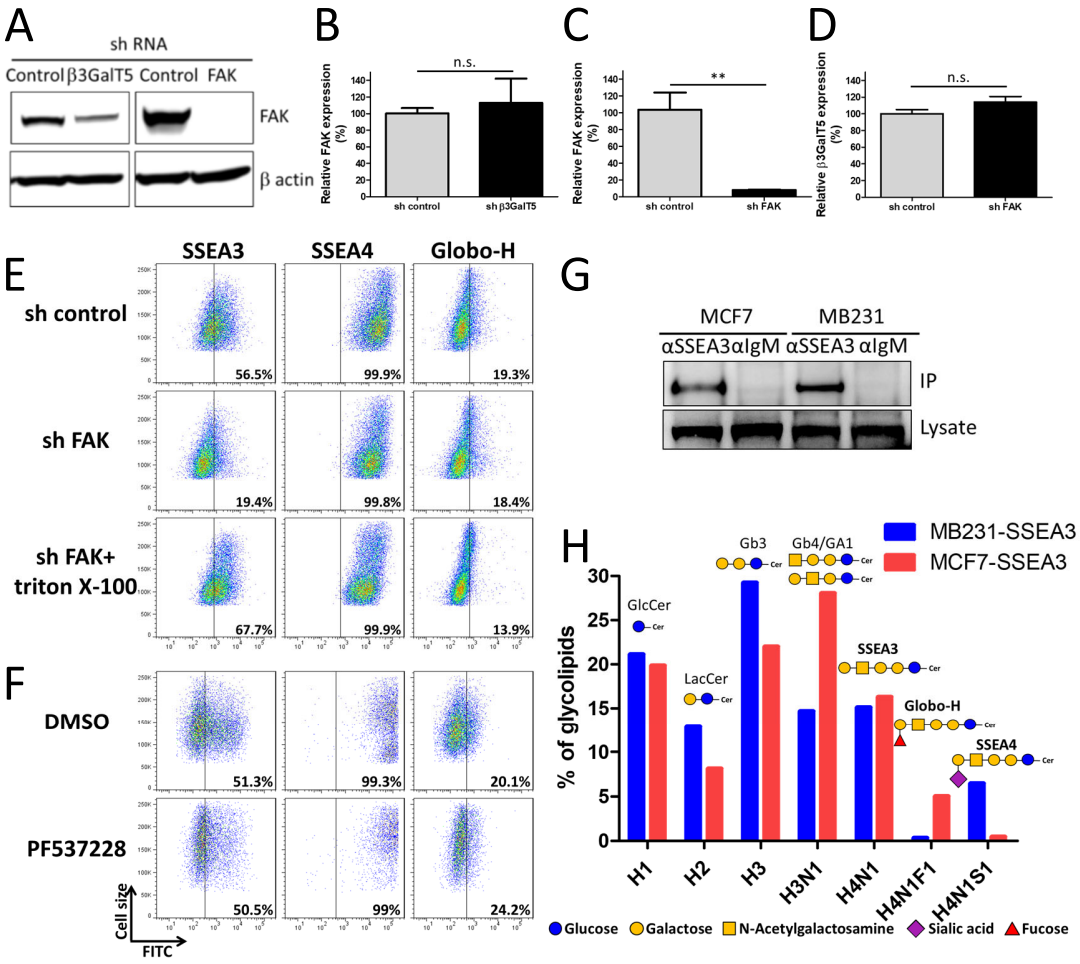

Fig. S5. Association of SSEA3 and FAK in breast cancer cells. (**A**) Expression of FAK in MDA-MB-231 cells with β3GalT5 or FAK knockdown. (**B**) The mRNA expression of *FAK* in β3GalT5 knockdown cells. (**C**) The mRNA expression of *FAK* in FAK knockdown cells. (**D**) The mRNA expression of *β3GalT5* in FAK knockdown cells. (**E**) The expression of SSEA3 in MDA-MB-231 cells knocked down with FAK. FAK knockdown cells showed decreased SSEA3 expression on the cell surface, and the staining intensity of anti-SSEA3 restored when intracellular staining was performed. (**F**) The expression of SSEA3, SSEA4, and Globo-H in MDA-MB-231 cells treated with or without PF537228, a phosphorylation inhibitor of FAK tyr397. MDA-MB-231 cells were stained and analyzed by flow cytometry. Representative data from triplicated experiment is shown. (**G**) Co-immunoprecipitation of FAK by anti-SSEA3 antibody. (**H**) Identification of SSEA3-associated glycolipids by LC-MS/MS. All the glycolipids determined were linked with C16:0 ceramide. (H: hexose, N: N-acetyl-hexosamine, S: sialic acid, F: fucose)

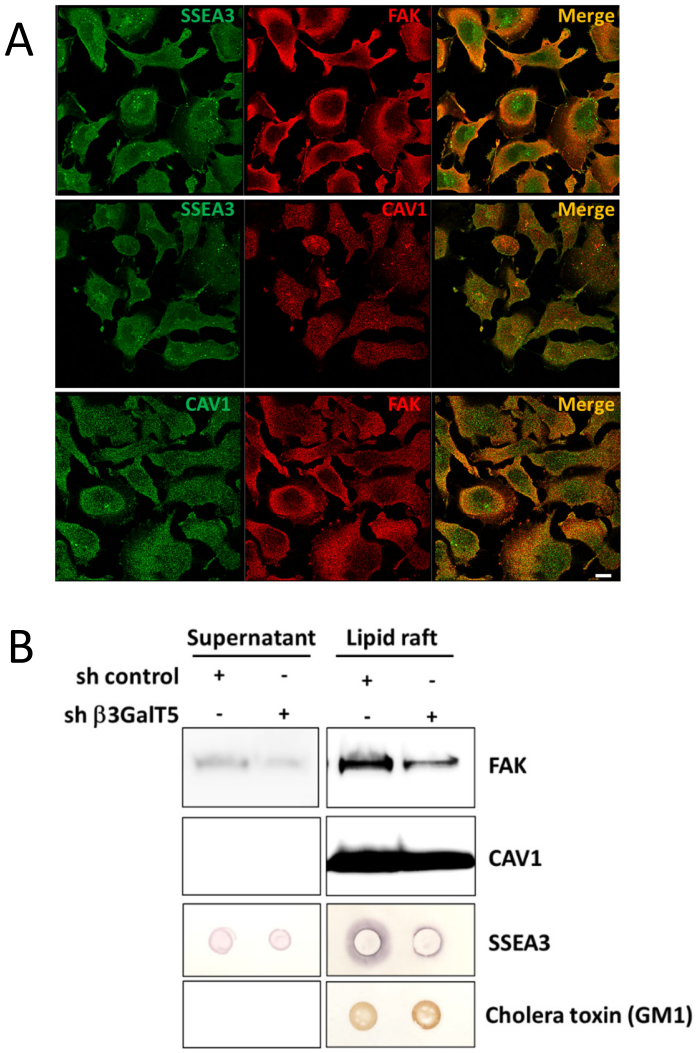

Fig. S6. SSEA3, FAK and CAV1 formed a complex in lipid raft microdomain. (**A**) Co-localization of SSEA3, FAK and CAV1 as examined by confocal microscopy (Scale bars, 10 μm). (**B**) Examination of SSEA3, FAK and CAV1 expression in lipid raft fractions purified from MDA-MB-231 cells with or without β3GalT5 knockdown.

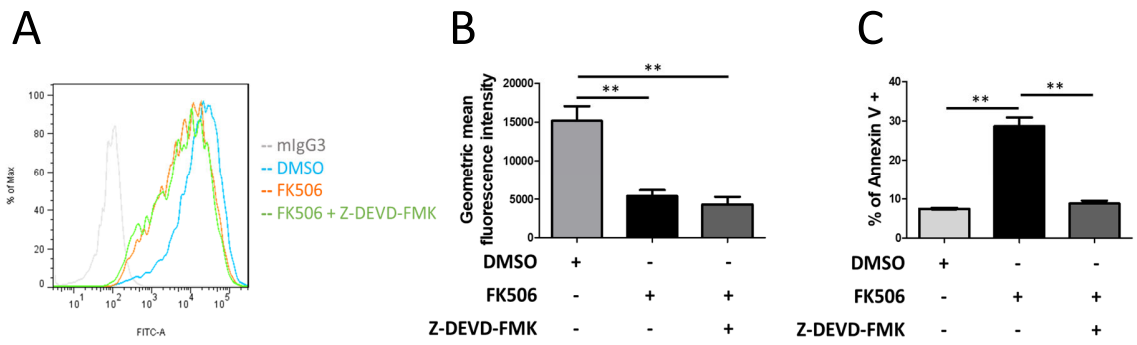

Fig. S7. FK506 suppressed SSEA4 transport to cell surface and induced cell apoptosis. (**A**) Flow cytometric analysis of SSEA4 surface expression in MDA-MB-231 cells treated with FK506. Representative data among triplicated experiments is shown. (**B**) The geometric mean fluorescence intensity of cell stained with anti-SSEA4 antibody. (**C**) Apoptosis of MDA-MB-231 cells were treated with FK506, Z-DEVD-FNK or DMSO. Representative data among triplicated experiments is shown. * $p < 0.05$; ** $p < 0.01$.

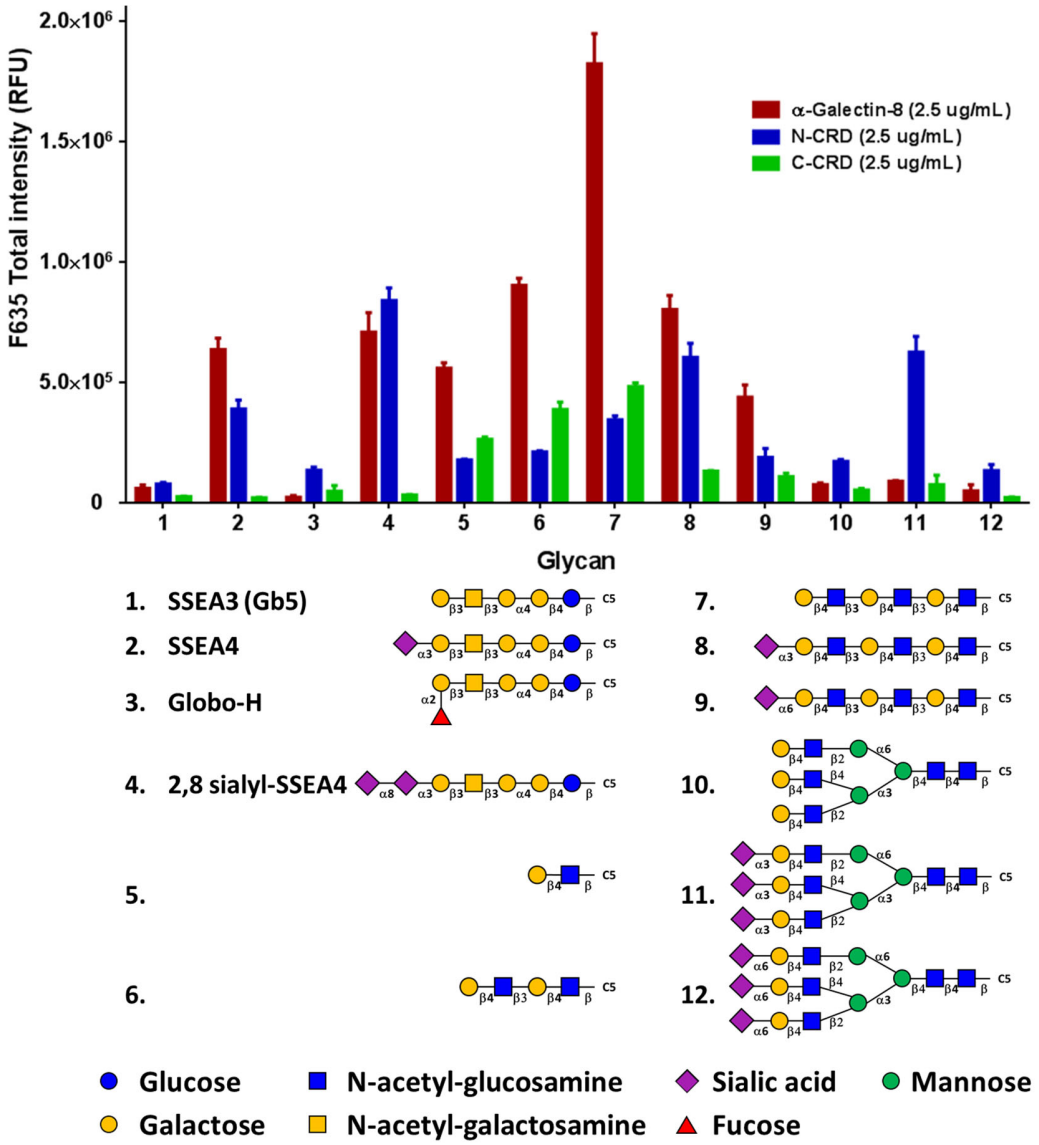

Fig. S8. Glycan binding patterns of galectin-8 and its N- or C-terminal carbohydrate recognition domains (CRDs). Binding of Galectin-8 and its N- or C-CRDs with glycans were analyzed by glycan array. RFU, relative fluorescence units. Error bars represent mean ± S.D.

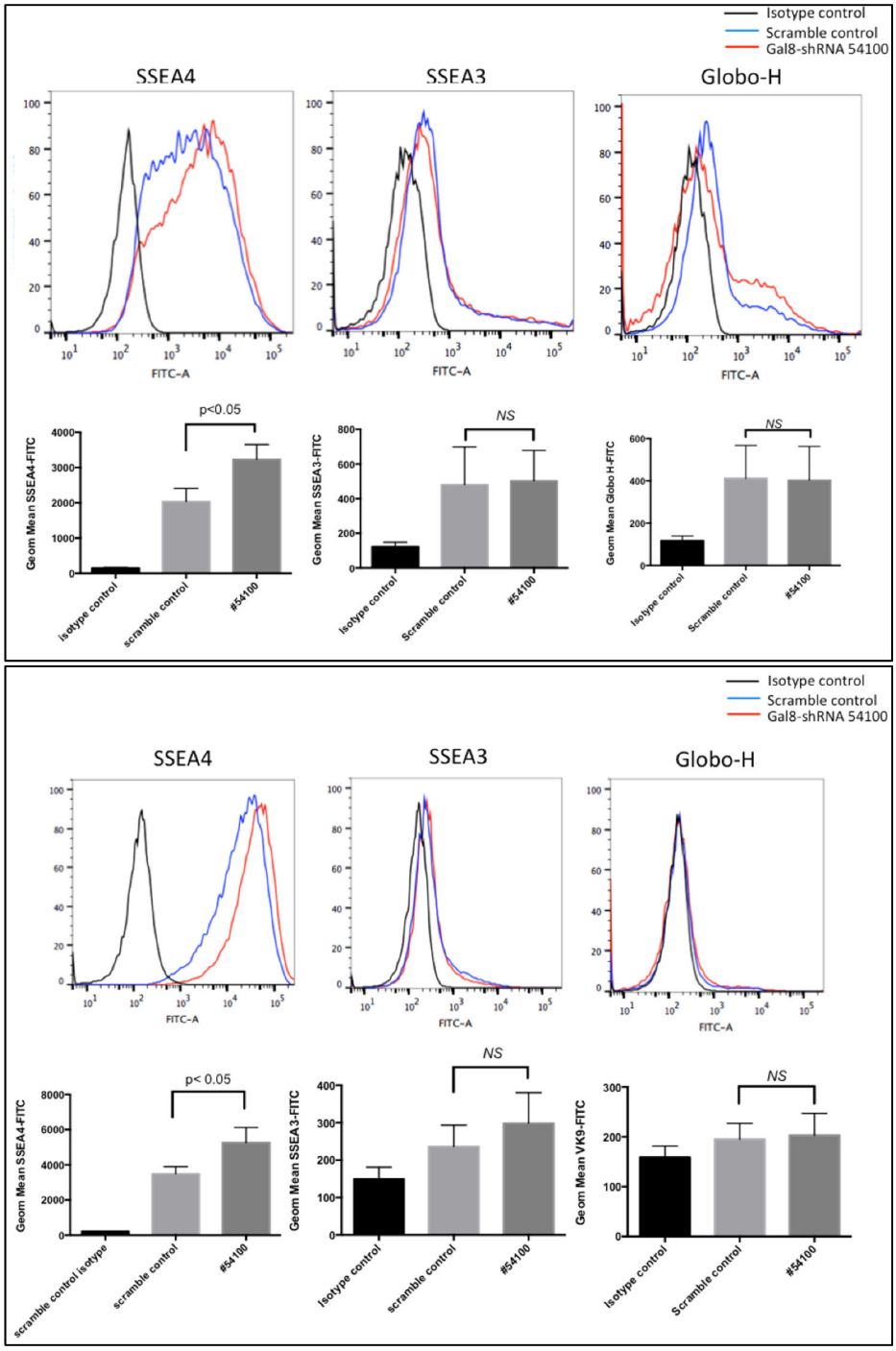

Fig. S9. Knockdown of galectin-8 specifically increased SSEA4 expression. Knockdown of galectin-8 increased expression of SSEA4, but not SSEA3 and Globo-H, on MCF7 (upper) and MDA-MB231 (lower) cell surface as detected by flow cytometry.

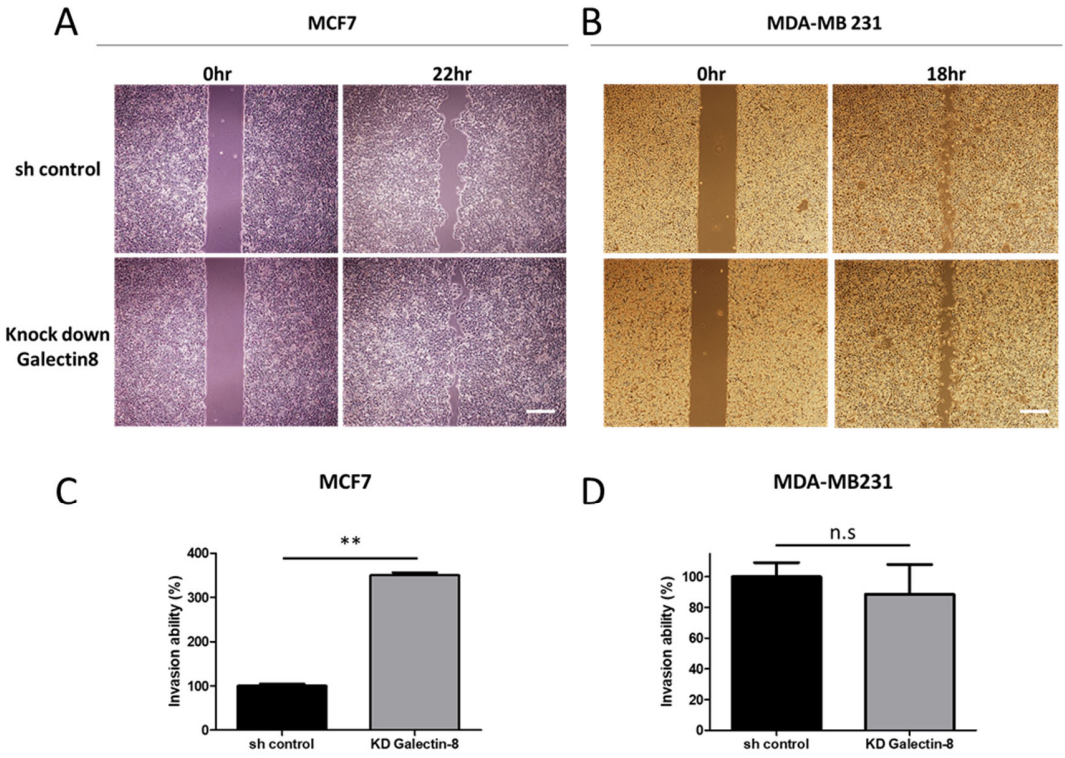

Fig. S10. Knockdown of galectin-8 in breast cancer cells. (A-B) The migration abilities of MCF7 cells (**A**) and in MDA-MB-231 cells (**B**) analyzed by wound healing assay (Scale bars, 250 μm). **(C-D)** The invasion abilities of MCF7 cells (**C**) and MDA-MB-231 cells (**D**) measured by transwell assay.

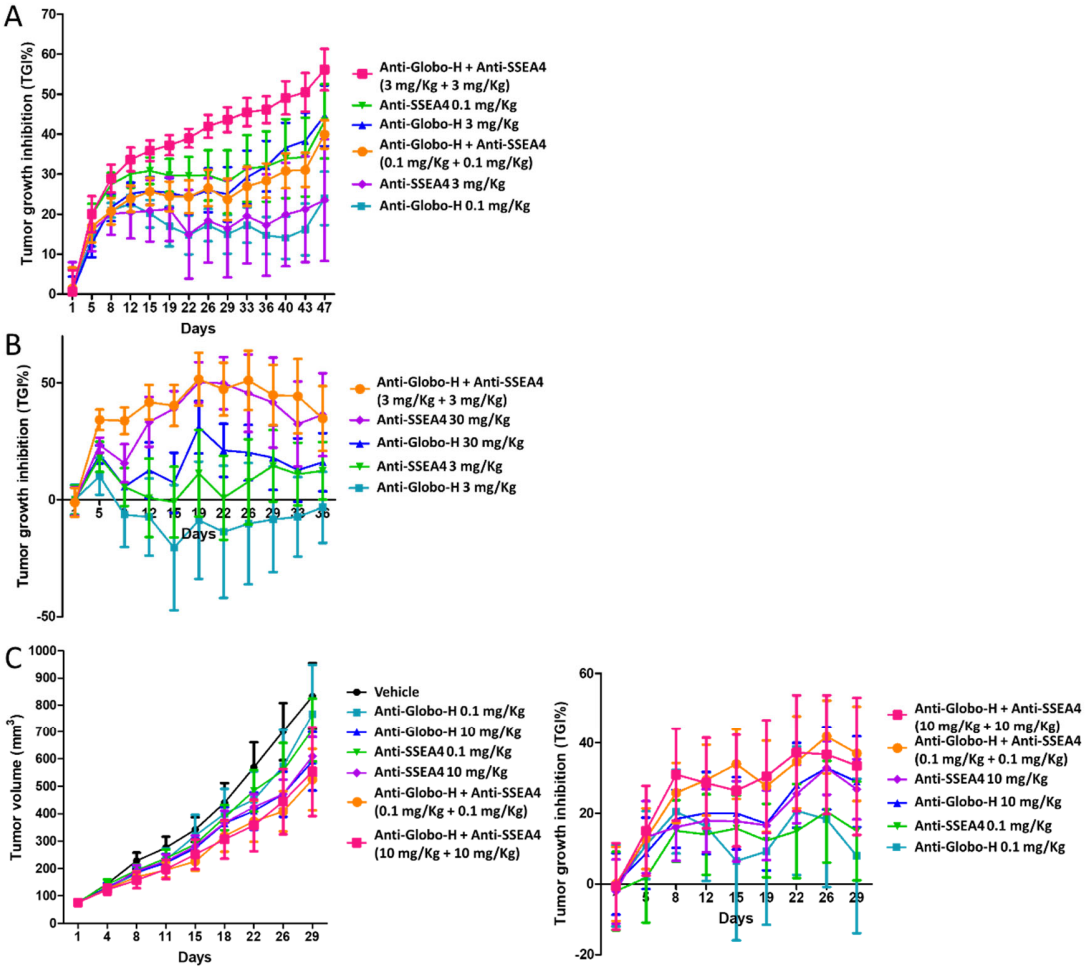

Fig. S11. Synergistic or additive effect observed in the combination of anti-Globo-H antibody and anti-SSEA4 antibody in human cancer cells implanted nude mice. (**A**) Tumor growth inhibition (TGI) of anti-Globo-H and anti-SSEA4 antibodies in MCF7 cells. Anti-Globo-H antibody (0.1 and 3 mg/kg, IV) or anti-SSEA4 antibody (0.1 and 3 mg/kg, IV) as single agent was active against breast cancer MCF7 xenograft, and the combination of both antibodies (3 mg/kg + 3 mg/kg, IV) showed a greater anti-tumor activity (56% of TGI) compared to individual antibodies. (**B**) Tumor volume and TGI of anti-Globo-H and anti-SSEA4 antibodies in HCC1428 cells. Anti-Globo-H antibody (3 and 30 mg/kg, IV) or anti-SSEA4 (3 and 30 mg/kg, IV) antibody as single agent was active against breast cancer HCC1428 xenograft, and the combination of both antibodies (3 mg/kg + 3 mg/kg, IV) showed a greater anti-tumor activity (35% of TGI) compared to individual antibodies. (**C**) Tumor volume and TGI of anti-Globo-H and anti-SSEA4 antibodies in HPAC cells. Anti-Globo-H antibody (0.1 and 10 mg/kg, IV) or anti-SSEA4 (0.1 and 10 mg/kg, IV) antibody as single agent was active against pancreatic cancer HPAC xenograft, and the combination of both antibodies (0.1 mg/kg + 0.1 mg/kg, IV) showed a greater anti-tumor activity (37% of TGI) compared to individual antibodies. The tumor volume in each group (n = 8) was measured at different time points and is shown as mean ± SD. $p < 0.0001$ and $p = 0.0286$ was determined by two-way RM ANOVA.

Table S1. Expression of SSEA3, SSEA4, and Globo-H on normal primary cells.

Cell category	Cell line	Full name	FACS analysis		
			SSEA3	SSEA4	Globo H
normal	**HCMEC**	Human Cardiac Microvascular Endothelial Cells	-	-	-
normal	**HCoEpiC**	Human Colonic Epithelial Cells	-	-	-
normal	**HH**	Human Hepatocytes	-	-	-
normal	**HHsteC**	Human Hepatic Stellate Cells	-	-	-
normal	**HK**	Human Keratocytes	-	-	-
normal	**OKB2**	Human Oral Buccal Keratinocytes	-	-	-
normal	**OKG4**	Human Oral Ginvival Keratinocytes	-	-	-
normal	**HOK**	Human Oral Keratinocytes	-	-	-
normal	**primary NHOK D1**	Human Normal Oral Keratinocytes	-	-	-
normal	**HMEpiC**	Human Mammary Epithelial Cells	-	-	-
normal	**HMF**	Human Mammary Fibroblasts	-	-	-
normal	**HOEpiC**	Human Ovarian Surface Epithelial Cells	+	+	+
normal	**HA**	Human Astrocytes	-	+	-
normal	**HN-MB**	Human Neurons-Midbrain	-	-	-
normal	**HBEpiC**	Human Bronchial Epithelial Cells	-	-	-
normal	**HPAEpiC**	Human Pulmonary Alveolar Epithelial Cells	-	-	-
normal	**HPF**	Human Pulmonary Fibroblasts	-	-	-
normal	**HPaSteC**	Human Pancreatic Stellate Cells	-	-	-
normal	**HPNE**	Human Pancreatic Epithelial Nestin-Expressing (	-	-	-
normal	**HPrEpiC**	Human Prostate Epithelial Cells	-	-	-
normal	**HREpiC**	Human Renal Epitheial Cells	+	+	+
normal	**HRGEC**	Human Renal Glomerular Endothelial Cells	-	+	-
normal	**HRPTEpiC**	Human Renal Proximal Tubular Epithelial Cells	+	+	+
normal	**HRPEpiC**	Human Retinal Pigment Epithelial Cells	+	+	+
normal	**HS**	Human Synoviocytes	-	-	-
normal	**HC-a**	Human Chondrocytes-articular	-	+	-
normal	**HO-f**	Human Osteoblasts-femural	-	-	-
normal	**HSkmC**	Human Skeletal Muscle Cells	-	-	-
normal	**HTEC**	Human Testicular Endothelial Cells	-	-	-
normal	**HUC**	Human Urothelial Cells	-	-	-
normal	**HAEC**	Human Aortic Endothelial cells	-	-	-
normal	**HUVEC**	Human Umbilical Vein Endothelial Cells	-	-	-
normal	**HuvmC**	Human Umbilical Vein Smooth Muscle Cells	-	+	-
normal	**HUVSMC**	Human Umbilical Veon Smooth Muscle cells	-	+	-
stem cell	**HMSC-ad**	Human Adipose-derived Mesenchymal Stem Ce	-	-	-
stem cell	**HMSC-bm**	Human Mesenchymal Stem Cells-bone marrow	-	+	-
stem cell	**HMSC-he**	Human Liver-derived Mesenchymal Stem Cells	-	-	-
stem cell	**HPMSC**	Human Pulmonary Mesenchymal Stem Cells	-	+	-

Table S2. Correlation of β3GalT5 expression and clinical characteristics in 67 patients with breast carcinoma.

	β3GalT5 staining score		
	0 (n=29)	**3+ (n=38)**	**P value***
Age	53.07±2.323	50.74±1.719	0.4122
Stage			
0+I	10	2	0.003
II+III	19	36	
Tumor status (T)			
T1+T2	24	30	0.7637
T3+T4	5	8	
lymph node metastasis (N)			
N0	20	15	0.0259
N1-N3	9	23	
Distal metastasis status (M)			
M0	28	37	1
M1	1	1	
Estrogen receptor (ER)			
Positive	13	16	1
Negative	16	22	
Progesterone receptor (PR)			
Positive	14	21	0.627
Negative	15	17	
Human Epidermal growth factor Receptor 2 (HER2)			
Positive	13	18	1
Negative	16	20	
Recurrences status			
Yes	7	13	0.4282
No	22	25	

*Statistical significance *p* value was analyzed by χ^2 test