Supplementary Information

A Chimeric Signal Peptide-Galectin-3 Conjugate Induces Glycosylation-Dependent Cancer Cell-Specific Apoptosis

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1. Abbreviations

Gal-3 = galectin-3; sGal-3 = engineered secreted galectin-3 with N-terminal tPA tag; CM = conditioned medium; WCE = whole cell extract; thrombospondin 1 = TSP-1; vascular endothelial growth factor = VEGF; PARP = poly (ADP-ribose) polymerase; MGAT5 = N-acetylglucosaminyltransferase V; β4GALT1-8 = β1,4-galactosyl transferases 1 to 8; β3GNT1-4 = β1,3-acetylglucosaminyl transferases 1 to 4.

2. Author contributions

Author contributions: Project concept, experimental design, data interpretation and manuscript were developed by SHL, FWK and EGVM. FWK found that sGal3 could induce cancer cell-specific killing and SHL identified the related mechanism. KT prepared modified rGal3, BY provided integrin expertise and performed flow cytometry analysis, SO performed immunofluorescence, FWK and AZ generated tet-inducible sGal3 cells, AZ, NSD and SHL performed mice tumorigenicity experiments, ZZ and JJO performed intracranial injections, MK and CH provided glioblastoma neurosphere cultures and RDC provided experimental advice. All authors read the manuscript.

3. Extended Material and Methods

Chemicals, Cell lines, and primary cells. Recombinant human galectin-3 was purchased from PeproTech (Rocky Hill, NJ). Lactose, sucrose, and melibiose were purchased from Sigma-Aldrich (St. Luis, MO). Caspase-3 inhibitor (Ac-DEVD-CHO) was purchased from BD Bioscience (San Jose, CA). Caspase-8 inhibitor (Z-IETD-FMK)

and caspase-9 inhibitor (Z-LEHD-FMK) were purchased from MBL international (Woburn, MA). Kifunensin (N-linked glycosylation inhibitor) and Benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (O-linked glycosylation inhibitor) were purchased from Sigma-Aldrich. LY294002 (PI3K/Akt inhibitor) was purchased from Sigma-Aldrich. Cpd22 (ILK inhibitor) was purchased from EMD-Millipore (Burlington, MA). MDL28170 (Calpain inhibitor III) and Verapamil hydrochloride (calcium channel blocker) were purchased from Enzo Life Sciences (Farmingdale, NY). Transfection reagents (GenePorter and GeneSilencer) were purchased from Genlantis (San Diego, CA). Unconjugated Phaseolus Vulgaris Leucoagglutinin (PHA-L), agarose-bound L-PHA, and agarose bound Ricinus Communis Agglutinin (RCA) were purchase from VectorLab (Burlingame, CA). Human glioblastoma cell lines LN-Z308 (p53 null) parental (1) have been previously described. Glioma cell lines LN229, clone LN229-L16 (rtTA expressing) (2), U87MG and SF767 (3), 293HEK (embryonic kidney cells), breast (MCF7 and MD468), lung (A549, H1299), colon (HCT116) and prostate (LnCaP) cancer cell lines, and human foreskin fibroblasts (HFF1) were grown in DMEM supplemented with 5% tetracycline-free FCS (Gibco, Grand island, NY). Primary cultures of human dermal fibroblasts (HDF) were grown in FibroLife basal medium (LIFELINE cell technology, Frederick, MD) with supplements and 2% FCS (Gibco). Human dermal microvascular endothelial cells (HDMEC) were grown in VascuLife basal medium (LIFELINE cell technology) with growth factor supplements and 2% FCS (Gibco). Human normal breast epithelial cells (MCF10) were grown in DMEM supplemented with 5% FCS (Gibco), EGF (Sigma, 20ng/ml), insulin (Sigma, 10μg/ml), and hydrocortisone (Sigma 500ng/ml). Human astrocytes were grown in supplemented astrocyte medium

(ScienCell Research Laboratories, Carlsbad, CA) and 2% FCS (Gibco). Non-tumor cell lines or primary cells were we used between passages 4-8. sGal-3 expression was induced by modulation with 2 μg/ml of doxycycline (dox) for 48 hr. The neurospheres (N08-74) were generated by mincing and digesting a GBM tumor tissue for 30 minute at 37 °C with 1 mg/ml collagenase/Dispase (Roche, Indianapolis, IN) followed by a Ficoll gradient. They were cultured in Neurobasal A-medium (GIBCO, INVITROGEN), with N-2 and B-27 supplement (Invitrogen, Waltham, MA), 10ng/ml human recombinant bFGF (STEMCELL technologies, Cambridge, MA) and 20 ng/ml EGF (STEM CELL technologies) at 37 °C with 5% CO_2. FACS-mediated cell sorting was used to isolate a CD133+ population, using anti CD133 /1 (AC133)-phycoerythrin (PE) coupled antibody (Miltenyi Biotech, Auburn, CA) with a FACS Vantage SE (Becton Dickinson, Franklin Lakes, NJ).

Transient cDNA or siRNA transfection studies. For β1 integrin knockdown studies, cells were transfected with 100 nM of two independent β1 integrin siRNAs (sc-35674, sc-44310) or control siRNAs (RNA-A; sc-37007; Santa Cruz BioTechnology) for 72 hrs with siRNA-specific transfection reagent (Gene Silencer, Genlantis) after which the cells were seeded at 5,000 cells per well in 96-well plates. β1 integrin knockdown was confirmed by western blot at the 72 hr time point. Twenty-four hours after cell splitting, 200 μl of 1x control or sGal-3 CM were added to each well. For MGAT5 overexpression or knockdown studies, the pCXN2-MGAT5 (kind gift from E. Miyoshi, Osaka University, Osaka, Japan) and pSUPER-MGAT5 (kind gift from M. Pierce, University of Georgia, Athens, GA) expression vectors were used (4, 5). Briefly, 2 x 10^6 cells/ 10 cm plates were cultured 6 hrs before transfection. For transfection, 10 μg of each plasmid

DNA with each 30 μl of GenePorter transfection reagent (Genlantis) were co-incubated for 6hrs with serum free condition. Fetal bovine serum (10%) was added for another 48 hrs and the transfection efficiency was confirmed through MGAT5 RT-PCR analysis.

Generation of sGal-3 expression vectors. The 753 bp *Gal-3* cDNA was generated by RT-PCR from SF767 glioma cell RNA, sequence verified and cloned into the XbaI cloning site of the pcDNA3.1 myc His expression vector (Invitrogen). Similarly, *LGALS3* cDNA without the start codon was amplified and cloned into the EcoR1 site of pUMVC7 plasmid (Aldevron, Fargo, ND) containing a classical secretion signal peptide from the tissue plasminogen activator, to generate plasmid pUMVC7-sGal-3 that constitutively secretes Gal-3 (sGal-3) from a CMV promoter. To construct doxycycline-inducible secreted Gal-3, the fragment containing sGal-3 (secretion signal plus Gal-3 transcript) was excised from this plasmid with BssHII and Not 1, and cloned into the pTRE2 expression vector (Clontech, Mountain View, CA). To prepare purified His-sGal3, sGal-3 insert (t-PA secretion signal peptide plus Gal-3 transcript) was excised from pUMvc7-sGal-3 with NdeI and BamHI, and cloned into the pET15b expression vector (Genescript, Piscataway, NJ).

Generation of cells with stable transfection. Doxycycline-inducible sGal-3 transfected clones were generated in LN229-L16 glioma cells (Tet-on clone derived from LN229)(6). Cells (2 x 10^6) were plated in 10 cm dishes and 24 hrs later they were transfected with 4 μg of pUMVC7-sGal-3, 1 μg of pcDNA3.1 containing the geneticin (G418)-resistance cassette and 5 μl of GenePORTER reagent (Genlantis). Individual clones were selected in G418 (1,200 μg/ml) and assessed for expression of sGal-3 after 48 hr dox induction by western blot analysis. sGal-3 expression was induced by modulation with 2 μg/ml of

doxycycline (dox) for 48 hr. MGAT5-stable transfection of 293 cells were prepared as described (7). Cells (5 x 10^5) were plated in each well of 6 well plate and 24 hrs later they were transfected with 1 μg of pCXN2-MGAT5, and 5 μl of GenePORTER reagent (Genlantis). Individual clones were selected in G418 (800 μg/ml) and assessed for expression of MGAT5 by western blot analysis.

Production of sGal-3. 293 cells were transiently transfected using GenePORTER reagent (Genlantis) with the pUMVC7-sGal-3 expression vector or pCMV-LacZ as control, and switched to serum free media 16 h later. The CM was collected after 48 hrs, floating cells removed through centrifugation at 1000 g, filtered through a 0.45 μm filter (Corning; NY, USA) and it was either stored in frozen aliquots at –20°C or used undiluted (1x) on target cells in cell viability assays. To produce larger amounts of sGal-3 CM, we treated LN229-L16 sGal-3 Tet-on inducible cells (clone #11) with 1μ/ml of doxycycline for 96 hrs and harvested the CM. About 500 ng/ml of sGal-3 was produced in 2x CM after 96 hrs based upon quantified with human Gal-3 ELISA kit (eBioscience, San Diego, CA; Raybiotech, Norcross, GA). Concentrated sGal-3 CM was prepared through Amicon Ultra centrifugal filters (Millipore-Sigma, Danvers, MA). Purification of sGal-3 from CM was performed using a lactosyl-sepharose column as described (8). Purification of His-sGal3 from bacterial cell extract lysate was followed as described (9). Briefly, pET-15b-sGal-3-transformed bacteria (BL21(DE3), NEB, Ipswich, MA) were grown in a shaker at 37°C until O.D. reached ~0.8. After adding 0.5 mM IPTG bacteria were further incubated at 17°C under shaking for another 24 hrs. Bacterial were lysed in phosphate lysis buffer (50 mM Na_2HPO_4, 300 mM NaCl, 0.5% Triton-X, pH 8.0) and lysates were loaded on an Ni-NTA agarose column (Qiagen, Germantown, MD). Nickel-

bound proteins were eluted with a concentration-gradient of imidazole buffer (phosphate lysis buffer + imidazole 75-300 mM, pH 7.0). Eluted fractions were tested by Coomassie brilliant blue staining and anti-Gal-3/His-tag western blotting to identify those containing His-sGal-3, and were further concentrated through Amicon ultra centrifugal filter units (30-kDa, Millipore-Sigma). The concentration of His-sGal-3 was analyzed with anti-His-tag ELISA kit (Genescript, Piscataway, NJ). Commercial rGal-3 was purchased from PeproTech (Rocky Hill, NJ).

Protein Extraction and Western Blot Analysis. Immunoblots were performed on cells lysed in 20 mM Tris-HCL, 150 mM NaCl, 1mM Na_2EDTA, 1mM EGTA, 0.5% NP-40 with protease inhibitor cocktail tablets (Roche). For protein analysis in the CM, the CM (1ml CM/lane) was precipitated with 15% TCA for 30 min at 4ºC, washed twice with ice-cold acetone, and then resuspended in 1x Laemmli buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue and 50mM Tris HCl, pH approx. 6.8). Electrophoresis and blotting were performed using the Criterion system (BioRad, Hercules, CA). Western blots were probed with antibodies for Gal-3 (1:500-1:1000; ab14364, A3A12, or H-160; Santa Cruz Biotechnology, Santa Cruz, CA), caspase-3 (1:3000; cat#9662; Cell Signaling Technology, Danvers, MA), cleaved caspase-3 (1:1000; Cell Signaling Technology), TSP1 (MS-421-P1-Ab-4; NeoMarkers, CA; 1:1000), PARP (1:3000; cat#9542; Cell Signaling Technology), cleaved PARP (1:1000; cat#5625; Cell Signaling Technology), Bax (1:1000; sc-493; Santa Cruz, 1:1000 cat#2772; Cell Signaling Technology), survivin (1:1000; sc-47750, Santa Cruz), integrin α3 (1:1000; sc-6588, Santa Cruz), integrin α5 (1:1000; sc-10729, Santa Cruz), integrin β1 (1:1000; sc-6622, sc-374429, Santa Cruz), integrin β1 clone 12G10 (active form

identification)(1:1000; sc-59827, Santa Cruz), phospho-Akt (ser473, 1:1000; #9271, Cell Signaling Technology), Akt (1:3000; #9272, Cell Signaling Technology), phospho-GSK3β (1:1000, sc-373800, Santa Cruz), GSk3α/β (1: 2000, sc-7291, Santa Cruz), MGAT5 (1:1000; MAB5469, R&D Systems, Minneapolis, MN), and Talin (sc-7534, Santa Cruz). β-Actin was used as a loading control (1:1500; sc-1615, 1:3000; sc-47778, Santa Cruz). Blots were visualized by SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific, Rockford, IL).

RT/PCR analyses. Total cellular RNA was isolated with Trisol (Invitrogen). Two hundred fifty ng of total RNA were subjected to RT-PCR reaction with FideliTaq RT-PCR master mix (ThermoScientifc). All RT-PCR products were analyzed through the loading in 1.2% agarose gels. All RT-PCR experiments were repeated 3-4 times independently and the intensity of PCR bands was quantified with NIH Image J software. For the MGAT5 RT-PCR, human MGAT5 primers were used as described (10). For the β3GnT2 RT-PCR the primers (sense: 5'-GAGAGTGAGAAGCACCAAG-3'; antisense: 5'-CTTAAAATGGCCAAACCAG-3') were used as follows: 95ºC (3 min); 95ºC (30'), 56ºC (45'); 72ºC (60'), 35 cycles; 72ºC 10 min. For the β4GalT1 RT-PCR the primers (sense: 5'-AATCGTGCTAAGCTCCTCAATGTTGGC-3'; antisense: 5'-CTCGGTGTCCCGATGTCCACTGTGAT-3') were used as follows: 95ºC (3 min); 95ºC (30'), 56ºC (45'); 72ºC (60'), 35 cycles; 72ºC 10 min. For the β4GalT2 RT-PCR the primers (sense: 5'-CGCTGGAGCGCGTCTGCAAGGC-3' antisense: 5'-ACAAGACCAGGTGGCGAGTCA-3') were used as follows: 95ºC (3 min); 95ºC (30'), 61ºC (45'); 72ºC (60'), 35 cycles; 72ºC 10 min. For the β4GalT5 RT-PCR the primers (sense: 5'-GCTGCTGTACTTCGTCTATGTGGCGC-3'; antisense: 5'-

GCCTCGGCATCTGTCCACATCC-3') were used as follows: 95ºC (3 min); 95ºC (30'), 58ºC (45'); 72ºC (60'), 35 cycles; 72ºC 10 min. The PCR parameters used were: For control reactions, G3PDH primers (sense: 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3'; antisense: 5'-CATGTGGGCCATGAGGTCCACCAC-3') or β-actin primers (sense: 5'-ATGGGTCAGAAGGATTCCTAT-3'; antisense: 5'-GCGCTCGGTGAGGAT-CTTCAT-3'), were used. For G3PDH and β-actin, the reaction conditions were: 95ºC (3 min); 95ºC (30'), 55ºC (45'); 72ºC (60'), 30 cycles; 72ºC 10 min.

Antibody-mediated activity blocking assays. Neutralization of β1 integrin on cells was achieved by addition of 5 μg/ml anti-human β1 integrin inhibitory antibodies (clone P5D2 and AIIB2 The Developmental Studies Hybridoma Bank, Iowa city, IA) or control immunoglobulins (normal mouse IgG, Santa Cruz) before sGal-3 CM treatment.

GST-Gal-3 pulldown assays. Recombinant GST-Gal-3 constructs (kindly provided by Dr. W. Stallcup, Sanford Burnham Prebys Medical Discovery Institute, San Diego, CA) were used for the production of rGal-3 in bacteria and used for pulldown assays as described with some modification (11). Briefly, 750 μg whole cell extracts at 1 μg/μl were mixed with 15 μl of glutathione agarose beads (ThermoScientific) for each of GST-Gal-3F, GST-Gal-3N, or GST-Gal-3 CRD (50% W/V), and rocked at 4ºC for 3 hrs +/- lactose competition (25 mM). The beads were pulled down by centrifugation, washed three times with PBS buffer, boiled in Laemmli sample buffer and the proteins analyzed by western blot. GST-Gal-3 beads alone were used to analyze for non-specific background and Ponceau staining was performed to verify equal amounts of GST-Gal-3 proteins were used. The experiments were performed three or more times independently.

Co-immunoprecipitation assays. To examine the interaction of sGal-3 with tumor cell surface integrins, sGal-3 CM was applied to live cultured cells. Briefly, 3 ml of 2x sGal-3 CM was added to 5 x10^6 cells in 10 cm diameter culture dishes (serum starved for 24 hrs) grown in 10 cm petri dishes and rocked for 4 hrs at room temperature. After sGal-3 CM removal, cells were washed three times with PBS and lysed with CHAPS buffer (30 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1mM EDTA, 0.25% CHAPS (A.G. Scientific, San Diego, CA)) with a protease inhibitor cocktail (Roche). Aliquots of 750 μg of whole cell extracts at 1μg/μl were pre-cleared with protein G agarose beads at 4°C for 20 min. Subsequently, anti-human galectin-3, β1 integrin, α5 integrin antibodies or non-specific immunoglobulins (1.5 μg/ml; Santa Cruz Biotechnology) were used for immunoprecipitation overnight at 4°C. Protein G agarose beads (20 μL; 50% (W/V), Roche) were added and incubated for another 4 hrs at 4°C. Beads were recovered by centrifugation, washed 3 times with lysis buffer, boiled in Laemmli sample buffer and the immunoprecipitated proteins analyzed by western blot. For the detection of MGAT5 synthesized glycans on cell surface integrin β1, L-PHA-cell surface integrin β1 co-immunoprecipitation assays were performed as above except the cultured cells were pre-treated with a pure L-PHA solution (2.5μg/ml)(Vector Labs, Burlingame, CA) for 2 hrs at room temperature. After washing 3x with PBS, a cell extract was prepared by lysis in CHAPS buffer and anti-L-PHA antibodies (1.5 μ/ml Vector Labs) used to immunoprecipitate the L-PHA-bound glycan residues, followed by Western blot for β1 integrin.

Plant lectin pull down assays. For the detection of glycans synthesized by MGAT5 on β1 integrin, L-PHA agarose-integrin β1 pulldown assays were performed. Briefly, 500 μg

of whole cell lysate was rocked for 2 hrs with 20 μl of L-PHA agarose beads slurry (50% (W/V), Vector Labs) at 4°C. The beads were recovered by centrifugation, washed 3x with PBS, boiled in loading buffer and the pulled-down proteins analyzed by Western blot with anti-β1 integrin antibodies. For the detection of β4GalT-transferred glycan branches, *Ricinus communis* agglutinin (RCA)-agarose-β1 integrin pulldown assays were used with RCA agarose beads (Vector Lab). All pulldown assays were performed at least three independent times and the intensities of western blots were quantified with NIH image J software.

Analysis of DNA Content and BrdU Incorporation. LN229 glioma cells were induced with 2 μg/ml doxycycline (DOX) for 48 hrs before 30 min of BrdU (10 μM) labeling. Cell-incorporated BrdU and total DNA content (7-AAD) were analyzed by flow cytometry. The percentages of G0/G1, S, and G2/M cells were identified. Parental LN229, clone LN229-L16 (tet-on), L11 (LN229-L16-derived cells stably expressing inducible sGal3 plasmid) were compared. Dot plots show percentages of cells in different phases of the cell cycle.

Immunofluorescence Staining. LN229 glioma cells were grown on glass-bottom slides (ibidi, 81506) and treated with control or sGal-3 CM for 24 hrs before proceeded to immunofluorescent staining protocol with fluorophore conjugated antibodies: Gal3-FITC (sc-53127) and integrin β1-594 (sc-13590) and counterstained with Hoechst for DNA. Confocal microscopy images were obtained on a Leica SP-8 microscope (Leica Microsystem Inc. Buffalo Grove, IL).

Apoptosis assays. For apoptosis analysis, cells were treated with either CM containing secreted Gal-3 or with purified sGal-3, 100 nM AC-DEVD-CHO caspase-3/7 specific

inhibitor (BD Bioscience), 20 μM of Z-IETD-FMK caspase-8 inhibitor, and 20 μM of Z-LEHD-FMK caspase-9 inhibitor (MBL international).

Caspase-3/7 or -9 Glo assays (Promega, Madison, WI) was performed on sGal-3-treated cells as per the manufacturer's instructions (Promega). The luminescence value (RLU, blank subtracted) was converted to fold induction and the values from control vector-transfected CM-treated cells were considered as 1.

Calpain GLO protease assays. For calpain protease activity analysis, cells were treated with either CM containing sGal-3 or sGal-3 with 500 nM of calpain inhibitor III (MDL28170, Cayman Chemical, CA). For additional sGal-3 control purpose, cells were treated with rGal-3 and sGal-3 CM was pretreated with 25 mM lactose or 25 mM melibiose for 30 min. Calpain GLO protease assays (Promega) was performed on sGal-3-treated cells as per the manufacturer's instructions (Promega). The luminescence value (RLU, blank subtracted) was converted to fold induction and the value from 0h sample was considered as 1. All assays were triplicated.

Calcium colorimetric assays. For calcium influx accumulation analysis, cells were pre-treated with 50 μM of verapamil (calcium channel blocker, Sigma Aldrich) for 24 hrs before treating with sGal-3 CM. In controls, sGal-3 CM was pretreated with 25 mM lactose for 30 min. Calcium colorimetric assay was performed on sGal-3-treated cells as per the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). The accumulated cytosolic calcium concentration was quantified by acquiring absorbance at 590 nm using a spectrophotometer and extrapolating from calcium concentration standard curve. Assays were performed in triplicate.

Crystal Violet cytotoxicity assays. Cells were plated at 5,000 cells/well in 96-well plates and treated with 1x control of Dox-induced sGal-3 CM (~500 ng/ml sGal-3) for 24 to 120 hrs. Thereafter, the cells were fixed in a crystal violet (0.2%) /ethanol (2%) solution for 10 min., washed in water and solubilized in 1% SDS. Relative cell number was quantified by acquiring absorbance at 575 nm using a spectrophotometer.

Soft-agar Colony Formation assays. Six-well plates were layered with 2 ml of 1% agar in DMEM medium supplemented with 10% Tet-free serum. This bottom layer was overlaid with 5,000 cells mixed in 0.33% agar with DMEM and 10% Tet-free serum. One ml of 10% Tet-tested serum containing media +/- 5 μg/ml of Dox was added to the top every 72 hrs. After 21 days the colonies were fixed using 100% methanol and visualized using Giemsa stain according to the manufacturer's protocol (Sigma). The plates were air-dried to flatten the agar discs, the colonies counted and photographed at 20x. The experiment was repeated three times in triplicate (n=3).

***In vivo* tumorigenicity experiments.** For the subcutaneous tumor growth experiment 6-week old female athymic nude mice (NCI) (n=8/ group) were injected subcutaneously with $5x10^6$ cells of the indicated cell lines. 2 mg/ ml Dox was delivered orally in drinking water containing 5% sucrose to induce expression of sGal-3 one week post injection of tumor cells until termination of the experiment. Tumor volume was calculated in mm^3 = (length x $width^2$)/2.

For the orthotopic brain tumor experiments, 6-week old female athymic nude (NCI) or C57BL6 mice were injected intracranially with 5×10^5 LN229-L16 sGal-3 Tet-on cells (clone #11) and divided into two groups (+/- Dox) of 11 mice each. Sixty-three days after the intracranial tumor injection, 10 nM of IR-labeled 2-deoxyglucose (2-DG)

(LI-COR, Lincoln, NE) was tail-vein injected and the intensity of dye-stained brain tumor was analyzed 24 hrs later with Olympus FV-1000 microscopy (IR wavelength = 750 nm). Mice were terminated as per Institutional Animal Care and Use Committee (IACUC) criteria. The Kaplan-Meier survival curve was established using SPSS and MedCalc statistical software.

For the *in vivo* tumor growth prevention experiments, 5 x 10^6 lung cancer cells (H1299 and A549) were pre-incubated with Ni-NTA agarose column-purified His-sGal-3 (500 nM) for 20 minutes at room temperature and subcutaneously co-injected at 1:1 ratio with Matrigel (Corning Life Sciences, Tewksbury, MA) in the flanks of athymic nude mice. Tumor cells treated with identical fractions of Ni-NTA agarose columns loaded with extracts from bacteria transfected with empty vector were used as negative control. Weekly tumor volume (length x $width^2$)/2 was measured by a caliper till the 8th week post tumor cell injection.

Statistics. Statistical analysis was performed using GraphPad Prism v6.01 software (GraphPad Software). Results are presented as mean ± SEM. For comparison of sample versus control, unpaired t-test was used. For Kaplan-Meir survival study, p-value was calculated by Logrank test. A p-value less than or equal to 0.05 was considered significant. For results p-values are presented as follows: *<0.05, **<0.01, ***<0.001, and ****<0.0001.

Study approval. All animal work was performed according to the guidelines for animal experimentation and welfare and approved by the Emory University Institutional Animal Care and Use Committee (IACUC).

4. Supplementary Figure Legends

Suppl. Figure 1: Secreted Gal-3 inhibits tumor cell viability *in vitro*.

A) Structure of engineered Gal-3 constructs: CRD, carbohydrate binding domain; FL, full length Gal-3; EL, N-terminal elongated Gal-3 with extra collagen-like sequences; sGal3, secreted form of Gal-3 with conjugated N-terminal t-PA signal peptide.

B) Western blot showing expression of sGal-3 is not affected by post-translational glycosylation. 293 cells were pre-treated with N- (NGI: 100 μM kifunensine) and O- (OGI: 2 mM benzyl-N-acetyl-α-D-galactosamide) glycosylation inhibitors for 24 hrs, and then co-transfected with sGal-3 and β1 integrin.

C) Crystal violet staining showing relative sensitivity of LN229 cells to 48 h. treatment with sGal3 or rGal-3 (125, 250 or 500 ng/ml; quantified by human Gal-3 ELISA; Ray Biotech). Representative pictures are shown. Scale bar represents 100 μm.

D) Western blot showing induction of PARP cleavage by 500 ng/ml of different engineered forms (CRD, FL, EL) of rGal-3 versus sGal3 in LN229 cells 12 hrs after treatment. Note only sGal-3 induces PARP cleavage.

E) Crystal violet cell viability assay showing cytotoxicity of conditioned media (CM) from 293 cells supplemented with different engineered forms (CRD, FL, EL) of rGal-3 versus sGal3-CM in LN229 cells (*** $p < 0.001$ compared to Ctrl. CM; unpaired *t*-test). Note only sGal-3 reduces cell viability. Right panel, representative images are shown (Scale bar = 100 μm).

F) Cytotoxicity of engineered Gal-3 forms in T-cell leukemia cells. Left panel: crystal violet assay testing the cytotoxicity of each form of engineered Gal-3 in T-cell leukemia cells (Jurkat) after 48 hrs. of treatment. (N=3). Ctrl, DMEM medium; Ctrl CM,

conditioned medium from empty pcDNA3 vector transfected 293 cells; Gal-3, thrombin-cleaved Gal-3 from bacterially expressed GST-Gal-3 solubilized in DMEM; rGal-3, recombinant Gal-3 from a commercial source (Peprotech) solubilized in DMEM; sGal-3, sGal-3 CM from pUMVC7-Gal-3 transiently transfected 293 cells. Gal3 concentrations were measured by ELISA (Ray Biotech). (N=3, *** p<0.001, ****p<0.0001; unpaired *t*-test). Right panel: Potency comparison between rGal-3 and sGal-3 for induction of PARP cleavage in Jurkat cells. Whole cell extracts were analyzed 12 hrs after treatment by Western blot.

G) Crystal violet assay showing lactose neutralization of sGal-3-mediated tumor cell specific killing. Pretreatment of sGal-3 CM with lactose (25 mM; "lactose to sGal-3") neutralized sGal-3 and prevented tumor cell death at 48 hrs. In contrast, tumor cell pretreatment with lactose ("lactose to cells") did not protect from sGal-3-mediated cell death. Representative cell images (left) and quantification (right) (N=3, *** p<0.001; unpaired *t*-test). Scale bar represents 100 μm.

Suppl. Figure 2: Gal-3 reduces the tumorigenicity of tumor cells *in vitro* and *in vivo*.

A) Cell cycle analysis of LN229 glioma cells with tet-on sGal3 expression. Cells were induced with 2 ug/ml doxycycline (DOX) for 48 hrs before 30 min of BrdU (10 μM) labeling. Cell-incorporated BrdU and total DNA content (7-AAD) were analyzed by flow cytometry. The percentage of G0/G1, S, and G2/M cells were quantified. Parental LN229, and LN229-L16 (tet-on) were used as controls.

B) Imaging of intracranial LN229-sGal3 tumors. Near-infrared (NIR, 750 nm) imaging 24 hrs after i.v. injection of a fluorescent 2-deoxy-glucose probe (10 nM; LI-

COR) shows decreased tumor burden in dox-treated mice (2 mg/ml in 4% sucrose drinking water) versus controls (no dox induction) at day 63.

C) Doxycycline treatment does not affect survival of mice (12/group) carrying intracranial LN229-L16 parental glioma cells (stably transfected with rtTA; no sGal3 expression).

D) Bacterially generated his-tagged sGal-3 (His-sGal-3) displays *in vitro* cytotoxicity against a variety of tumor cells. Left panel: Coomassie blue staining of Ni-NTA-purified His-sGal-3. Ni/B, His-sGal3-bound Ni-NTA agarose column fraction before imidazole elution; Ni/A, same fraction after imidazole elution; W, wash with wash buffer; E3,4,5, imidazole-eluted His-sGal-3 fractions 3, 4, and 5. Right panel: Crystal violet assay showing His-sGal3 (500 ng/ml)-mediated tumor cell killing after 48 hr. of treatment in glioma (LN229) and lung cancer cells (H1299, A549). (N=3, *** $p<0.001$; unpaired *t*-test)

E) His-sGal-3 prevents *in vivo* lung cancer growth. One million lung carcinoma cells (H1299 and A549) were detached from plates, pre-treated with His-sGal3 (500 ng/ml) at room temperature for 20 minutes, admixed with Matrigel (1:1 vol.) and injected s.c. in athymic nude mice. Tumor volume (1/2 x L x W^2) was derived from weekly caliper size measurements. For control groups, Ni-NTA-eluted lysates from empty-vector transfected bacteria were used. The H1299 study had 10 mice/group, while the A549 study had 5 mice/group. (****$p<0.0001$ and ***$p<0.001$).

Suppl. Figure 3: Secreted Gal-3 selectively induces apoptosis in tumor cells:

A) Western blot showing purified sGal-3-mediates apoptosis in LN229 cells. Conditioned medium of 293 cells transfected with sGal-3 was purified using a lactosyl-sepharose column and sGal-3 quantified by ELISA. DMEM medium was used as negative control. Cleaved PARP (89-kDa) and cleaved caspase-3 (17/19-kDa) were increased 6-12 hrs after sGal-3 (500 ng/ml) treatment.

B) Western blot analysis of HFF-1 fibroblasts. sGal-3 does not cause any PARP or caspase-3 cleavage. C3i, caspase 3 inhibitor.

C) Western blot showing caspase-7 cleavage in caspase-3-deficient MCF7 breast cancer cells after 24 hrs sGal-3 treatment.

D) Proteome Profiler Human Apoptosis Array Kit (R&D system) was used to analyze changes in apoptosis-related proteins after 6 hrs 2x sGal-3 CM treatment of LN229 cells. Note increased expression of Bax and cleaved caspase-3 and decreased survivin.

E) Western blot showing decreased survivin expression in LN229 cells after sGal-3 treatment.

F) Western blot showing increased Bax expression in LN229 cells after sGal-3 treatment.

Suppl. Figure 4: **β1 integrin is needed for sGal-3-mediated cell death and shows preferential sGal-3 interaction in cancer cells.**

A) RNA interference knockdown of β1 integrin expression renders cancer cells resistant to sGal-3-activated cell death. Cells were pre-treated with β1 or control siRNAs (200 nM; 72 h.), then seeded at 5,000 cells/well in 96-well plates. 24 hrs later, cells were exposed to 200 μl of 1x control or sGal-3 CM and cell viability examined 72

hrs later by crystal violet assay. Knockdown of β1 expression was confirmed by western at the 72 h. time-point (right panel). N=3 (triplicates). **p < 0.01; unpaired *t*-test

B) Caspase-3/7 GLO assay shows that β1 integrin siRNA inhibits induction of caspase-3/7 cleavage by sGal-3 in LN229 cells.

C) Crystal violet cell survival assay showing that 24 hr pre-treatment with anti-β1 integrin blocking antibodies (P5D2; 5 μg/ml) prevents sGal-3-mediated tumor killing in LN229 cells. For antibody control, same concentration of mouse normal antibody was used (Santa Cruz Biotech). Cell viability was measured 48 hrs after sGal-3 CM treatment. N=3 (triplicates). ***p < 0.001; unpaired *t*-test

D) Immunofluorescence shows co-localization of sGal3 with β1 integrin at cancer cell surface. LN229 cells were grown on a glass-bottom slide (ibidi, 81506) and treated with control or sGal-3 CM for 24 hrs before proceeded to immunofluorescence staining with fluorophore conjugated antibodies: Gal3-FITC (sc-53127) and integrin β1-594 (sc-13590) and counterstained with Hoechst for DNA. Confocal microscopy images were obtained on a Leica SP-8 microscope (Leica Microsystem Inc. Buffalo Grove, IL).

E) GST-Gal-3 pull down experiment shows that transient transfection of HEK293 cells with α5 and β1 integrin expression vectors leads to a robust increase in Gal-3 binding.

F) Western blot shows that transient transfection of α5 and β1 integrins strongly sensitizes HEK293 cells to sGal-3-induced apoptosis as evidenced by cleavage of caspase 3 and PARP.

G) Western blot showing expression of α5 and β1 integrin subunits in LN229 and HFF-1 cells. Note expression level of α5/β1 integrin is roughly equivalent between both cell

lines, but β1 size in increased in LN229 cells and a fraction of β1 is in immature form in HFF-1 cells.

H) Detection of cell-bound exogenous sGal-3 by western blot. Whole cell extracts were harvested before (left panel) and after (right panel) sGal-3 CM (500 ng/ml, 4 hrs. at 37C°) treatment. Cell-bound exogenous sGal-3 (34-kDa) and endogenous Gal3 (monomer, 26.5-kDa and dimer, 52-kDa) are detected.

Suppl. Figure 5: **β1 integrin is needed for sGal-3-mediated cell death and shows preferential sGal-3 interaction in cancer cells.**

A) Crystal violet cell viability assay showing that $Ca2^{+}$ channel (Verapamil) and Calpain (inhibitor III) inhibitors abrogated sGal-3-mediated cell death. ILK (Cpd22), and PI3K/Akt (Ly294002) inhibitors had no effect. LN229 cells were treated with sGal-3 for 60 hrs. N=5; *p<0.05, ****p<0.0001 (unpaired *t*-test).

B) Western blot showing sGal-3-mediated cleavage of PARP was inhibited by Verapamil and calpain inhibitor III, but not by Cpd22 and Ly294002. LN229 cells were treated with sGal-3 for 9 hrs.

Suppl. Figure 6. **Alteration in MGAT5 expression modulates complex N-glycan formation on β1 integrin, sGal-3-β1 integrin interaction and sGal-3-mediated cell killing.**

A) Upper panel: MGAT5-stably transfected 293 cells were pretreated with kifunensine for 24 hrs. Then α5β1 integrin subunits were transiently transfected for 48 hrs and cells treated with 2x sGal3 CM during the last 12 hrs. Note kifunensine blocked

sGal-3-mediated PARP cleavage in α5β1 integrin transfected cells. Lower panel: MGAT5/α5β1 co-transfected 293 cells are more sensitive to sGal-3-mediated PARP cleavage than α5β1 integrin transfected controls, and this effect is abrogated by kifunensine. The transfection scheme used for both experiments is presented at top.

B) MGAT5 transfection increases β1 integrin activation as well as β1 integrin-bound talin (immunoprecipitation with 12G10 antibody and GST-Gal-3 pulldown assay). Left panel: Input fractions showing MGAT5 transfection efficiency and total levels of β1 integrin (6622 antibody) in both cell types. Middle panel: co-immunoprecipitation of active β1 integrin with 12G10 antibody shows increased active β1 integrin and binding to talin in MGAT5-transfected cells. Right panel: GST-Gal-3 pulldown confirms increased talin interaction in MGAT5-transfected cells. Lower graphs: Quantification of blots with each normalization (MGAT: MGAT5/β-actin, Talin (Co-IP): Talin Co-IP/Talin Western blot, Talin (Pulldown): Talin (Pulldown)/β1 integrin/Talin)

C) Western blot showing MGAT5 transfection increases the fraction of active β1 integrin (12G10 antibody) in β1 integrin co-transfected 293 cells.

Suppl. Figure 7. **Tumor cells display increased expression of N-glycosyltransferases that promote formation of complex N-glycans with a poly-N-acetyl-lactosamine structure recognized by Gal-3.**

A) RT-PCR analysis of glycosyltransferases (βGnT2, β4GalT5, and MGAT5) mRNA expression shows increased expression in tumor cells.

B) Densitometry analysis of the RT-PCR results of **A)**. Upper panel: relative expression ratios of glycosyltransferase/β-actin mRNA levels are shown) and normalized to the least

expressed sample (astrocyte) set at 1. (N=3) (*** $p < 0.001$ compared to fold induction for LN229; unpaired *t*-test). Lower panel: cumulative differential expression.

C) RT-PCR analysis of glycosyltransferases mRNA expression shows higher expression in patient-derived glioblastoma stem-like cells (GSC; CD133+) than in normal neural progenitors (NPG).

D) Pull-down assays show increased formation of complex N-glycans on β1 integrin in tumor vs. normal cells. The GST-Gal-3-β1 integrin pull-down assay detected binding of Gal-3 to the N-acetyllactosamine branches of β1 integrin. The RCA agarose-β1 integrin pull-down assay detected binding of RCA to the β1,4-galactose residues transferred by β4GalTs on the complex N-glycan branches of β1 integrin. The L-PHA agarose-β1 integrin pull-down assay detected binding of L-PHA to the β1,6-N-acetylglucosamine residues transferred by MGAT5 on the N-glycans of β1 integrin. β1 integrin western blots were performed on pulled down proteins and input fractions. β1-m, mature; β1-i, immature form of β1.

E) Densitometry analysis of the pull-down results of **D)**. Upper panel: relative expression ratios of glycosylated β1/input β1 protein levels in cell extracts are shown. N=3 (*$p<0.05$, ***$p<0.001$ compared to the fold induction for LN229; unpaired t-test). Lower panel: cumulative graph showing that N-glycosylation of β1 is increased in tumor vs. normal cells.

5. Supplementary References

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