Supplementary Materials and Methods

Cell Culture. U87-MG and T98G cell lines were obtained from ATCC (Manassas, VA, USA) and cultured according to ATCC protocol supplemented with 10% fetal bovine serum (FBS), 5% 10 000 Units/mL Penicillin-10 000 µg/mL Streptomycin, and 5% 200 mM L-Glutamine. Primary GBM WHO grade IV cell lines SN186 (76 yr old male), SN275 (58 yr old male) and SN310 (78 yr old female) were provided as a kind gift by P. Hothi (Swedish Neuroscience Institute, Seattle, WA) and have been previously described (49). Primary GBM cell lines were cultured in Neurobasal-A Medium (Gibco; Gaithersburg, MD) supplemented with 1x B27 Supplement (Gibco), 1 mM sodium pyruvate, 0.5 mM L-glutamine, 1x Antibiotic-Antimycotic (Gibco), 0.02 mg/mL insulin solution, 20 ng/mL EGF, and 20 ng/mL FGF-2. For crystal violet staining, 5 x 10^4 cells were plated and subjected to the treatments as described in the individual figures and then stained with 0.5% crystal violet prepared in a 1:1 methanol-water solution followed by PBS washes. All cell lines used in this study were selected as pools following infection and selection.

Animal models of cancer. Nu/Nu athymic 6-8 week old mice from Charles River Laboratories (Wilmington, MA, USA) were immobilized using the Just for Mice ^{TM} Stereotaxic Frame (Harvard Apparatus, Holliston, MA, USA) and injected intracranially with 5 µl of a 20 000 U87-MG-Luciferase cells/µl solution. Tumor growth was monitored via bioluminescence imaging on the IVIS 200 system (Perkin Elmer) and results analyzed using Living Image software (Caliper Life Sciences, Waltham, MA, USA). For survival studies, mice were injected and health monitored until in moribund state. All animal protocols were approved by the Institutional Animal Care and Use Committee.

Human samples. GBM tumor tissue samples were obtained from 36 patients diagnosed with craniotomy resection of the tumor. All patients received standard 6-week radiation treatment with concurrent temozolomide. Specimens from multiple resections were included in TMA including 26 samples from

36 patients that were isolated in the first resection, 30 samples from same 36 patients from second resections, and 13 samples from the 36 patients were isolated from third resections thus total of 69 samples were analyzed. Histology was evaluated according to the World Health Organization (WHO) classification for CNS tumors. For survival analysis Kaplan–Meier curves were generated using the online database UALCAN (http://ualcan.path.uab.edu/analysis.html) (50) to determine the relevance of CDK5 mRNA expression in patients with GBM.

Reagents. Anti-actin (Santa Cruz Biotechnology; Dallas, TX, USA), anti-OGT, anti-ACSS2, anti-Cdk5, anti-K48 polyubiquitin, anti-HA-tag, anti-p35/p25, anti-Rb, anti-phospho Rb-S780, anti-cleaved-caspase-3, IgG-conjugated sepharose beads (Cell Signaling Technology; Danvers, MA, USA), anti-O-GlcNAc (Sigma Aldrich; St. Louis, MO, USA) and anti-thiophosphate ester (Abcam; Cambridge, U.K.). Protein G sepharose 4B beads (Invitrogen; Carlsbad, CA, USA). Plasmids used were pReceiver N-Flag OGT, pReceiver HA-ACSS2, HA-ACSS2-S267A, HA-ACSS2-S267D (Genecopoeia; Rockville, MD, USA). Cdk5-HA was a gift from Sander van den Heuvel (Addgene plasmid # 1872). HA-Ub and HA-Ub-K48R was a gift from Edward Hartsough (Drexel University). Ac-5S-GlcNAc and NButGT were a kind gift from David J. Vocadlo and previously described (24). Cycloheximide (Sigma Aldrich, St. Louis, MO, USA), Dinaciclib (SCH727965) (Selleckchem, Houston, TX, USA), lactacystin (Calbiochem, San Diego, CA, USA), D-luciferin potassium salt (Perkin Elmer, Waltham, MA, USA), and crystal violet (Sigma Aldrich, St. Louis, MO, USA). pReceiver.HA-ACSS2-WT, S267A, and S267D plasmid were provided by Genewiz (South Plainfield, NJ, USA) and confirmed via Sanger sequencing.

Rabbit polyclonal antibody recognizing phosphorylated ACSS2 S267 was created by YenZym Antibodies (South San Francisco, CA, USA). A peptide containing ACSS2 pS267 was injected into rabbits and serum was collected and purified using affinity column conjugated with nonphosphorylated ACSS2 S267 peptide to exclude antibodies recognizing ACSS2 nonphosphorylated form, followed by an affinity column conjugated with phosphorylated ACSS2 S267 peptide to purify the ACSS2 S267 phospho-specific antibody. This antibody was the eluted and used at concentration of 0.22 μg/ml.

RNA interference. Stable cell lines for shRNA knockdowns were generated as previously described (11). Briefly, HEK-293T cells were grown to ~75% confluency and transfected. Prior to transfection, 20 μg of shRNA or overexpression plasmid DNA, 10 μg VSVG, 5 μg RSV, and 5 μg RRE were gently mixed and incubated in 1.5 mL of Optimem for 5 minutes. Concurrently, 105 μl of PEI was added dropwise to 1.5 mL of Optimem and incubated for 5 minutes. Following the 5 minute incubation, the PEI solution was added dropwise to the DNA solution and incubated for at least 30 minutes. The PEI-DNA solution was then added dropwise to the HEK-293T cells already plated with 5 mL of Optimem and the cells were incubated overnight in the transfection media. Approximately 16-18 hours later, the transfection media was replaced with normal growth media. Viral supernatants were collected at 24 and 48 hours following the media change. These supernatants were passed through a 0.45 μm filter and portioned into 1 mL aliquots to be stored at -80°C if not for immediate us.

Control shRNA was acquired from Addgene (plasmid 1864), from D. Sabatini (Massachusetts Institute of Technology). Control-scrambled shRNA sequence used was: CCTAAGGTTAAGTCGCCCTCGCTCTAGCGAGGGCGACTTAACCTT. All other shRNA constructs were acquired from Sigma and shRNA sequence used: for OGT-1, GCCCTAAGTTTGAGTCCAAATCTCGAGATTTGGACTCAAACTTAGGGC, and for OGT-2, GCTGAGCAGTATTCCGAGAAACTCGAGTTTCTCGGAATACTGCTCAGC. Cdk5 shRNA sequence used: for Cdk5-1, CCGGGTGAACGTCGTGCCCAAACTCCTCGAGGAGTTTGGGCACGACGTTCACTTTTTTG, and for Cdk5-2, CCGGCAGAACCTTCTGAAGTGTAACCTCGAGGTTACACTTCAGAAGGTTCTGTTTTTTG. ACSS2 shRNA sequence used: CCGGGCTTCTGTTCTGGGTCTGAATCTCGAGATTCAGACCCAGAACAGAAGCTTTTTG.

mRNA expression. RNA was isolated from cells using RNEasy products and protocols (Qiagen, Valencia, CA, USA). Taqman gene expression assay primer probes for OGT (Hs00269228_m1), ACSS2 (Hs00218766_m1), and GAPDH (Hs02758991_g1) were purchased from Applied Biosystems (Foster City, CA, USA). qRT-PCR was performed as previously described (26) using Brilliant II qRT-PCR Master Mix 2 Kit (Stratagene, San Diego, CA, USA) using Applied Biosystems 7500 machine.. Briefly, isolated RNA concentrations were determined by measuring on a NanoDrop and all samples were diluted to match the sample with the lowest concentration. A mastermix was then generated for the individual primers using 8.8 µl dH_2O, 12.5 µl Brilliant PCR master mix, 0.4 µl diluted dye (0.5 µl dye into 250 µl dH_2O), 0.1 µl reverse transcriptase, and 1.25 µl primer per sample. A total reaction volume of 25 µl was used by gently mixing 2 µl of sample RNA and 23 µl of the mastermix into the reaction tubes, being careful to avoid bubbles. The samples were then loaded into the Applied Biosystems 7500 machine and the experimental setup was as follows: acquire experiment as "Quantitation ΔΔCT", targets and samples were assigned, the initial holding stage adjusted to 50°C for 30 minutes, followed by a secondary holding stage at 95°C for 10 minutes, then 40 cycles consisting of 95°C for 15 seconds and cooling to 60°C for 1 minute were completed. Data were exported from the Applied Biosystems software and expression levels were analyzed using Data Assist v2.0 (Life Technologies, Grand Island, NY, USA).

Immunoblotting. Immunoblotting protocols have been previously described (12). Briefly, cell lysates from 1-5 x 10^6 cells were prepared in radioimmune precipitation assay buffer (150 mM NaCl, 1% NP40, 0.5% DOC, 50 mM Tris HCL at pH 8, 0.1% SDS, 10% glycerol, 5 mM EDTA, 20 mM NaF, and 1 mM Na_3VO_4) supplemented with 1 µg/ml each of pepstatin, leupeptin, aprotinin, and 200 µg/ml PMSF. Lysates were cleared by centrifugation at 16 000 x *g* for 15 minutes at 4 °C and analyzed by SDS-PAGE and autoradiography with chemiluminescence. Proteins were analyzed by immunoblotting using primary antibodies indicated above.

Immunohistochemical staining. The slides containing glioblastoma tumors were deparaffinized by Xylene and subsequent rehydration was done by decreasing concentrations of ethanol-water mixture. Antigen retrieval was done by citrate buffer immersion and steaming the slides for 45 minutes. Tissue was treated with 200-400 ul 1% BSA+5% serum PBS solution for 1hr. Primary incubation was done at 4º C overnight using OGT, O-GlcNAc RL2 (Santa Cruz 1:50), p-S267 ACSS2 (1:100) antibodies. Secondary antibody incubation was done for an hour at RT with respective antibodies at 1: 200 dilution. The stain was developed using the DAB kit by Vectastain (Vector Labs, Burlingame, CA, USA). Finally, slides were mounted and imaged under a light microscope. The staining patterns of tumor tissues of GBM were assessed by a board-certified pathologist.

Immunoprecipitation. Cells were lysed and extracted with radioimmune precipitation assay buffer (150 mM NaCl, 1% NP40, 0.5% DOC, 50 mM Tris HCL at pH 8, 0.1% SDS, 10% glycerol, 5 mM EDTA, 20 mM NaF, and 1 mM Na3VO4) supplemented with 1 µg/ml each of pepstatin, leupeptin, aprotinin, and 200 µg/ml PMSF. For each sample, 1 mg of protein lysate was added to an Eppendorf tube and precleared with 15 µl of prewashed protein G sepharose beads by rotating at 4°C for 30 minutes followed by a 1-minute centrifugation at 2,500 x g at 4°C. These precleared supernatants were transferred to fresh tubes and were incubated with the indicated antibodies and lysates overnight at 4^0C. The following day, 30 µl of prewashed protein G sepharose beads were added to the immunoprecipitations and incubated for 2-3 hours by rotating at 4°C. The immunoprecipitation reactions were centrifuged at 2 500 x g at 4°C for 5 minutes followed by a wash with 750 µl of 1X PBST and this process was repeated three more times. After the final wash, the antibody-protein-bound beads were collected via centrifugation at 4°C and removal of supernatant. Beads were resuspended in 5x SDS loading buffer, boiled for 5 min and loaded. Immunoprecipitated proteins were resolved by SDS-PAGE sent for mass spectrometry or transferred to a PVDF membrane. Immunoblotting protocol was followed as per methods above.

LC-MS/MS Analyses and Data Processing. Quantitative comparisons of post-translational modifications via mass spectrometric analysis on immunoprecipitated HA-ACSS2 treated with DMSO or 100 μM NButGT was performed by The Wistar Institute Proteomics & Metabolomics facility (Philadelphia, PA, USA). Briefly, liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis was performed using a Q Exactive Plus mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) coupled with a Nano-ACQUITY UPLC system (Waters). Samples were digested in-gel with trypsin and injected onto a UPLC Symmetry trap column. Tryptic peptides were separated by reversed phase HPLC on a BEH analytical column. Eluted peptides were analyzed by the mass spectrometer. Peptide sequences were identified using MaxQuant 1.5.2.8 (51). MS/MS spectra were searched against a UniProt human protein database. Consensus identification lists were generated with false discovery rates of 1% at protein, and peptide levels.

Soft-Agar Colony Formation Assay and Neurosphere Assay. Soft agar assays have been previously described (12). Briefly, cells (1 x 10^4/well) infected with lentiviral shRNA or overexpression vectors, selected and plated in triplicate on top layers consisting of growth medium containing 0.3% agarose. The top layer was overlaid with 1 ml of medium. The cells were grown for 14 days, having the media changed every other day. After 14 days, the colonies were stained with 1 ml of 0.05% p-iodonitrotetrazolium violet overnight, and colonies measuring > 50 μm were counted manually. For neurosphere assays, SN310 primary glioblastoma cells (1 x 10^3) infected with shRNA, selected and were plated in polyhema-coated (1.2%) 24-well plates with the number of neurospheres formed being determined at Day 14 and neurosphere **measuring > 50 μm** were counted manually.

In Vitro Kinase Assay. Wildtype ACSS2 and S267A mutant were cloned into a pET28a vector into the NotI and BamH1 sites and transformed into BL21 cells. The BL21 cultures were induced with 1mM IPTG at 37°C for 3 hours. Cells were centrifuged and resuspended in His Lysis buffer (50mM Tris, pH 8.0, 5 mM imidazole pH 8.0, 500mM NaCl), supplemented with protease inhibitors (1 μg/mL each of

pepstatin, leupeptin, and aprotinin and 200 μg/mL phenylmethylsulfonylfluoride (PMSF) and 100mg/ml lysozyme. The lysate was sonicated on ice and centrifuged at 15000 x g for 15 minutes at 4°C. The supernatant was rotated over Nickel NTA Agarose beads (Gold Biotechnology, Cat# H-350-25) in a column (Gold Biotechnology, Cat# P-301) for 2 hours at 4°C. Upon collecting flow-through, beads were washed with wash buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 40 mM imidazole, pH 8.0), and eluted with His Elution buffer (500mM NaCl, 50mM Tris, pH 8.0, 250 mM imidazole, pH 8.0). Dialysis was performed in Slide-A-Lyzer G2 Dialysis Cassette (Thermo Scientific, Prod# 88251) for 2 hours at 4°C in dialysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM DTT). Recombinant CDK5/p25 (60 ng) (EMD Millipore, Burlington, MA, USA) was combined with purified ACSS2 (2ug) in kinase buffer (50 mM HEPES, pH 7.5, 0.625 mM $MgCl_2$, 0.625 mM $MnCl_2$, 12.5 mM NaCl) and 1mM ATPγS. The kinase reaction incubated for 5 minutes at 30°C, then was alkylated with 2.5mM PNBM. The alkylating reaction proceeded for 1 hour at room temperature, followed by the addition of 6× boiling sample buffer to stop the reaction. Reactions were then resolved by SDS–PAGE, followed by immunoblotting with the indicated antibodies.

Nile Red Staining of Cells. Cells were fixed using 4% Formalin and washed in 1xPBS prior to staining. Working solution (5 μg/ml) in PBS was gently added to cells and incubated for 30 minutes away from light. Cells were then washed three times in 1x PBS and visualized and photographed on EVOS FL (Life technologies) using Texas Red filter.

Free Fatty Acid and Acetyl-CoA Extraction and Quantification. Free Fatty Acid concentrations were measured with the Free Fatty Acid Quantification Kit (BioVision Inc., Milpitas, CA, USA) according to the manufacturer's protocol. Acetyl-CoA concentrations were measured with the Acetyl-Coenzyme A Assay Kit (Sigma Aldrich) according to the manufacturer's protocol.

$^{13}C_2$-Acetate Labeling. Cells were grown to ~80% confluency in a 6 cm dish in normal growth medium. 24 hours prior to labeling cells were grown in serum-free DMEM. Cells were incubated for 4 hours in 100 μM $13C_2$-sodium acetate. Cells were collected in cold PBS then centrifuged at 1 000 rpm for 5 minutes at 4 °C. Acetyl-CoA was then extracted from the cell pellets using 80% methanol – 20% dH2O and the samples were then analyzed via LC/MS.

For measuring acetate incorporation into lipids, cells were plated in 10 cm plates in standard culture media until 90% confluency was achieved. Confluent cells were grown in labeling media containing phenol red free (Gibco A1443001), 10 mM glucose, 4 mM glutamine, 200 uM acetate with one metabolite ^{13}C labeled, dialyzed FBS. Media was changed every 24 hours for 48 hours. Unlabeled control samples were cultured in the same media conditions, without ^{13}C acetate. After 48 hours, cells were washed with ice cold DPBS and trypsinized. The trypsin reaction was stopped using cold 10% fatty acid free BSA in DPBS to remove any exogenous fatty acids. The cells were then washed twice with ice cold DPBS and the cell pellet was frozen at -80C until extraction.

Analysis of fatty acid methyl esters (FAME). Lipids were extracted as previously described (53). The cell pellet was resuspended in 2 ml ice cold methanol, followed by addition of 0.7 mL ice cold glass distilled water. 10 uL of 1 mM heptadecanoic acid in methanol was added to each sample as an internal standard. Cell suspensions were sonicated using a Branson Sonifier 250 at an output of 2.5 and a duty cycle of 20% for 15 pulses. Following sonication, 1 mL of ice-cold chloroform was added and the suspension was mixed by vortexing. An additional 0.7 mL of ice-cold chloroform and 0.7 mL of ice-cold glass distilled water was added to the mixture and vortexed to mix. The suspension was centrifuged at 8000 RCF for 10 minutes at 4C. The chloroform fraction was transferred to a new tube and the original suspension was re-extracted with 0.7 mL ice cold chloroform and centrifuged at 8000 RCF for 10 minutes at 4C. The chloroform fraction from both extractions were pooled and 0.1 mL ice cold water was added and the sample was vortexed to mix. The sample was centrifuged at 8000 RCF for 10 minutes at 4C and the chloroform fraction was transferred to a new tube and dried under nitrogen at 40C.

Lipids were derivatized to methyl-esters by first resuspending the dried lipid extracts in 2 mL methanol: toluene (80:20) containing butylated-hydroxy toluene (5 mg in 50 mL). 3 uL acetyl-chloride was added and the samples were heated to 95C for 1 hour. Following heating, 5 mL 6% potassium carbonate was added and the samples were centrifuged at 8000 RFC for 10 minutes at 4C. The toluene layer was transferred to a new tube and centrifuged at 10000 RPM for 5 minutes at room temperature. The toluene layer was transferred to a glass GC/MS vial with a volume reducing insert. Fatty acid methyl esters were analyzed by GC/MS on an Agilent GC/MS 7890A/5975A with a DB-5 column. Acetate enrichment into fatty acids was determined using Fluxfix (54).

***Ex vivo* brain slice model.** *Ex vivo* tumor brain slice model was prepared as previously described (52) with some modifications. Briefly, adult mice (6-8 week) or mice after 12 days following intracranial GBM injection, as described above, were decapitated and their brains rapidly removed into ice-cold (4°C) sucrose-aCSF composed of the following (in mM): 280 sucrose, 5 KCl, 2 MgCl2, 1 CaCl2, 20 glucose, 10 HEPES, 5 Na^+-ascorbate, 3 thiourea, 2 Na^+-pyruvate; pH=7.3. Brains were blocked with a sharp scalpel and sliced into 250 µm sections using a McIlwain-type tissue chopper (Vibrotome inc). Four to six slices were placed onto each 0.4 µm Millicell tissue culture insert (Millipore) in six-well plates, 1 ml of medium containing the following: Neurobasal medium A (Gibco), 2% Gem21-Neuroplex supplement (Gemini), 1% N2 supplement (Gibco), 1% glutamine (Invitrogen), 0.5% glucose, 10 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen), placed underneath each insert. One-third to one half of the media was changed every 2 d. Tumor growth was monitored via bioluminescence imaging on the IVIS 200 system (Perkin Elmer) and results analyzed using Living Image software (Caliper Life Sciences, Waltham, MA, USA). For MTS assay, individual brain slices were transferred to a 96-well plate and subjected to Promega CellTiter 96® Aqueous One Solution (Cat: G3582) mixed in a 1:5 ratio with culture media and treated as previously described (52). Tissues were incubated at 37℃, 5% CO_2 for 4 hours and absorbance at 490nm was measured with Tecan Spark Microplate reader.

Click Chemistry. Detection of potential O-GlcNAcylation was carried out by CuAAC "click" labeling assay as previously described (55). In brief, 250ug of cell lysate protein from each sample was washed to remove free UDP-GlcNAc residues by chloroform-methanol extraction. Protein pellets were then dissolved in 100mM HEPES buffer, pH 7.9, containing 1% SDS. Dissolved protein samples were rotated overnight at 4ºC with 25 μM of UDP-GalNAz (Biorbyt), in the presence of 5.5 mM of MnCl2 and 0.1 mg/mL of Y298L galactotransferase (GalT1), a kind gift from Dr. Kelley Moremen, University of Georgia. The samples were then washed by chloroform-methanol extraction to remove exceed UDP-GalNAz, and then "click" with 100μM of alkyne-biotin (Click Chemistry Tool) in the presence of Cu^{++} for one hour at room temperature. Exceed biotin residues were removed by chloroform-methanol extraction. Labeled protein samples were then incubated at room temperature for two hours with NeutrAvidin-agarose bead (Thermo), followed by extensive washing to remove non-O-GlcNAc targets. Samples were then boiled for 15 minutes and analyzed by SDS-PAGE and immunoblot.

Statistical Analysis and reproducibility. All results shown are results of at least three independent experiments and shown as averages and presented as mean ± s.d. *P* were calculated using a Student's two-tailed test (* represents $P \leq 0.05$ or **P ≤ 0.01 or as marked in figure legend). Statistical analysis of growth rate of mice was performed using ANCOVA. *$P < 0.05$.

Supplementary Figure Legends

Supplementary Figure S1. Inhibition of OGT or O-GlcNAcylation impedes glioblastoma growth. **(a)** Representative images from anchorage-independent growth assay comparing the colony formation of control or OGT shRNA expressing U87-MG (top) and T98G (bottom) cells. **(b)** U87-MG cells were treated with 100 μM and 200 μM of OGT inhibitor, Ac-5S-GlcNAc, for 24 hours and then lysates were collected for immunoblot analysis with the indicated antibodies (top) or stained with crystal violet (bottom). **(c)** T98G cells were treated with 100 μM and 200 μM of OGT inhibitor, Ac-5S-GlcNAc, for 24 hours and then lysates were collected for immunoblot analysis with the indicated antibodies (top) or stained with crystal violet (bottom). **(d)** SN-186 cells were treated with 100 μM and 200 μM of OGT

inhibitor, Ac-5S-GlcNAc, for 24 hours and then lysates were collected for immunoblot analysis with the indicated antibodies (top) or placed in neurosphere assay and representative image shown (bottom). **(e)** Cell lysate from U87-MG cells expressing luciferase and control or OGT shRNA were collected for immunoblot analysis with the indicated antibodies. **(f)** Representative colony formation stained with crystal violet (top) from clonogenic assay of U87-MG cells stably expressing shSCR, shOGT RNA and supplementation of shOGT expressing cells with exogenous 50 μM oleic acid (OA). Graphical representation of relative colony formation (bottom) quantified as average relative to control. Student's t-test reported as mean ± SD. * = p-value < 0.05.

Supplementary Figure S2. Targeting OGT expression inhibits lipid metabolism. (a) Cell lysates from of U87-MG cells stably expressing control shRNA or shOGT were collected for immunoblot analysis with the indicated antibodies. **(b)** Measurement of relative free fatty acids in U87-MG cells stably overexpressing shControl or shOGT and quantified as average relative to control. Student's t-test reported as mean ± SD. * = p-value < 0.05. **(c)** Measurement of acetyl-CoA extracted from U87-MG cells stably overexpressing shControl or shOGT. Student's t-test reported as mean ± SD. * = p-value < 0.05. **(d)** Cell lysates from T98G cells stably expressing control shRNA or shOGT were collected for immunoblot analysis with the indicated antibodies. **(e)** Measurement of relative free fatty acids in T98G cells stably overexpressing shControl or shOGT and quantified as average relative to control. **(f)** Measurement of acetyl-CoA extracted from T98G cells stably overexpressing shControl or shOGT. **(g)** Cell lysate from T98G cells that were treated with control (DMSO) or 100 μM OGA inhibitor (NButGT) for 48 hours were collected for immunoblot analysis with the indicated antibodies (top) and measurement of acetyl-CoA extracted from the treated T98G cells is shown (bottom). Student's t-test reported as mean ± SD. * = p-value < 0.05. **(h)** Graphical representation of the concentration of $^{13}C_2$-acetate remaining in the media following 1 and 4 hours of exposure to 100μM $^{13}C_2$-acetate supplemented serum-free media in U87-MG cells stably overexpressing control or OGT. Student's t-test reported as mean ± SD. * = p-value < 0.05. **(i)** U87-MG control and OGT overexpressing cells were labeled with 200 μM 13C2-acetate for 48 hours. Measurement of the percent enrichment for unlabeled (M+0) and labeled (M+2, M+4) palmitic acid. Student's t-test reported as mean ± SD. * = p-value < 0.05. **(j)** U87-MG control and OGT overexpressing cells were labeled with 200 μM 13C2-acetate for 48 hours. Measurement of the percent enrichment for unlabeled (M+0) and labeled (M+2, M+4) stearic acid. For both (i) & (j) isotopologues up to m+16 were analyzed, and minimal enrichment was seen above m+4. Student's t-test reported as mean ± SD. * = p-value < 0.05.

Supplementary Figure S3. Elevated OGT/O-GlcNAcylation increases ACSS2 protein levels. (a) Total RNA was collected from U87-MG cells stably expressing shControl or shOGT. Quantification of qRT-PCR performed on RNA extracts analyzing OGT and ACSS2 gene expression normalized to GAPDH and quantified as average relative to control. Student's t-test reported as mean ± SD. * = p-value < 0.05. **(b)** Graphical representation of densitometry from three independent experiments comparing ACSS2 protein expression normalized to actin in lysate from control and OGT overexpressing U87-MG cells as shown in Fig. 3a**.** Student's t-test reported as mean ± SD. * = p-value < 0.05. **(c)** Cell lysates from SN310 cells overexpressing control or OGT were collected for immunoblot analysis with indicated antibodies. **(d)** Cell lysates from U87-MG cells treated with OGA inhibitor (NButGT) for indicated times and dose were collected for immunoblot analysis with the indicated antibodies. (**e)** Whole lysate and avidin pulldown from CuAAC "click" labeling assay of U87-MG cells expressing control and HA-ACSS2 blotted with anti-HA antibody and positive control OGT to determine O-GlcNAcylation. **(f)** Cell lysate collected from U87-MG cells stably overexpressing control or HA-ACSS2 and treated with either DMSO or 100 μM NButGT for 48 hours. The lysates were then subjected to immunoprecipitation with isotype control IgG or HA-tag antibodies and analyzed via SDS-PAGE for further analysis via western blotting and mass-spectrometry. **(g)** Scansite analysis of the ACSS2 protein sequence. **(h)** Non-radioactive *in vitro* kinase assay performed with increasing concentrations of recombinant ACSS2 with recombinant CDK5. **(i)** Non-radioactive *in vitro* kinase assay performed with either wild-type or S267A purified ACSS2 in presence or absence of recombinant CDK5. **(j)** Immunoprecipitation was performed with indicated antibodies from U87-MG cells stably expressing wild-type (WT) or S267A HA-ACSS2. **(k)** In vitro immunoprecipitation of recombinant CDK5 in presence of purified ACSS2 and immunoblot analysis with the indicated antibodies. **(l)** Immunoprecipitation of endogenous CDK5 from U87-MG glioblastoma cells treated with 100 μM NButGT for 48 hours and immunoblot analysis with the indicated antibodies.

Supplementary Figure S4. ACSS2-serine 267 phosphorylation in GBM cells and neuronal tissue. (a) Cell lysates from U87-MG cells were subjected to immunoprecipitation with isotype control IgG or phospho-ACSS2-S267 antibody and analyzed via SDS-PAGE for further analysis via western blotting with indicated antibodies. **(b)** Cell lysates from normal human astrocytes, T98G or U87-MG cells treated with control (DMSO) or OGA inhibitor (NButGT) (100 μM, 48 hrs) were collected for immunoblot analysis with the indicated antibodies. **(c)** Cell lysates from *ex vivo* brain slice from P10 mice were treated with control (DMSO) or OGA inhibitor (NButGT) (100 μM, 48 hrs) were collected for immunoblot analysis with the indicated antibodies. Cell lysate from U87-MG cells stably expressing control or CDK5 shRNA and treated with DMSO or 100 μM NButGT for 24 hours were collected for

immunoblot analysis with the indicated antibodies. **(d)** Graphical representation of densitometry from three independent experiments comparing expression of p-S267 ACSS2/total ACSS2 shown in Figure 4f. Student's t-test reported as mean ± SD. * = p-value < 0.05. **(e)** Graphical representation of densitometry from three independent experiments comparing ratio of K48 polyubiquitination to HA from immunoprecipitation experiments of WT-, S267A-, and S267D-ACSS2 shown in Figure 5b and quantified as average relative to WT-Ub WT-ACSS2. Student's t-test reported as mean ± SD. * = p-value < 0.05, **= p-value < 0.01, ***= p-value < 0.005. **(f)** Cell lysate from U87-MG-Luciferase cells stably expressing shRNA against endogenous ACSS2 (left) and overexpressing HA-ACSS2-WT or HA-ACSS2-S267A (right) were collected for immunoblot analysis with the indicated antibodies.

Supplementary Figure S5. CDK5 suppression inhibits GBM tumorigenic potential. (a) Kaplan-Meier overall survival curve for GBM cancer patients according to CDK5 high (n=38) or low (n=114) expression levels. **(b)** Representative images from anchorage-independent growth assay (top) comparing the colony formation of control or CDK5 shRNA expressing U87-MG cells. Data are quantified and presented as average from three independent experiments (bottom). Student's t-test reported as mean ± SD. * = p-value < 0.05. **(c)** Cell lysates from T98G cells expressing control or CDK5 shRNA were collected for immunoblot analysis with the indicated antibodies (top). Representative images from cells stained with crystal violet (bottom). **(d)** Cells were placed in anchorage-independent growth assay (top) comparing the colony formation of control or CDK5 shRNA expressing T98G cells. Data are quantified and presented as average from three independent experiments (bottom). Student's t-test reported as mean ± SD. * = p-value < 0.05. **(e)** Cell lysates from SN186 cells expressing control or CDK5 shRNA were collected for immunoblot analysis with the indicated antibodies. **(f)** Neurospheres were quantified for SN310 cells expressing control or CDK5 shRNA. Student's t-test reported as mean ± SD. * = p-value < 0.05. **(g)** Representative colony formation stained with crystal violet (top) from clonogenic assay of U87-MG cells stably expressing shSCR, shCDK5 RNA and supplementation of shCDK5 expressing cells with exogenous 50 μM oleic acid (OA). Graphical representation of relative colony formation (bottom) quantified as average relative to control. Student's t-test reported as mean ± SD. * = p-value < 0.05.

Supplementary Figure S6. CDK5 is required for OGT-mediated effect on GBM growth. (a) Cell lysate from U87-MG cells stably overexpressing control or OGT and control or CDK5 shRNA were collected for immunoblot analysis with the indicated antibodies. **(b)** Representative images from anchorage-independent growth assay (top) comparing the colony formation of control and OGT overexpressing cells expressing control or CDK5 shRNA U87-MG cells. Data are quantified and

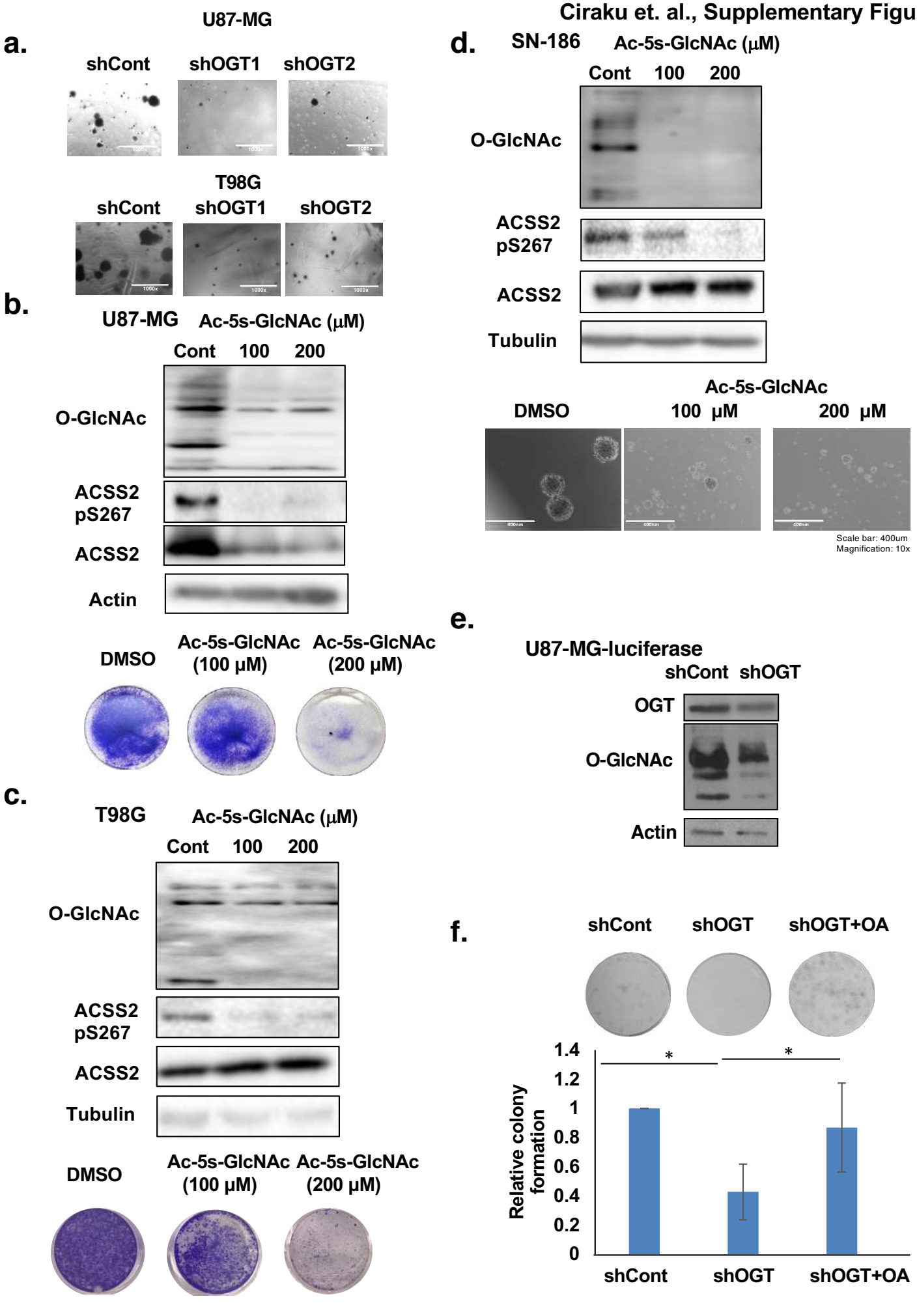
presented as average from three independent experiments (bottom). Student's t-test reported as mean ± SD. * = p-value < 0.05. **(c)** Cell lysate from U87-MG cells stably overexpressing control or OGT and control or CDK5 shRNA were collected for immunoblot analysis with the indicated antibodies. **(d)** Measurement of acetyl-CoA extracted from U87-MG cells stably overexpressing control or OGT and control or CDK5 shRNA. Student's t-test reported as mean ± SD. * = p-value < 0.05. **(e)** Cell lysate from U87-MG cells that were treated with control (DMSO) or 100 μM OGA inhibitor (NButGT) for 48 hours stably expressing control or CDK5 shRNA were collected for immunoblot analysis with the indicated antibodies. **(f)** Measurement of acetyl-CoA extracted from cells treated with control (DMSO) or 100 μM OGA inhibitor (NButGT) for 48 hours and stably expressing control or CDK5 shRNA. Student's t-test reported as mean ± SD. * = p-value < 0.05**. (g)** Representative images of nile red staining of U87-MG cells stably expressing ACSS2-WT, ACSS2-S267A and ACSS2-S267D mutants and expressing shCDK5 as shown in Fig. 6g.

Supplementary Figure S7. ACSS2-S267D mutant rescues CDK5 and OGT-mediated inhibition of GBM growth. (a) Representative images of nile red staining of U87-MG cells stable expressing ACSS2-WT, ACSS2-S267A and ACSS2-S267D mutants and overexpressing shControl or shOGT as shown in Figure 7a. **(b)** Cell lysates from U87-MG cells stably overexpressing control or ACSS2-S267D mutant and shRNA against control or OGT were collected for immunoblot analysis with the indicated antibodies. **(c)** Measurement of acetyl-CoA extracted from U87-MG cells stably overexpressing control or ACSS2-S267D mutant and shRNA against control or OGT. Student's t-test reported as mean ± SD. * = p-value < 0.05. **(d)** Cell lysate from U87-MG-luciferase cells stably expressing HA-ACSS2-S267A and HA-ACSS2-S267D and control or OGT shRNA were collected for immunoblot analysis with the indicated antibodies. (**e)** Cell lysates from *ex vivo* brain slice from adult mice were treated with control (DMSO) or OGT inhibitor (Ac-5S-GlcNAc) (200 μM, 48 hrs) were collected for immunoblot analysis with the indicated antibodies. (**f)** *Ex vivo* brain slice as treated in Fig. 8A (without tumor) were collected at day 6 and analyzed for cell viability (MTS assay). Student's t-test reported as mean ± SD. * = p-value < 0.05.

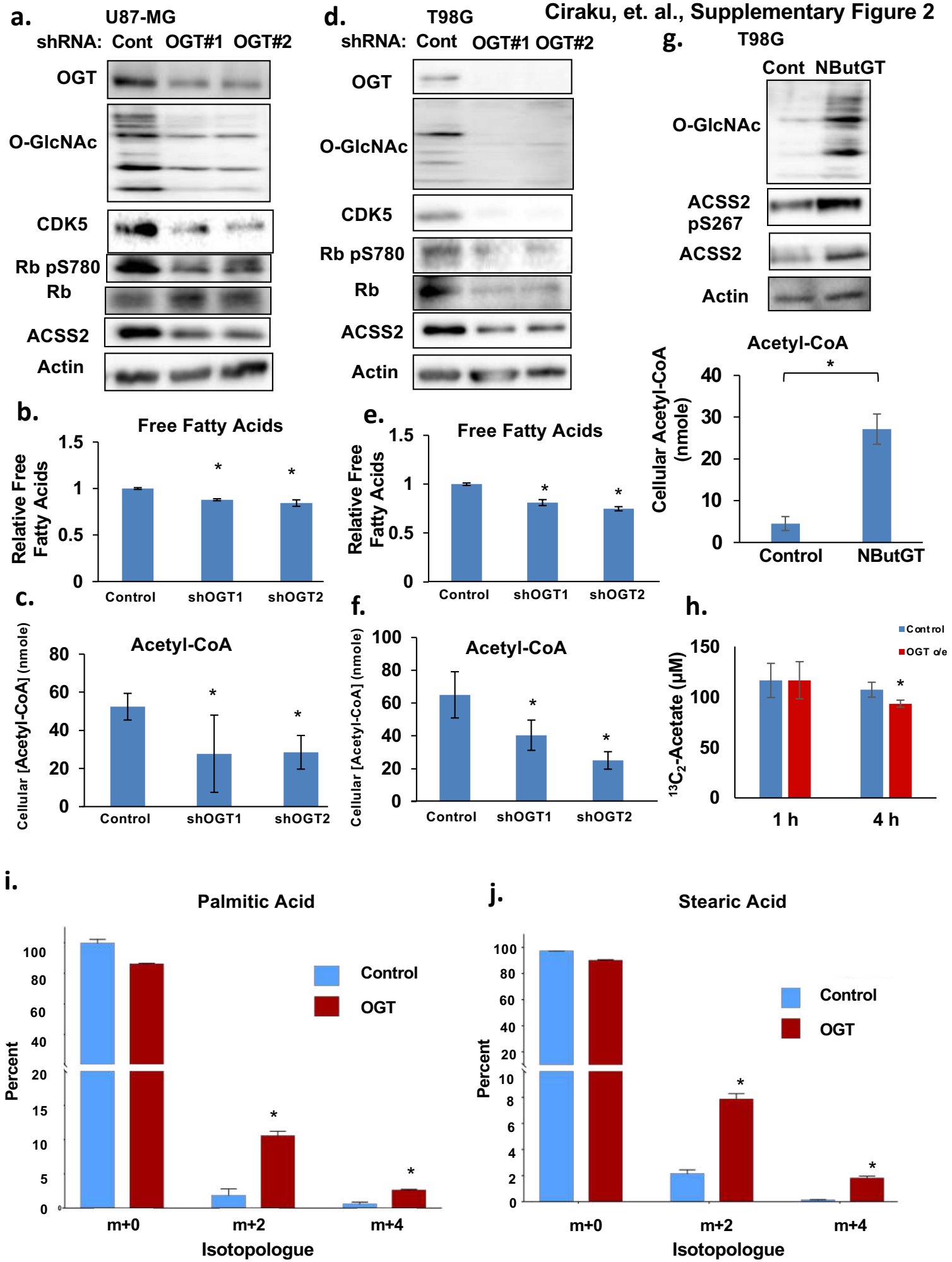
Supplementary Figure S8. Dinaciclib treatment of GBM cells blocks ACSS2-Ser267 phosphorylation, neurosphere formation, and tumor growth *ex vivo*. (a) Lysates from SN310 primary GBM cells treated for 48 hours with indicated concentrations of dinaciclib or vehicle (DMSO) were collected for immunoblot analysis with the indicated antibodies. **(b)** Representative images from SN310 neurosphere formation (top) and quantification of neurosphere formation of primary SN310 GBM cells treated with dinaciclib in (a) (bottom) quantified as average relative to control (DMSO).

Student's t-test reported as mean ± SD. * = p-value < 0.05. **(c)** Lysates from U87-MG cells treated for 48 hours with indicated concentrations of dinaciclib or vehicle (DMSO) were collected for immunoblot analysis with the indicated antibodies. **(d)** *Ex vivo* brain slice were treated with indicated dose of dinaciclib or vehicle (DMSO) as in Fig. 8c (without tumor) were collected at day 2 and analyzed for cell viability (MTS assay). PFA-fixed slices used as negative control. Statistical analysis was calculated using two-way ANOVA analysis for statistical significance as mean ± SD. * = p-value < 0.05.

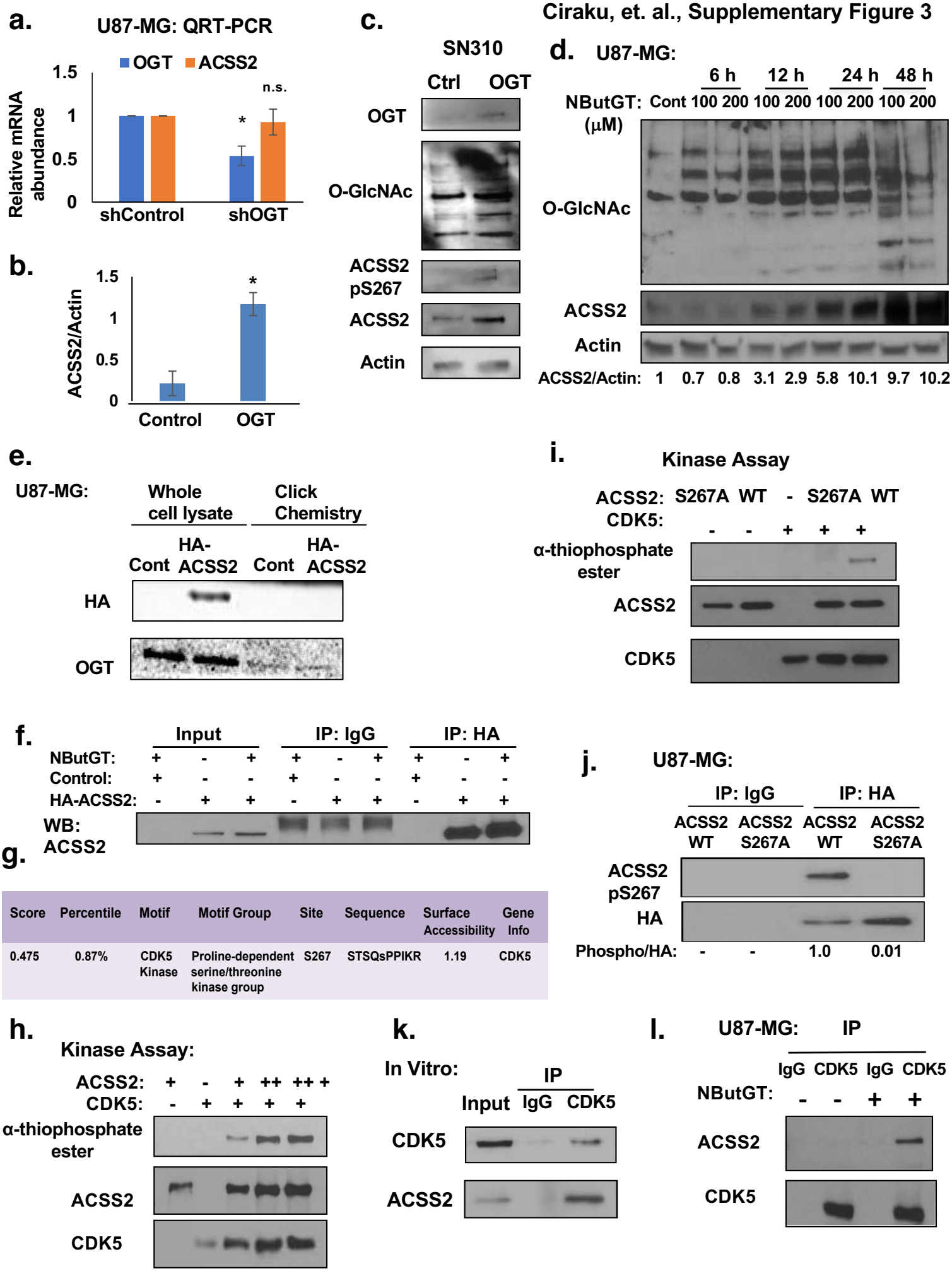
Ciraku et. al., Supplementary Figure 1



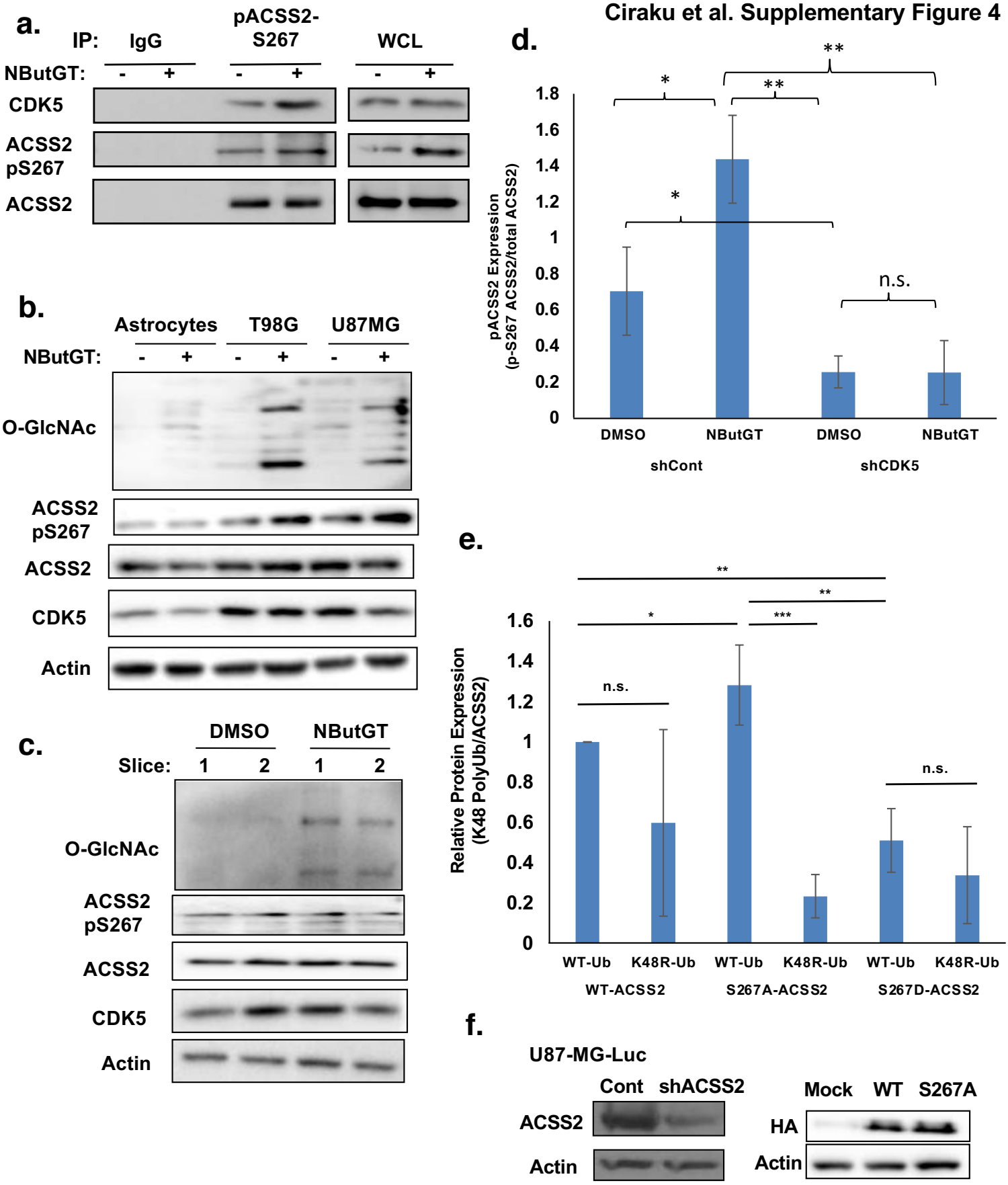
Ciraku, et. al., Supplementary Figure 2



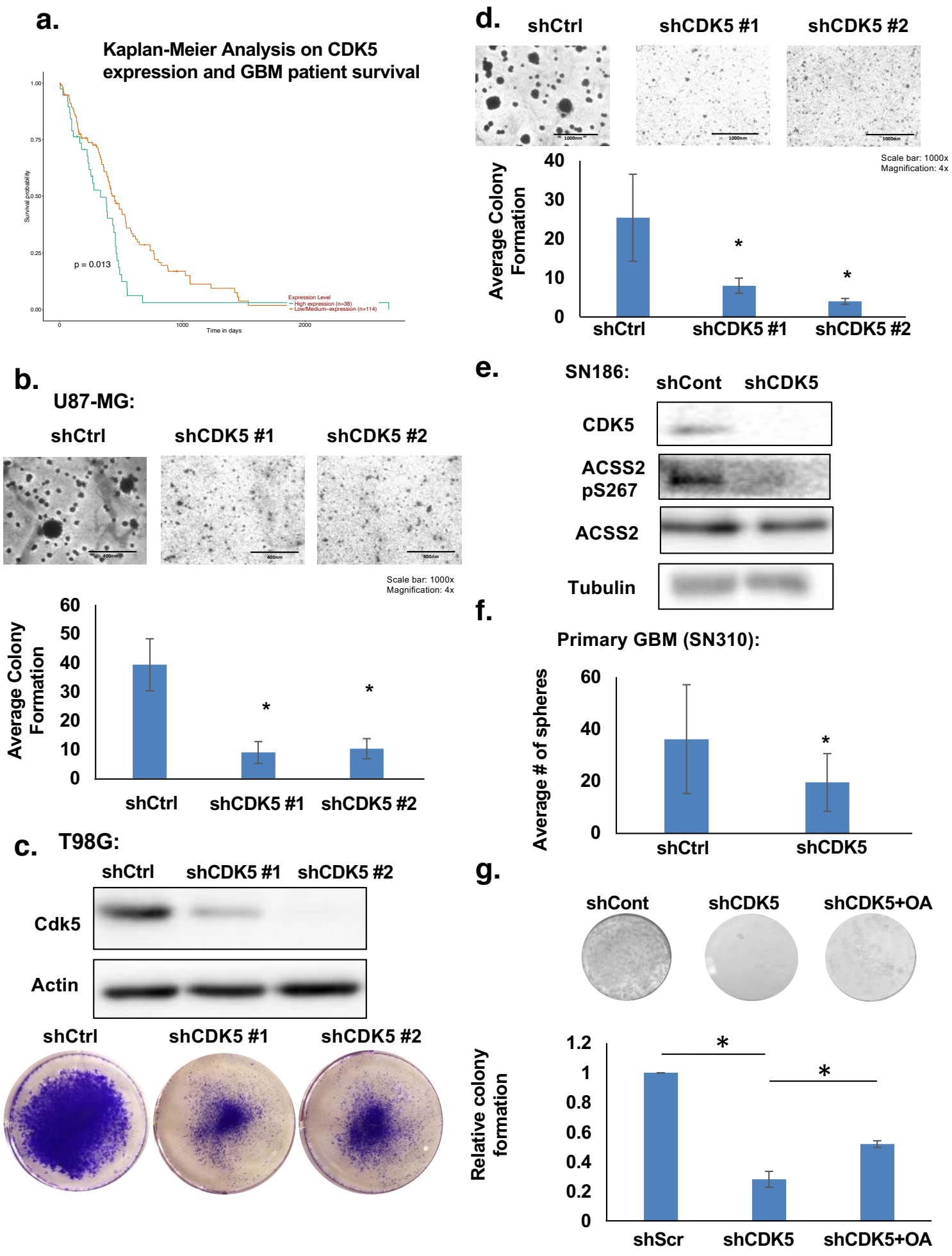
Ciraku, et. al., Supplementary Figure 3



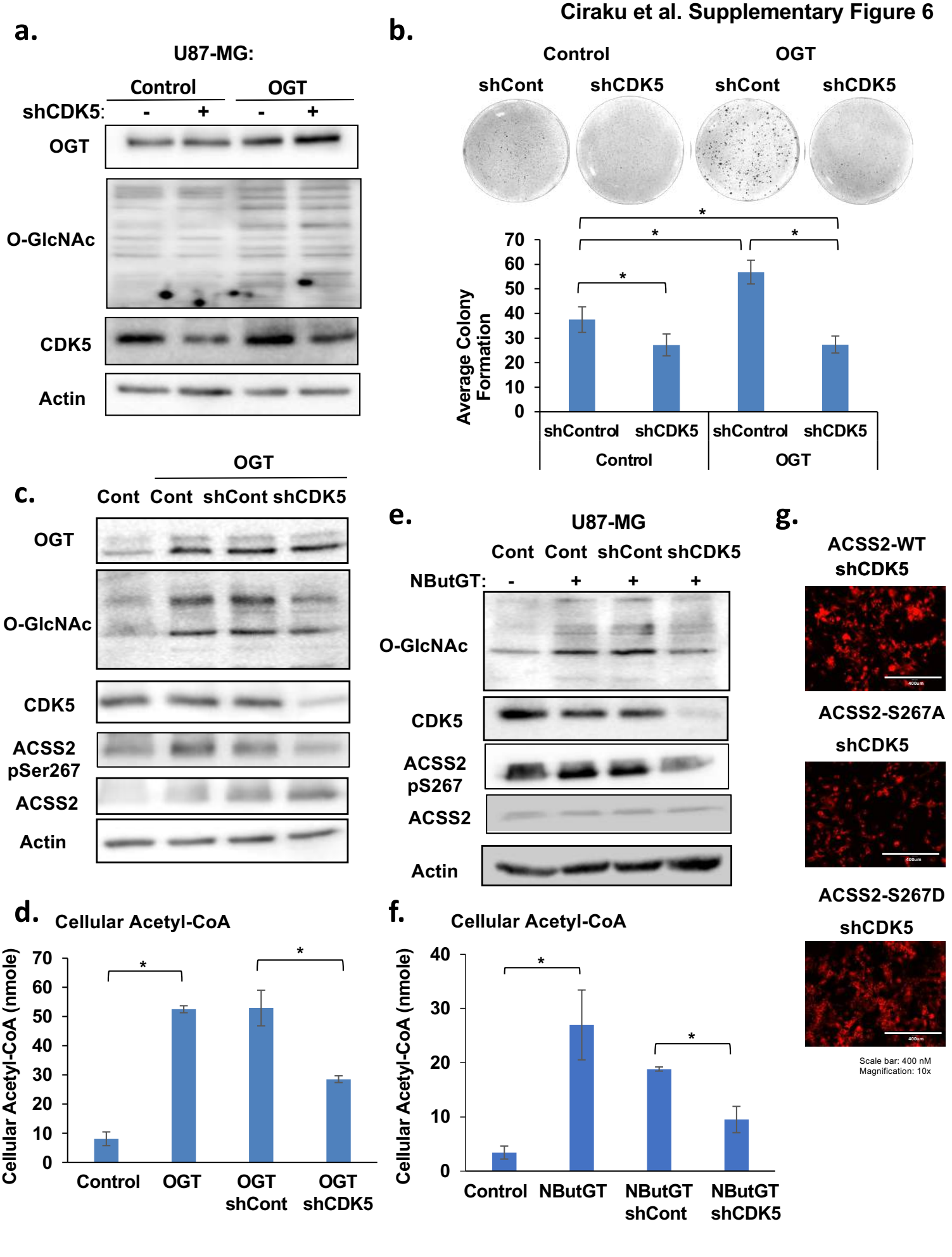
Ciraku et al. Supplementary Figure 4

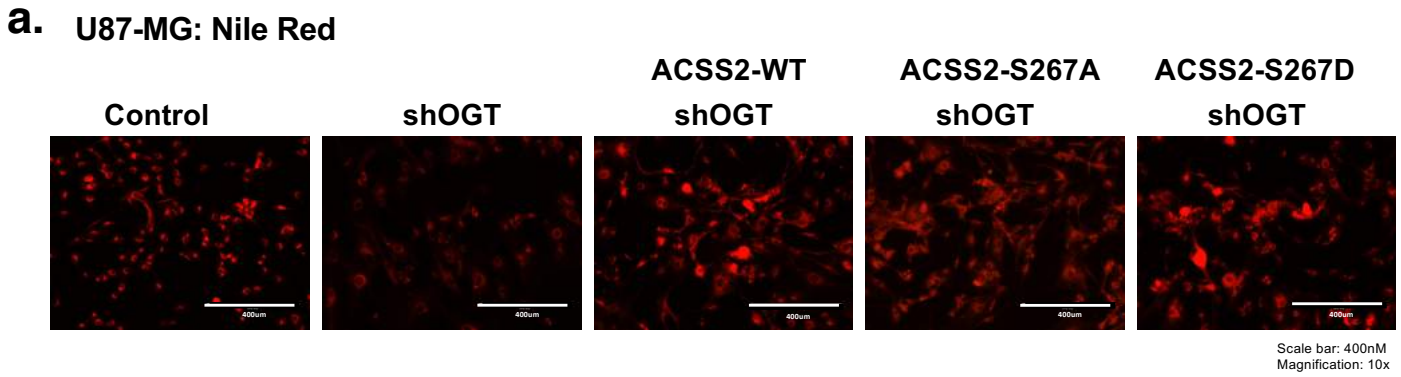


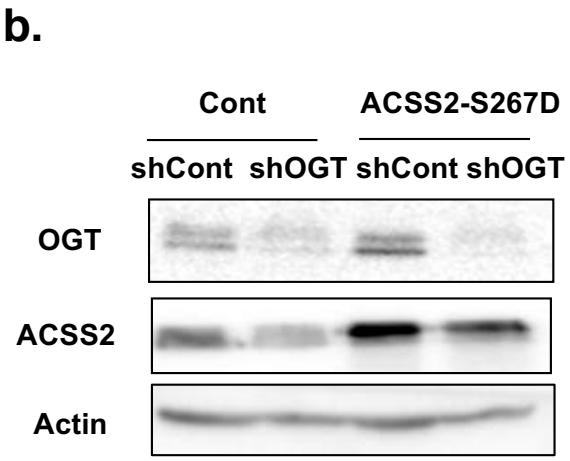
Ciraku et al. Supplementary Figure 5



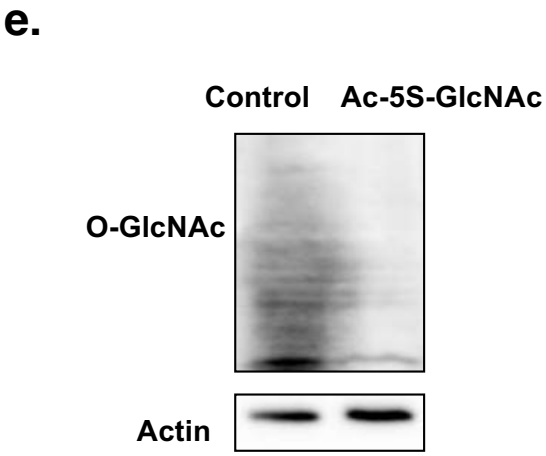
Ciraku et al. Supplementary Figure 6

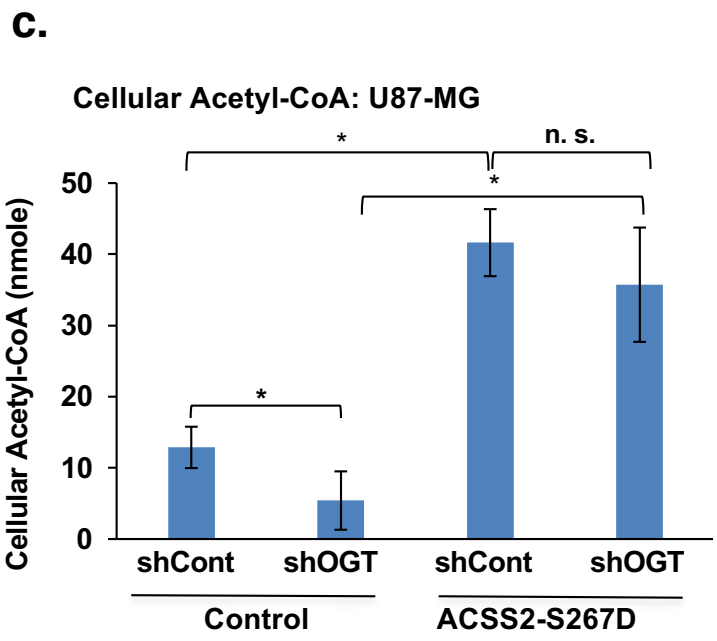


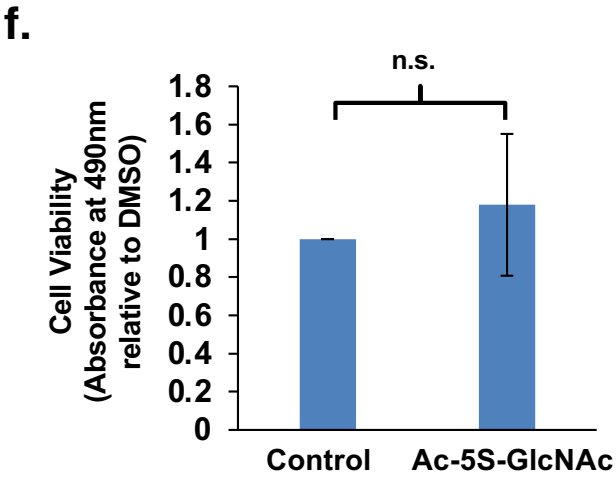

a.
U87-MG: Nile Red
Control
shOGT
ACSS2-WT
shOGT
ACSS2-S267A
shOGT
ACSS2-S267D
shOGT
Scale bar: 400nM
Magnification: 10x


b.
Cont
ACSS2-S267D
shCont
shOGT
shCont
shOGT
OGT
ACSS2
Actin


d.
U87-MG-Luc
shOGT
Ctrl
Ctrl
S267A
S267D
OGT
HA
Actin


e.
Control
Ac-5S-GlcNAc
O-GlcNAc
Actin


c.
Cellular Acetyl-CoA: U87-MG
*
n. s.
*
*
50
40
30
20
10
0
Cellular Acetyl-CoA (nmole)
shCont
shOGT
shCont
shOGT
Control
ACSS2-S267D


f.
n.s.
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Cell Viability
(Absorbance at 490nm
relative to DMSO)
Control
Ac-5S-GlcNAc

Ciraku et al. Supplementary Figure 8