Supplemental Experimental Procedures

Materials- O-GlcNAcase inhibitor NButGT (Macauley et al., 2005) and OGT inhibitor Ac-5SGlcNAc (Gloster et al., 2011) have been previously described. α-ketoglutarate was obtained from TCI America. The antibodies used were anti-actin, RL2, PFK-1 and PFKFB-3 from Santa Cruz Biotechnology (Santa Cruz, CA); anti-OGT and anti-O-GlcNAc antibodies from Sigma; anti-LKB1, anti-phosphoserine 428 LKB1, anti-AMP kinase, anti- phosphothreonine 172 AMP kinase, anti-S6 kinase, anti-phosphothreonine 389 S6 kinase, anti-CHOP, anti-PERK, anti-phosphoserine 51 eIF2a, anti-eIF2a, anti-cleaved caspase-3, anti-cleaved caspase-8, anti-cleaved PARP, anti-HA tag, anti-PUMA and anti-ubiquitin lysine 48 from Cell Signaling (Danvers, MA); anti-HIF-1α from Abcam (mouse) and Novus (rabbit); anti-GLUT1 from Abcam; anti-VHL from BD Biosciences; anti-BimEL from Epitomics and anti-NOXA from IMGENEX.

Viral Transductions- Retroviruses were packaged as previously described (Caldwell et al., 2010) and used to stably transduce MDA-MB-231 cells with pMiT-HIF-1α-P402A,P546A (kindly provided by D. Plas, University of Cincinnati) (Tandon et al., 2011) and pBabe-GLUT1 (kindly provided by R. Govers, INSERM U626) (Berenguer et al., 2010). Lentiviruses were packaged as previously described (Caldwell et al., 2010) and used to stably transduce MCF-7 cells with pLenti4-HA-OGT (kindly provided by K. Vosseller, Drexel University College of Medicine). MEFs-$OGT^{F/Y}$ cells were transduced with Adenovirus expressing β-gal or Cre-recombinase driven by the CMV as previously described (Ryan et al., 2000).

Metabolic Assays- Lactate production was measured with the Lactate Assay Kit II (BioVision, Milpitas, CA), by assaying conditioned media, according to the manufacturer's protocol. ATP levels were measured with the ATP Assay Kit (Calbiochem, San Diego, CA), by assaying lysed cells, according to the manufacturer's protocol. Glucose uptake was measured by treating cells with 100 μM 2-NBDG (Invitrogen, Carlsbad, CA) for 2 hours and quantified mean fluorescence via flow cytometry. Alpha-ketoglutarate levels were measured using the Alpha-Ketoglutarate Colorimetric/Fluorometric Assay Kit (BioVision, Milpitas, CA) according to the manufacturer's protocol and levels were normalized to total amount of cellular protein per sample. All other assays were normalized according to manufacturer's protocol.

shRNA Transfections- shRNA lentiviral particles were generated as previously described (Caldwell et al., 2010). Briefly, pLKO-Puro vectors carrying control (scramble sequence) shRNA, and two different OGT shRNA sequences (OGT-1 or OGT-2). shRNA were packaged into VSVG-pseudotyped lentiviruses, through co-transfection of HEK-293T packaging cells with 10 μg of vector DNA and appropriate packaging vectors. Control shRNA was acquired through Addgene (plasmid 1864), from D. Sabatini (Whitehead Institute for Biomedical Research, MIT) and the sequence used was: CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG. OGT shRNA constructs were acquired from Sigma and the sequences used for OGT-1: GCCCTAAGTTTGAGTCCAAATCTCGAGATTTGGACTCAAACTTAGGGC and for

OGT-2:

GCTGAGCAGTATTCCGAGAAACTCGAGTTTCTCGGAATACTGCTCAGC.

Immunoprecipitation– Cells subjected to normoxic (21% O_2) or hypoxic (1% O_2) conditions (where indicated) were lysed and extracted with radioimmune precipitation assay buffer (150 mM NaCl, 1% NP40, 0.5% DOC, 50 mM TrisHCL 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. Protein G Sepharose beads were pre-cleared in 1% bovine serum albumin (BSA) and lysates were incubated with indicated antibodies overnight at 4^0C. The next day, samples were subjected to immunoprecipitation using previously cleared protein G Sepharose beads followed by washes in 1% phosphate buffered saline + Tween 20. Immunoprecipitated proteins were resolved by SDS-PAGE and transferred to a PVDF membrane. Immunoblotting protocol was followed as per instructions above.

3D morphogenesis assay and indirect immunofluorescence- Assays were performed as described previously (Caldwell et al., 2010). Briefly, MDA-MB-231 cells, in respective media containing 2% Matrigel, were placed in an 8-well chamber slide (BD Biosciences) coated with 50 μl of Matrigel. The number of cells was counted in two chambers at indicated time points and the mean of each determined. Immunofluorescence of 3D structures was performed as described previously (Caldwell et al., 2010) using antibodies to cleaved caspase-3 then stained with 4′,6-diamidino-2-phenylindole. Fluorescent secondary antibodies coupled with Alexa-Fluor dyes (Molecular Probes, Carlsbad, CA,

USA) were used. Confocal analysis was performed by using the Leica DM6000B Confocal Microscope (Leica). Images were generated using the Leica Imaging Software (Wetzlar, Germany) and converted to Tiff format.

Quantitative RT-PCR (qRT-PCR)- Total RNA was isolated using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol for monolayer animal cell RNA extraction. Levels of OGT, HIF-1α, adrenomedullin (ADM), BCL2/adenovirus E1B 19 kd-interacting protein (BNIP3L), glucose transporter 1 (GLUT1), lactate dehydrogenase (LHDA), cyclophilin A PPIA) were determined using the Stratagene Mx3000P QPCR device, with Brilliant II QRT-PCR Master Mix Kit (Strategene), according to manufacturer's protocol. Data analysis was performed with the MX Pro software package (Stratagene). TaqMan gene expression assay primer/probes were purchased from Applied Biosystems (Foster City, CA) for cyclophilin A (Hs99999904_m1), OGT (Hs00914634_g1), HIF-1α (Hs00936371_m1), ADM (Hs00181606_m1), BNIP3L (Hs0018949_m1), GLUT1 (Hs00892681_m1), LDHA (Hs00855332_g1), PFK1-L (Hs00160027_m1), and PFK1-M (Hs00175997_m1). Expression levels were normalized to cyclophilin A.

Mouse Xenografts-Female athymic nude Nu/Nu mouse (Charles River, Wilmington, MA, USA) (5–6 weeks old) were anesthetized with 4% isoflurane and inoculated with 1.5 x 10^6 MDA-MB-231 cells stably expressing luciferase and either pBabe-Control or pBabe-GLUT1 and containing either Control or OGT shRNA. Cells were injected in 100 μl of 1% PBS containing 20% matrigel (Invitrogen) using a 271/2-gauge needle into the fourth

inguinal mammary fat pad. Mice were injected intraperitoneally with 200 μl of D-luciferin solution (9 mg/ml; Caliper Life Sciences, Hopkinton, MA), and bioluminescence imaging was done 3 hours after the mammary fat pad injection to detect the distribution of breast cancer cells; mice were then imaged weekly. Images were acquired with an IVIS 200 imaging system, and results were analyzed using Living Image software (Caliper Life Sciences). After injection, tumors were measured weekly along and perpendicular to the longest dimension using digital calipers (Fowler Co., Inc., Newton, MA, USA). Tumor volumes were calculated as $V=(length)x(width)^2x0.52$. After 8 weeks, tumors were excised, weighed and photographed. All protocols involving the use of animals were approved by the Institutional Animal Care and Use Committee at Drexel University College of Medicine.

Immunoblotting- 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 TrisHCL 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 20 minutes at 4 °C and analyzed by SDS-PAGE and autoradiography. Proteins were analyzed by immunoblotting using primary antibodies indicated above.

HCI sample protein extraction and western blotting—Flash frozen, patient-derived xenograft tissue fragments maintained by the Huntsman Cancer Institute (HCI) breast tumor bank resource (DeRose et al., 2011) were a kind gift from Dr. Alana L. Welm

(Huntsman Cancer Institute, University of Utah). A summary of the features of the HCI breast tumor bank tissues may be found in Supplementary Table 1 of (same reference as previous sentence). High-salt enriched whole cell lysates were prepared from tumor fragments that were previously ground to a fine powder under liquid nitrogen as previously described (Schwab et al., 2012). HS-WCE (10 mg/lane) were resolved on 3-8% Tris-Acetate gels (Invitrogen) and transferred to PVDF membrane. Membranes were probed with primary antibodies to either HIF-1α (Novus Biologicals, NB-100-479), OGT or O-GlcNAc as previously described, or ß-tubulin (AbCam, 6046) as a loading control. Following incubation in the appropriate secondary horseradish peroxidase-conjugated antibodies (Bio-Rad or Santa Cruz), signals were visualized with Millipore ECL substrate.

Flow Cytometry—7 days post-infection with control or OGT shRNA lentivirus, cells were harvested and washed with PBS. Pellets were resuspended in 1X Annexin V Buffer as per Annexin V: FITC Apoptosis Detection Kit II (BD Biosciences) manufacturers protocol. All data were collected and analyzed using a Guava EasyCyte Plus system and CytoSoft (version 5.3) software (Millipore). Apoptosis data are presented as the percent fluorescence intensity of gated cells positive for Annexin V and Propidium Iodide.

Bioinformatics Analysis— The level of OGT and MGEA5 mRNA expression in clinical breast tumor subtypes was derived from the TCGA data portal (http://cancergenome.nih.gov/). Level three normalized data were used for mRNA expression, and samples (n=804 total tumors) were stratified by tumor subtype based on

the PAM50 method. Plots were generated using Prism 5.0 (GraphPad Software, Inc.) and *p*-values (ANOVA with Bonferroni correction) of 0.05 (or less) were considered significant. As the TCGA data set provides limited survival data, survival analysis was conducted using the van't Veer microarray dataset downloaded from Rosetta Inpharmatics (http://www.rii.com/publications/2002/vantveer.html). Normalized log-ratio expression values for OGT or MGEA5 were analyzed for frequency distributions and the top quartile (>75%) was chosen to represent "high" expression and the bottom quartile (<25%) was chosen to represent "low" expression. The y-axis (probability) is defined as the frequency of survival events. Data were analyzed using the survival package within WinSTAT® for Excel.

Metabolite Analysis— MDA-MB-231 cells were cultured and stably infected with control or OGT shRNA for 48 hrs as described above. Cells were counted, and approximately 107 cells per sample were spun down at 300g for 3 min in a polystyrene tube. Cells were washed twice with PBS and then snap-frozen on dry ice and stored at −80 °C until analysis by Metabolon (Durham, NC). The untargeted metabolic profiling platform employed for this analysis combined three independent platforms: ultrahigh performance liquid chromatography/tandem mass spectrometry (UHLC/MS/MS^2) optimized for basic species, UHLC/MS/MS^2 optimized for acidic species, and gas chromatography/mass spectrometry (GC/MS). Cells were homogenized in a minimum volume of water and 100µL withdrawn for subsequent analyses. Using an automated liquid handler (Hamilton LabStar, Salt Lake City, UT), protein was precipitated from the homogenized cells with methanol that contained four standards to report on extraction efficiency. The resulting supernatant was split into equal aliquots for analysis on the three platforms as described

previously (Reitman et al., 2011) (Evans et al., 2009). Aliquots, dried under nitrogen and vacuum-desiccated, were subsequently either reconstituted in 50µL 0.1% formic acid in water (acidic conditions) or in 50µL 6.5mM ammonium bicarbonate in water, pH 8 (basic conditions) for the two UHLC/MS/MS^2 analyses or derivatized to a final volume of 50µL for GC/MS analysis using equal parts bistrimethyl-silyl-trifluoroacetamide and solvent mixture acetonitrile:dichloromethane:cyclohexane (5:4:1) with 5% triethylamine at 60°C for one hour. In addition, three types of controls were analyzed in concert with the experimental samples: samples generated from pooled experimental samples served as technical replicates throughout the data set, extracted water samples served as process blanks, and a cocktail of standards spiked into every analyzed sample allowed instrument performance monitoring. For UHLC/MS/MS^2 analysis, aliquots were separated using a Waters Acquity UPLC (Waters, Millford, MA) and analyzed using an LTQ mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA) that consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The MS instrument scanned 99-1000 *m/z* and alternated between MS and MS^2 scans using dynamic exclusion with approximately 6 scans per second. Derivatized samples for GC/MS were separated on a 5% phenyldimethyl silicone column with helium as the carrier gas and a temperature ramp from 60°C to 340°C and then analyzed on a Thermo-Finnigan Trace DSQ MS (Thermo Fisher Scientific, Inc.) operated at unit mass resolving power with electron impact ionization and a 50-750 atomic mass unit scan range. Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (*m/z*), preferred adducts, and in-source fragments as

well as associated MS spectra and curated by visual inspection for quality control using software previously described (Dehaven et al., 2010). Experimental samples and controls were randomized across a one-day platform run. Any missing values were assumed to be below the limits of detection and for statistical analyses and data display purposes, these values were imputed with the compound minimum (minimum value imputation) after normalization to total protein for each sample. Statistical analysis of log-transformed data was performed using Array Studio software (Omicsoft, Inc) and R (http://cran.r-project.org/). Welch's t-tests were performed to compare data between experimental groups. Multiple comparisons were accounted for by estimating the false discovery rate (FDR) using q-values.

Supplemental References

Berenguer, M., Martinez, L., Giorgetti-Peraldi, S., Le Marchand-Brustel, Y., and Govers, R. (2010). A serum factor induces insulin-independent translocation of GLUT4 to the cell surface which is maintained in insulin resistance. PLoS One *5*, e15560.

Caldwell, S.A., Jackson, S.R., Shahriari, K.S., Lynch, T.P., Sethi, G., Walker, S., Vosseller, K., and Reginato, M.J. (2010). Nutrient sensor O-GlcNAc transferase regulates breast cancer tumorigenesis through targeting of the oncogenic transcription factor FoxM1. Oncogene *29*, 2831-2842.

Dehaven, C.D., Evans, A.M., Dai, H., and Lawton, K.A. (2010). Organization of GC/MS and LC/MS metabolomics data into chemical libraries. J Cheminform *2*, 9.

DeRose, Y.S., Wang, G., Lin, Y.C., Bernard, P.S., Buys, S.S., Ebbert, M.T., Factor, R., Matsen, C., Milash, B.A., Nelson, E.*, et al.* (2011). Tumor grafts derived from women with breast cancer authentically reflect tumor pathology, growth, metastasis and disease outcomes. Nat Med *17*, 1514-1520.

Evans, A.M., DeHaven, C.D., Barrett, T., Mitchell, M., and Milgram, E. (2009). Integrated, nontargeted ultrahigh performance liquid chromatography/electrospray ionization tandem mass spectrometry platform for the identification and relative quantification of the small-molecule complement of biological systems. Anal Chem *81*, 6656-6667.

Gloster, T.M., Zandberg, W.F., Heinonen, J.E., Shen, D.L., Deng, L., and Vocadlo, D.J. (2011). Hijacking a biosynthetic pathway yields a glycosyltransferase inhibitor within cells. Nat Chem Biol *7*, 174-181.

Macauley, M.S., Whitworth, G.E., Debowski, A.W., Chin, D., and Vocadlo, D.J. (2005). O-GlcNAcase uses substrate-assisted catalysis: kinetic analysis and development of highly selective mechanism-inspired inhibitors. J Biol Chem *280*, 25313-25322.

Reitman, Z.J., Jin, G., Karoly, E.D., Spasojevic, I., Yang, J., Kinzler, K.W., He, Y., Bigner, D.D., Vogelstein, B., and Yan, H. (2011). Profiling the effects of isocitrate dehydrogenase 1 and 2 mutations on the cellular metabolome. Proc Natl Acad Sci U S A *108*, 3270-3275.

Ryan, H.E., Poloni, M., McNulty, W., Elson, D., Gassmann, M., Arbeit, J.M., and Johnson, R.S. (2000). Hypoxia-inducible factor-1alpha is a positive factor in solid tumor growth. Cancer Res *60*, 4010-4015.

Schwab, L.P., Peacock, D.L., Majumdar, D., Ingels, J.F., Jensen, L.C., Smith, K.D., Cushing, R.C., and Seagroves, T.N. (2012). Hypoxia-inducible factor 1alpha promotes primary tumor growth and tumor-initiating cell activity in breast cancer. Breast Cancer Res *14*, R6.

Tandon, P., Gallo, C.A., Khatri, S., Barger, J.F., Yepiskoposyan, H., and Plas, D.R. (2011). Requirement for ribosomal protein S6 kinase 1 to mediate glycolysis and apoptosis resistance induced by Pten deficiency. Proc Natl Acad Sci U S A *108*, 2361-2365.