

## **Supplemental Methods**

### **Protein knockdown by siRNA transfection.**

Predesigned siRNA duplexes targeting GLUT3, GFPT1, GFPT2 and OGT, as well as control siRNA were obtained from Ambion. Other siRNA duplexes were chemically synthesized and purchased from Hokkaido System Science. The sequences are as follows: GAPDH, 5'-GAGCCACAUCGCUCAGACAdTdT-3' and 5'-UGUCUGAGCGAUGUGGCUCdGdG-3'; EPAC1, 5'-CCAUCAUCCUGCGAGAAGAdTdT-3' and 5'-UCUUCUCGCAGGAUGAUGGdTdT-3'; EPAC2, 5'-GAGUUAGCAGGUGUUCUCAdTdT-3' and 5'-UGAGAACACCUGCUAACUCdTdT-3'; sAC, 5'-AUGUAGCCUGGAGAUCCAUdTdT-3' and 5'-AUGGAUCUCCAGGCUACAUCdTdT-3'; PKM1/M2, 5'-GGACCUGAGAUCCGAACUGdTdT-3' and 5'-CAGUUCGGAUCUCAGGUCCdTdT-3'; PKM2 #1, 5'-AGGCAGAGGCUGCCAUCUAdTdT-3' and 5'-UAGAUGGCAGCCUCUGCCUdTdT-3'; PKM2 #2, 5'-CCAUAAUCGUCCUCACCAAdTdT-3' and 5'-UUGGUGAGGACGAUUAUGGdTdT-3'.

For transfection of each siRNA into T4-2 cells, Lipofectamine RNAi MAX (Invitrogen) was used, according to the manufacturer's instruction. Cells were trypsinized 36 hrs after transfection and cultured in 3D-OT IrECM for 3 days before harvest.

### **Assays of glucose uptake and lactate release.**

Cells suspended in 200 µl IrECM were seeded in 12 well glass-bottom plates (14 mm diameter; MatTek Corporation). Gels were overlaid with 500 µl H14 media and cultured for 10 days before each assay unless otherwise described.

For glucose uptake assay: Cells were incubated with 1  $\mu\text{M}$  of IRDye800CW-2DG (LI-COR Biosciences) for 1 hour, then fixed within gels with 4% paraformaldehyde (PFA) in PBS for 30 min. and stained with DRAQ5 (1:10000 dilution), an infrared dye that binds stoichiometrically to DNA, for another 30 min. Cells were extensively washed with PBS, then IRDye800-2DG uptake together with DRAQ5 staining were measured by using Odyssey Infrared Imaging System (LI-COR Biosciences).

For lactate release: On day 9, conditioned-media were harvested to measure lactate remaining in the 200  $\mu\text{l}$  of the gel (assuming equilibrium between medium and gel,  $C_0$  nmol/ $\mu\text{l}$ ). At this point 500  $\mu\text{l}$  fresh H14 medium was added. After 24 hours, total culture medium was harvested for measurement of final lactate concentrations ( $C$ ; nmol/ $\mu\text{l}$ ) and total volumes ( $V$ ;  $\mu\text{l}$ ) of the conditioned-media, and the cells still within the gel were fixed with PFA as above. Concentration of lactate in each sample was measured using a lactate assay kit (BioVision). Net lactate production within 24 hours ( $N$ ) was calculated:  $N = C \times (V + 200) - C_0 \times 200$ . Fixed cells were stained with DRAQ5, and the staining was measured as above.

For calculation of total cell number from DRAQ5 staining: S1 or T4-2 cells were seeded at arbitrary concentrations into gels, fixed immediately with PFA and stained with DRAQ5, and the DRAQ5 staining was measured as above. A calibration curve was made based on the staining intensity/cell number ratio, and the cell number in each well was used for calculation in glucose uptake or lactate release assays. Similarly, gels containing arbitrary concentrations of IRDye800-2DG were made, and a calibration curve was obtained based on the intensity/molarity ratio, to calculate the absolute amount of IRDye800-2DG taken up by the cells,

### **Measurement of relative oxygen consumption rate.**

Cells in 40  $\mu\text{l}$  3D IrECM gels were placed in OxoPlates (PreSens), at the bottom of which a mixture of an oxygen-sensing and a reference dyes is attached, and covered with 100  $\mu\text{l}$  culture

media containing chemical inhibitors or vehicle but not phenol red. Cells were cultured for 10 days to allow formation of acinar structures/colonies. Just before the assay, we made wells containing 140  $\mu$ l air-saturated or oxygen-removed (by  $\text{Na}_2\text{SO}_3$ ) water, and each well was covered with 100  $\mu$ l mineral oil (Sigma) to block diffusion of oxygen from the air. The fluorescence of the indicator (oxygen-sensing) and the reference dyes were measured by Alvo MX 1420 (PerkinElmer) every 10 minutes for 5 hours. Filter sets used were as follows: the indicator dye, F540/8 for excitation and F650/10 for emission; the reference dye, F540/8 for excitation and F590/10 for emission. The plate was kept at 37 °C throughout the assay. Background fluorescence intensity of mineral oil, water, culture medium or IrECM gel were measured after the assay and subtracted from the intensity of each well. Total cell number was also measured by DRAQ5 as described above. Oxygen concentration at each time point ( $\text{pO}_2$ ) was calculated as follows:  $\text{pO}_2 = 100 \times (k_0 / I_R - 1) / (k_0 / k_{100} - 1)$ , where  $I_R$  is the ratio of intensities of indicator and reference dyes,  $k_0$  and  $k_{100}$  are  $I_R$  of oxygen-removed and air-saturated water, respectively. Oxygen consumption rate in each well was obtained by collinear approximation of the oxygen concentration at each time point, divided by the total cell number and shown as relative value after normalizing the value obtained for S1 cells as 1.0.

**Measurement of fructose-6-phosphate, pyruvate, lactate, ATP and cAMP.**

Cells were cultured in 3D-OT IrECM for 3 or 4 days and washed twice and then incubated with the fresh culture medium containing no IrECM for 2 hours. For glucose deprivation, cells were washed twice and then incubated with the culture medium without any IrECM or glucose for the indicated times before the assay. For investigation of the effect of PEP metabolism, cells in 3D-on top (OT) of IrECM were cultured with 1.75 mM glucose for 3 or 4 days, washed twice and then incubated with the culture medium containing 1.75 mM glucose but no additional IrECM for 2 hours. Cells were then supplemented with 0.5 mM PEP and incubated for the indicated times.

After treatment described above, cells were quickly plunged in ice-cold PBS, and the gel and the medium were removed by centrifugation. Cell pellets were snap-frozen by liquid nitrogen and stored at -80 °C until each metabolite could be measured. Fructose-6-phosphate, pyruvate and lactate were measured by enzyme-based assays using kits for each metabolite (BioVision). Briefly, cell pellets were lysed in PBS or lysis buffers provided by the kits, and the lysates were sonicated and centrifuged at 4 °C. For measurement of the protein concentration, 5-10 µl of the lysate was stored separately. For measurement of the metabolites, protein fraction of the lysate was removed by centrifugal filters (MWCO 10 kDa, Pall), and the metabolite levels were measured by following the manufacturer's instruction. For measurement of ATP and cAMP, cells were treated and collected as above, and the cell pellets were lysed in PBS or 0.1 M HCl, respectively. Relative ATP level was measured by a luciferase-based kit (Wako), and absolute cAMP level was examined by an ELISA-based kit (Enzo). The amount/relative level of each metabolite, ATP or cAMP was normalized with that of protein in each sample.

### **Construction of plasmid DNAs.**

HIV 5' LTR sequence in pTRIPZ vector (Open Biosystems) was removed by FspI-BbvCI digestion to replace with sequence containing chimeric CMV promoter-HIV 5' LTR. CMV promoter was amplified by PCR from pcDNA3 (Invitrogen), fused to a sequence containing partial HIV 5' LTR sequence. The resulting intermediate vector, pTRIPZ-OY (optimal yield) was digested with BbvCI and NotI, to remove the sequence containing TRE-minCMV promoter, turboRFP, UbqC promoter and rtTA3 (described in the manual provided by the manufacturer). The sequences containing the EF1 $\alpha$ /HTLV hybrid promoter and multiple cloning sites were removed from pCDH-EF1-MCS-T2A-Puro vector (System Bioscience) by digestion with the same restriction enzymes, and ligated into the digested pTRIPZ-OY vector, resulting in pLETIP vector. GLUT3 cDNA fused to C-terminal HA tag was amplified from total cDNA of T4-2 cells by

PCR. The primers used were as follows: 5'-CGGGATCCGCCACCATGGGGACACAGAAGGTAC-3' and 5'-AACCCGGGTCAAGCATAATCTGGAACATCGTATGGATAGACATTGGTGGTGGTCTCCTTAGCA-3'. The amplified cDNA was digested with BamHI and XmaI, and ligated into the same sites of pBluescriptII SK(-) vector for validating the sequence. GLUT3-HA cDNA was then digested with BamHI and SmaI, and ligated into pLETIP vector digested with NotI, blunted with T4 polymerase and further digested with BamHI, resulting in pLETIP GLUT3-HA vector.

Full length cDNAs of PKM2 and sAC were amplified from total cDNA of T4-2 or MDA-MB-231 cells, respectively, by PCR. The primers used were as follows: PKM2, 5'-TTGGATCCGCCACCATGTCTGAAGCCCCATAGTGAAGCCGGGAC-3' and 5'-TAAGCGGCCGCTCACGGCACAGGAACAACACGCATGGTGTTG-3'; sAC, 5'-TTGGATCCGCCACCATGAACACTCCAAAAGAAGAATTCCAGGAC-3' and 5'-TTGCGGCCGCTTAGAAATGATTGTCCACGGTATTAGCTCTCAT-3'. The amplified cDNAs were digested with BamHI and NotI, and ligated into the same sites of pBluescriptII SK(-) vector for validating the sequence. cDNA fragment containing the PKM1-specific sequence was amplified from total cDNA of T4-2 by nested PCR. The primer used were as follows: 1st PCR, 5'-ATAGCTCGTGAGGCTGAGGCAGCCATGTTCCACCGC-3' and 5'-TAAGCGGCCGCTCACGGCACAGGAACAACACGCATGGTGTTG-3'; 2nd PCR, 5'-TGCGCATGCAGCACCTGATAGCTCGTGAGGCTGAG-3' and 5'-TAAGCGGCCGCTCACGGCACAGGAACAACACGCATGGTGTTG-3'. The amplified cDNA fragment was digested with SphI and NotI, and ligated with pBluescriptII-PKM2 digested with the same sites, by which the cDNA fragment containing PKM2-specific sequence was removed. PKM1 or PKM2 cDNAs were moved into pEBG vector (1) by using BamHI and NotI. To add V5-tag at the C-terminus of sAC, BamHI-Asel fragment of sAC was cut out from the pBluescriptII containing the full length sAC. Fragment of sAC cDNA was amplified by PCR using the following

primers: 5'-GACATTATCAGTCCCTCTGCAGACTTAG-3' and 5'-AAACCGGTGAAATGATTGTCCACGGTATTAGCTCTC-3'. The amplified cDNA fragment was digested with AseI and AgeI. cDNA fragment encoding V5 tag was obtained by phosphorylation and ligation of the following oligonucleotides: 5'-CCGGTAAGCCTATCCCTAACCTCTCCTCGGTCTCGATTCTACGTAGC-3' and 5'-GGCCGCTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTA-3'. Note that this fragment can be ligated with AgeI and NotI sites. These 3 cDNA fragments were ligated together with pcDNA3 vector digested with BamHI and NotI, resulting in pcDNA3-sAC-V5.

### **Establishment of S1 cells overexpressing GLUT3.**

The above-described pLETIP GLUT3-HA (or empty vector), together with plasmid vectors encoding VSV-G, HIVgp and Rev, were transfected into 293T cells, using polyethylenimine solution. Supernatants were collected and clarified with filtration (0.45 µm pore), and the virus particles were precipitated by ultracentrifugation. S1 cells were infected with the purified virus particles, using ExpressMag Transduction System (Sigma-Aldrich). After 48 hrs, cells were selected with 1 µg/ml puromycin for 7 days. Bulk clones were grown and expanded in H14 media (containing EGF) supplemented with 0.5 µg/ml puromycin for each assay.

### **Indirect immunofluorescence.**

Pieces of IrECM gel containing cell colonies were suspended together in approximately the same volume of phosphate buffered saline (PBS) and spread directly on glass slides. After brief air-drying, colonies were fixed with 3.7% paraformaldehyde (PFA) in PBS at room temperature (RT) for 10 min, washed with PBS containing 0.1 mM glycine, and permeabilized with 1:1 methanol/acetone at -20 °C for 10 min. After blocking with IF buffer (0.2% Triton X-100, 0.1% BSA [radioimmunoassay grade], 0.05% Tween 20, 7.7 mM NaN<sub>3</sub> in PBS) containing 10% goat

serum and 1% goat F(ab')<sub>2</sub> anti-mouse immunoglobulin G (IgG; Caltag) at room temperature for 30 min, colonies were stained with anti- $\alpha$ 6 integrin antibody (Chemicon International) at 4 °C overnight. Colonies were washed extensively with IF buffer, and stained with Alexa 568-labeled anti-rat IgG antibody at RT for 2 hr. Colonies were washed with IF buffer again, and nuclei were stained with DAPI at RT for 10 min. Slides were briefly washed with PBS and mounted with 50% glycerol in PBS.

### **Western blotting.**

Cell colonies were separated from IrECM gels by shaking in PBS containing 5 mM EDTA (but not trypsin) and the proteinase inhibitor cocktail set I (Calbiochem) on ice for 30 min. Cells were lysed in lysis buffer (1% NP-40, 1% deoxycholate, 2% SDS, 150 mM NaCl, 20 mM Tris-HCl [pH 7.4] plus 5 mM EDTA and protease inhibitor cocktail set I and phosphatase inhibitor cocktail set I [Calbiochem]) and sonicated three times for 30 sec each. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies against the following proteins: MEK1/2, phospho-MEK1/2 (Ser217/221), Akt, phospho-Akt (Ser473), AMPK $\alpha$ , phospho-AMPK $\alpha$  (Thr172), p70S6K, phospho-p70S6K (Thr398), 4EBP1, phospho-4EBP1 (Thr37/46), STAT3, phospho-STAT3 (Tyr705), CHOP, EPAC1, PKM2 (Cell Signaling),  $\beta$ 1 integrin, EGFR, activated EGFR, HIF-1 $\alpha$ , (Chemicon International), PFKP, ADCY10/sAC (Abgent), GAPDH, GLUT1, GLUT3, MEK5 (GeneTex), LDHA, PDHE1 $\alpha$ , DLAT, DLD, Rap1, sAC, EPAC2, OGT, Lamin A/C (Santa Cruz Biotechnology), Aldolase, muscle type pyruvate kinase (Polysciences), GFPT (PtoteinTech Group), HIF-2 $\alpha$ , phospho-Histone H3 (Thr11), GST, O-GlcNAc [clone RL2] (Abcam), Histone H3 (Monoclonal antibody laboratory), PKM1 (Sigma), V5-tag (Invitrogen) and HA-tag (Covance). After probing with HRP-conjugated secondary antibodies, blots were developed using SuperSignal West Femto chemiluminescence reagent (Pierce Biotechnology). Images were captured using FluorChem 8900 imager (Alpha Innotech).

Some membranes were probed with IRDye680- or IRDye800- conjugated secondary antibodies and scanned with Odyssey Infrared Imaging System (LI-COR). *For detection of HIF -1 $\alpha$  or -2 $\alpha$ , 50  $\mu$ g of each lysate was loaded due to their very low expression in the 3D cultures.*

### **Quantitative PCR.**

First strand cDNA pools of S1, T4-2 and reverted T4-2 cells were obtained from total RNA of each sample by using SuperScript II (Invitrogen). For each sample, reverse transcription was carried out with random hexamer and with oligo dT primer following the manufacturer's instruction, and the two reactions were mixed in a ratio of 9:1. Expression levels of *EGFR*, *ITGB1*, *SLC2A3* and 18S ribosomal RNA (used as a control) were quantified in LightCycler system (Roche) using QuantiTect SYBR Green PCR Kits (QIAGEN) and the reverse transcription mixtures. Expression levels of *EGFR*, *ITGB1*, *SLC2A3* were normalized to that of 18S ribosomal RNA.

### **Measurement of Rap1 activity.**

Rap1 activity was measured as described previously (Itoh et al., 2007). Briefly, cell colonies were extracted from IrECM as described above, and lysed in lysis buffer (50 mM Tris-HCl [pH 7.4], 500 mM NaCl, 1% NP40, 2.5 mM MgCl<sub>2</sub>, 10% glycerol, 10  $\mu$ g/ml aprotinin and 10  $\mu$ g/ml leupeptin) at 4 °C for 30 min. Lysates were cleared by centrifugation at 15,000  $\times g$  at 4 °C for 15 min. Supernatants (containing 300  $\mu$ g protein in 300  $\mu$ l) were incubated with 15  $\mu$ g of GST-tagged RalGDS-RBD fusion protein immobilized on Glutathione Sepharose 4B beads (GE Healthcare), at 4 °C for 1 hr. Beads were washed trice in lysis buffer and re-suspended in Laemmli buffer. Samples were separated by SDS-PAGE, followed by transfer to polyvinylidene fluoride membranes (0.2  $\mu$ m pore) and immunoblotting using anti-Rap1 antibody.



### **Immuno-precipitation.**

T4-2 cells were cultured in 3D-OT IrECM for 4 days. Cells were collected in ice-cold PBS, and the IrECM gel and culture medium were removed by centrifugation at 4 °C. Immuno-precipitation was performed as described previously (2) with slight modification. Briefly, cell pellets were lysed in NP-40 buffer (1% NP-40, 150 mM NaCl, 20 mM Tris-HCl [pH 7.4], 5 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 μM Na<sub>2</sub>MoO<sub>4</sub>, 2 mM NaF and protease inhibitor cocktail set [Calbiochem]) on ice for 10 minutes, and the soluble fraction was collected by centrifugation. Three μg of anti-sAC antibody or non-immune rabbit IgG, together with 5 μl of Protein A-bound sepharose beads (GE Healthcare), were added to 600 μg lysate dissolved in 300 μl lysis buffer and incubated at 4 °C for 2 hours. The precipitate was washed 4 times with lysis buffer and then subjected to SDS-PAGE and western blot. Co-precipitation of PKM1/M2 was examined by blotting with goat polyclonal antibody against muscle-type pyruvate kinase. To minimize the cross-reactivity to the rabbit IgG on the membrane, TrueBlot anti-rabbit secondary antibody (Rockland) was used for detection of sAC.

### **In vitro GST binding assay.**

The above-described pcDNA3-sAC-V5, pEBG-PKM1, pEBG-PKM2 or empty pEBG were transfected into 293T cells using polyethylenimine solution, respectively. Each transfectant was lysed in NP-40 buffer (see above), and GST-PKM1 or PKM2 or GST alone was purified from the lysate by using glutathione sepharose beads (GE Healthcare). The purified GST proteins were incubated with the crude cell lysate containing sAC-V5 for 1 hour at 4 °C, and the precipitate was washed 4 times with NP-40 buffer, then subjected to SDS-PAGE and western blot.

### **Measurement of invasive activity of cancer cells.**

Invasive activity was examined using BD Biocoat GFR (growth factor reduced)-Matrigel invasion chamber (BD Biosciences). Before the assay, lower surface of the membrane of the upper chamber was coated with collagen type I (10 µg/ml). MDA-MB-231 cells cultured on 2D plastic for two days- with H14 medium containing different concentrations of glucose (17.5, 1.75 or 0 mM) or supplemented with metabolic inhibitors (40 µM IA or 20 µM DON, with 17.5 mM glucose)- were detached by trypsinization, and 25,000 cells for each condition were seeded on the upper chambers. Both upper and lower chambers were filled with medium of the same formulations as that in each 2D cultures. After 16 hours, cells that had invaded were fixed with 4% PFA in PBS, stained with crystal violet and counted.

### **Statistics overview.**

In all experiments, statistical significance was determined by 2-tailed Student's t test. A P value of less than 0.05 was considered statistically significant.

The survival analysis of the microarray datasets of 295 human breast tumors with the associated clinical data (2; Figure 11), obtained from Rosetta Inpharmatics, was performed using Sigma Plot 11.0. For analyses based on the expression levels of each marker gene (*PFKP*, *GFPT1* or *GFPT2*) alone, patients were stratified into upper quartile (high), intermediate (medium) and lower quartile (low) for expression of each marker. For analysis based on the combined, multiple gene expression levels, patients were stratified into upper quartile (high) first, and then upper (medium) and lower (low) halves of the remaining, based on the expression of the markers. Patients of "*PFKP* -high AND [*GFPT1*-high OR *GFPT2*-high]" and "*PFKP* -low AND *GFPT1*-low AND *GFPT2* -low" were stratified as "high" and "low", respectively. The remaining populations were categorized as "medium". Survival curves were computed using the Kaplan and Meier method. Statistical significance was determined by the log-rank test.

## Supplemental References

1. Mayer, B.J., Hirai, H., and Sakai, R. 1995. Evidence that SH2 domains promote processive phosphorylation by protein-tyrosine kinases. *Curr Biol* 5:296-305.
2. Onodera, Y., Nam, J.M., Hashimoto, A., Norman, J.C., Shirato, H., Hashimoto, S., and Sabe, H. 2012. Rab5c promotes AMAP1-PRKD2 complex formation to enhance beta1 integrin recycling in EGF-induced cancer invasion. *J Cell Biol* 197:983-996.
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## Supplemental Figures and Legends

### Supplemental Figure 1

Signaling and intermediary metabolic properties of S1, T4-2 and phenotypically reverted T4-2 cells derived from gene expression arrays and biochemical measurements. **(A)** Western blots of signaling intermediates in 3D Ir-ECM cultures of S1, T4-2 and T4-2 cells reverted by different signaling inhibitors (see Figure 1A). **(B and C)** S1 and T4-2 cells in 3D-on top (OT) IrECM cultures were deprived of glucose. Intracellular levels of fructose-6-phosphate (F6P, **B**) and lactate (**C**) were measured at indicated time points. Data are shown as mean  $\pm$ SD of triplicate experiments. **(D and E)** S1 and T4-2 cells were cultured in the conditions shown in **(A)**. Expression of genes involved in glycolysis (**D**) and citric acid cycle (**E**) were shown. Data were retrieved from microarray analyses from Bissell laboratory and shown as relative expression (arbitrary units) compared to S1 cells. The array data can be found at GEO database.

### Supplemental Figure 2

Manipulation of GLUT3 expression leads to metabolic alterations in S1 and T4-2 cells cultured in 3D IrECM. **(A and B)** T4-2 cells transfected with control or GLUT3 siRNA were cultured in 3D-OT IrECM. Glucose uptake (**A**) and western blot of GLUT1 (**B**) were shown. **(C-E)** S1 cells infected with empty or GLUT3-HA-encoding lentivirus were cultured in 3D-OT IrECM with or without 17.5 mM glucose. Glucose uptake (**C**) and intracellular levels of F6P (**D**) and lactate (**E**) were measured. **(F)** Western blots of metabolic enzymes and GLUT1 in S1 cells infected with empty or GLUT3-HA-encoding lentivirus, cultured in 3D IrECM with 2DG (4 mM) or different concentration of glucose. In **(A)** and **(C-E)**, data are shown as mean  $\pm$ SD of triplicate experiments.

### Supplemental Figure 3

mTOR activity and HIF expression do not correlate with malignant phenotype of mammary epithelial cells cultured in 3D Ir-ECM. **(A)** Confocal IF images of 3D cultures of T4-2 cells treated with (a) vehicle or (b-d) pp242 (0.1, 0.3 or 1.0  $\mu$ M, respectively).  $\alpha$ 6 integrin (green), nuclei (red). Bars, 20  $\mu$ m. **(B-D)** Measurements were done in conditions shown in **(A)**: Cell numbers at the colony mid-section (**B**), percent colonies with basal polarity (**C**), western blots of signaling intermediates (**D**). **(E)** Full membrane of the western blots of HIF -1 $\alpha$  and -2 $\alpha$  shown in Figure

4F. (F) Western blots of GLUT3 and metabolic enzymes in T4-2 cells cultured in 3D-OT Ir-ECM. (G and H) S1 or T4-2 cells were cultured in 3D IrECM with different glucose concentrations of glucose as indicated. Activity of AMPK and mTOR pathways (G) and expression of HIF -1 $\alpha$  and -2 $\alpha$  (H) were measured. In (B) and (C), data are shown as mean  $\pm$ SD of triplicate experiments.

#### **Supplemental Figure 4**

Decrease in glycolytic metabolism, but not pyruvate metabolism nor mitochondrial ATP synthesis, is responsible for the phenotypic reversion. (A) Confocal IF images of T4-2 cells transfected with (a) control or (b) GAPDH siRNA, cultured in 3D-OT IrECM.  $\alpha$ 6 integrin (green), nuclei (red). Bars, 20  $\mu$ m. (B-D) Measurements were done in conditions shown in (A): Cell numbers at the colony mid-section (B), percent colonies with basal polarity (C), western blots of signaling intermediates (D). (E) Confocal IF images of T4-2 cells cultured in 3D IrECM with different concentrations of glucose and pyruvate, indicated as (i)-(v).  $\alpha$ 6 integrin (green), nuclei (red). Bars, 20  $\mu$ m. (F-H) Measurements were done in conditions shown in (E): Cell numbers at the colony mid-section (F), percent colonies with basal polarity (G), protein expression and/or activation of  $\beta$ 1 integrin and Akt (H). (I) Confocal IF images of S1 and T4-2 cells cultured with Oligomycin (OGM, 4 ng/ml), indicated as (1)-(4).  $\alpha$ 6 integrin (green), nuclei (red). Bars, 20  $\mu$ m. (J and K) Measurements were done in conditions shown in (I): Cell numbers at the colony mid-section (J), percent colonies with basal polarity (K). In (B), (C), (F), (G), (J) and (K), data are shown as mean  $\pm$ SD of triplicate experiments.

#### **Supplemental Figure 5**

Knockdown of sAC suppresses Rap1 activity and malignant phenotype. (A) Confocal IF images of T4-2 cells transfected with (a) control or (b) sAC siRNA, cultured in 3D-OT IrECM.  $\alpha$ 6 integrin (green), nuclei (red). Bars, 20  $\mu$ m. (B-D) Measurements were done in conditions shown in (A): Cell numbers at the colony mid-section (B), percent colonies with basal polarity (C), western blots of signaling intermediates (D). (E) Western blot of sAC in S1, T4-2 and T4-2 cells reverted by different signaling inhibitors (see Figure 1A). In (B) and (C), data are shown as mean  $\pm$ SD of triplicate experiments.

#### **Supplemental Figure 6**

Phosphorylation of STAT3 and Histone H3 is not correlated with glucose uptake and metabolism in 3D cultures of S1 and T4-2 cells. Levels of total and phosphorylated STAT3 and

Histone H3 were examined by western blot in 3D cultures of S1 and T4-2 cells in different glucose concentrations. MEK5 expression, which is upregulated by STAT3, is shown also.

### **Supplemental Figure 7**

Knockdown of GFPT or OGT induces phenotypic reversion. **(A)** Confocal IF images of T4-2 cells transfected with (a) control, (b) GFPT1/2 or (c) OGT siRNA, cultured in 3D-OT IrECM.  $\alpha 6$  integrin (green), nuclei (red). Bars, 20  $\mu\text{m}$ . **(B-D)** Measurements were done in conditions shown in **(A)**: Cell numbers at the colony mid-section **(B)**, percent colonies with basal polarity **(C)**, western blots of signaling intermediates **(D)**. In **(B)** and **(C)**, data are shown as mean  $\pm$ SD of triplicate experiments.

### **Supplemental Figure 8**

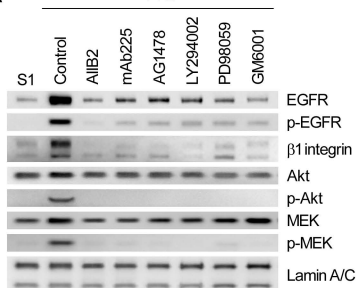
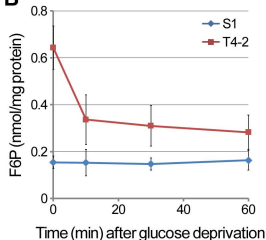
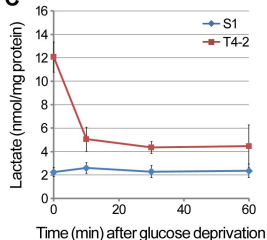
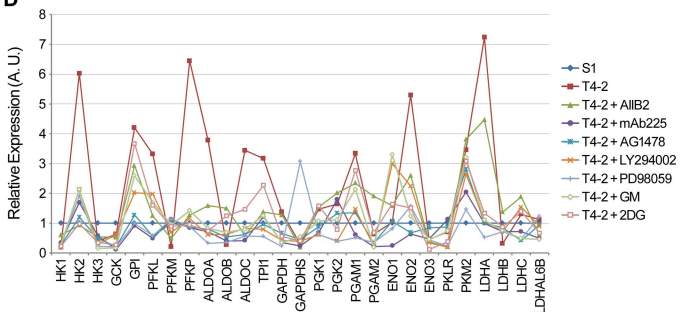
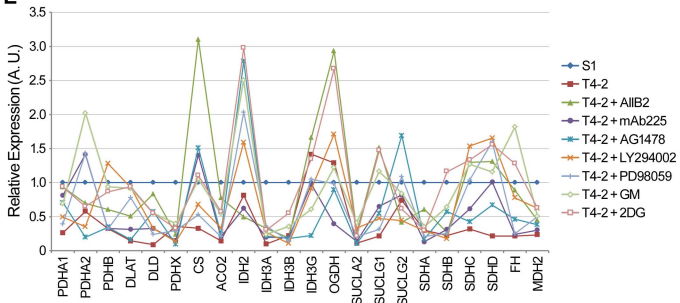
Expression levels of enzymes mediating the reciprocal interaction between glucose metabolism and oncogenic signaling in GLUT3-overexpressing S1 cells. Western blots of PKM2, sAC, GFPT, OGT and total O-GlcNAc in S1 cells infected with empty or GLUT3-HA-encoding lentivirus, cultured in 3D IrECM with 2DG (4 mM), or with different concentration of glucose. Asterisks indicate the O-GlcNAc bands specifically increased in T4-2 cells (see Figure 6).

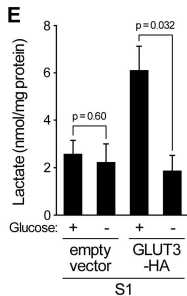
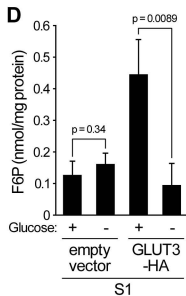
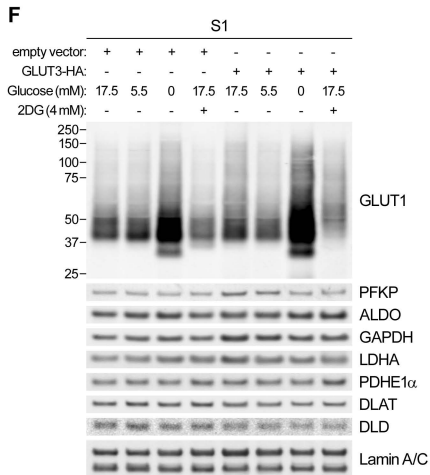
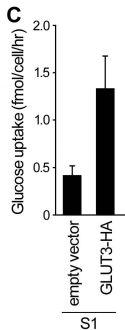
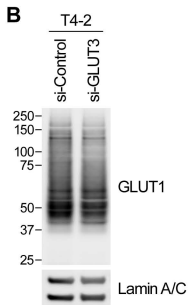
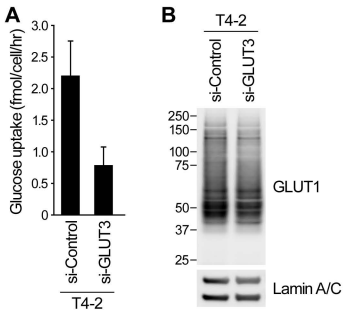
### **Supplemental Figure 9**

Reciprocity between metabolic pathways and other signaling pathways is lost in 2D cultures of S1 and T4-2 cells. **(A)** Protein expression and activation of signaling pathways involved in oncogenesis in 2D cultures of S1 and T4-2 cells treated with metabolic inhibitors used in this study. **(B)** Glucose uptake in cells from **(A)**. Data are shown as mean  $\pm$ SD of triplicate experiments.

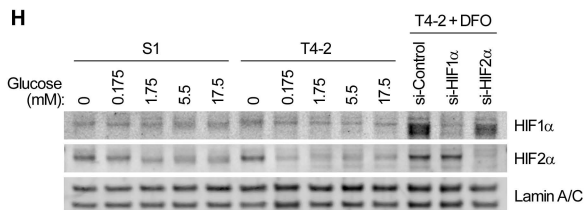
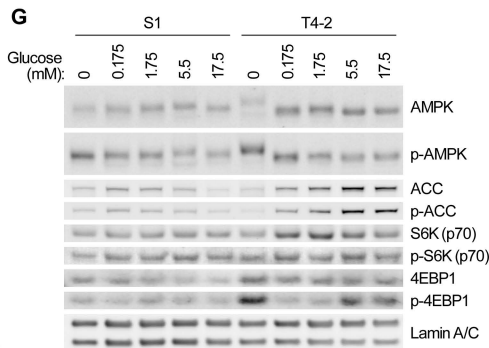
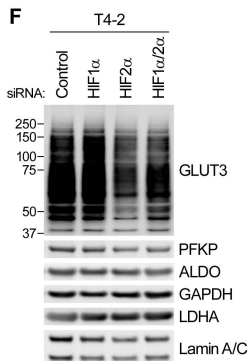
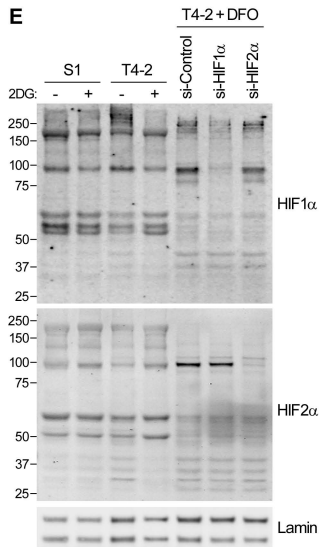
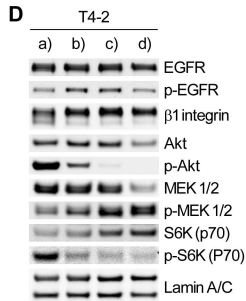
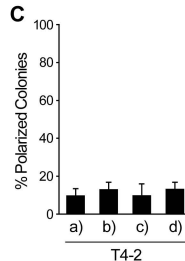
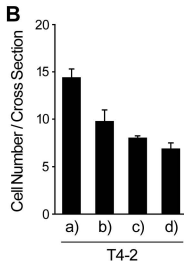
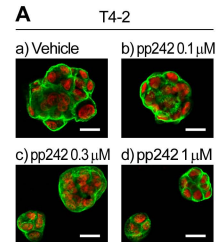
**A**

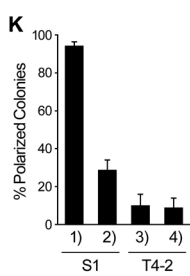
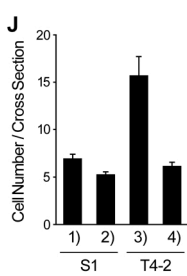
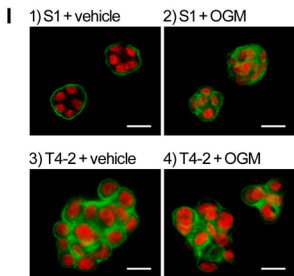
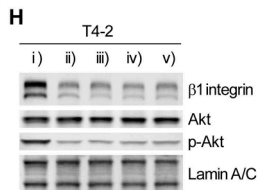
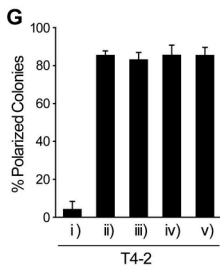
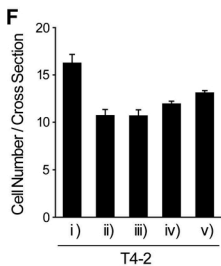
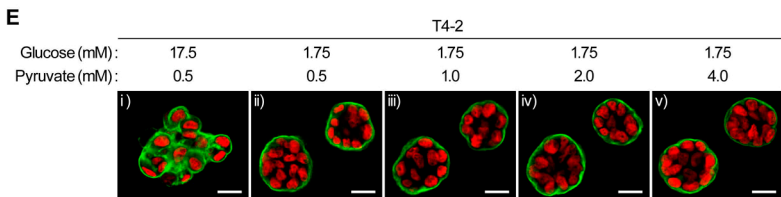
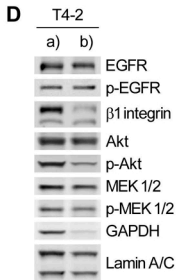
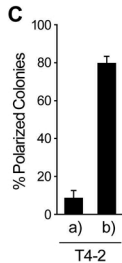
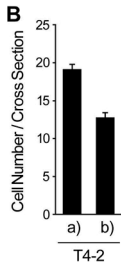
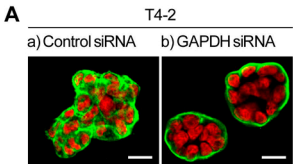
T4-2

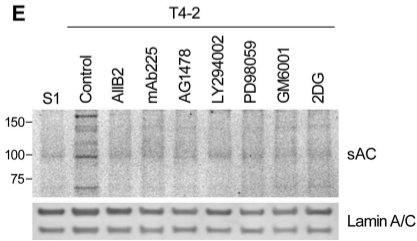
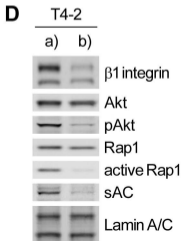
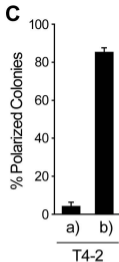
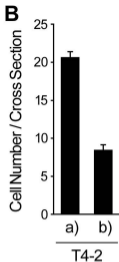
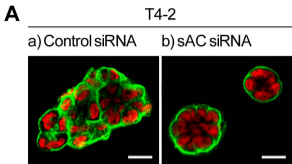
**B****C****D****E**













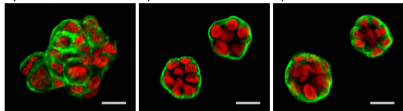
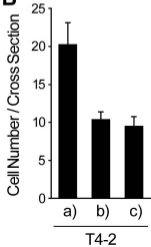
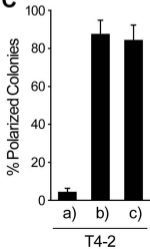
**A**

T4-2

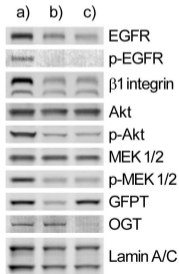
a) Control siRNA

b) GFPT1/2 siRNA

c) OGT siRNA

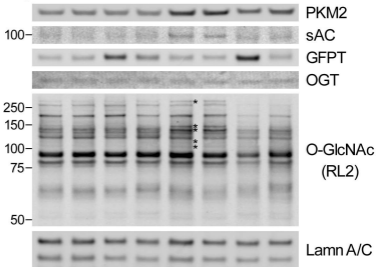
**B****C****D**

T4-2



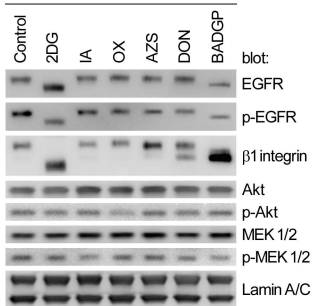
## S1

empty vector:	+	+	+	+	-	-	-	-
GLUT3-HA:	-	-	-	-	+	+	+	+
Glucose (mM):	17.5	5.5	0	17.5	17.5	5.5	0	17.5
2DG (4 mM):	-	-	-	+	-	-	-	+



**A**

T4-2 (monolayer)

**B**