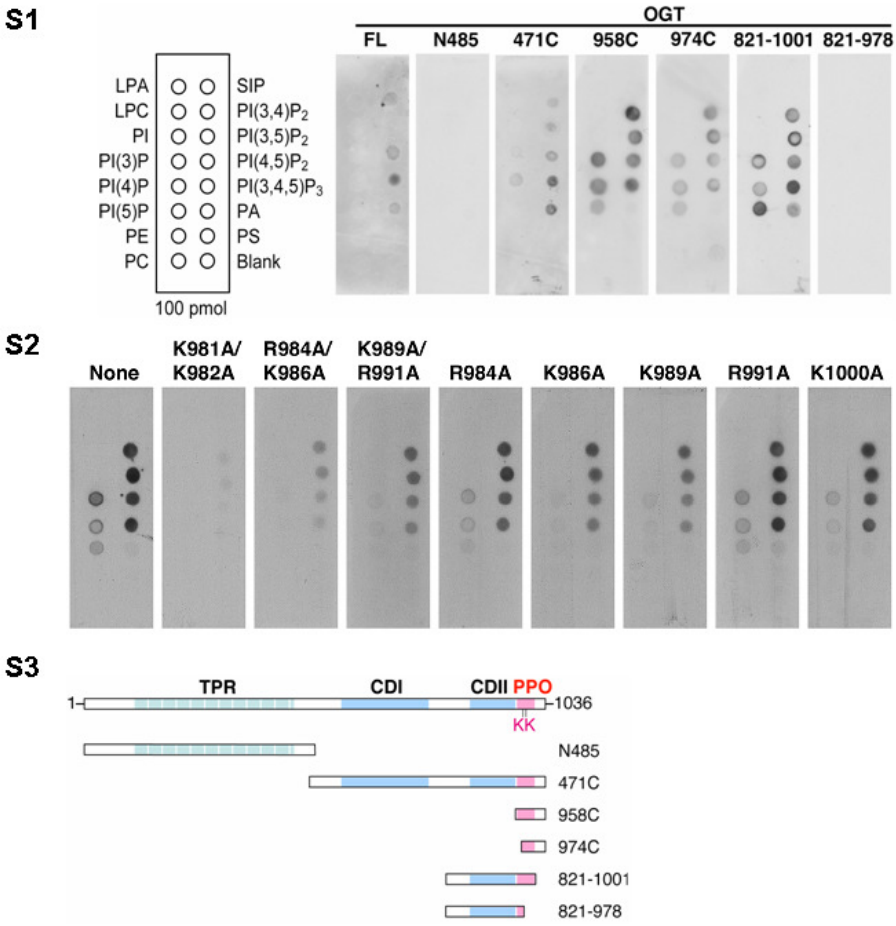
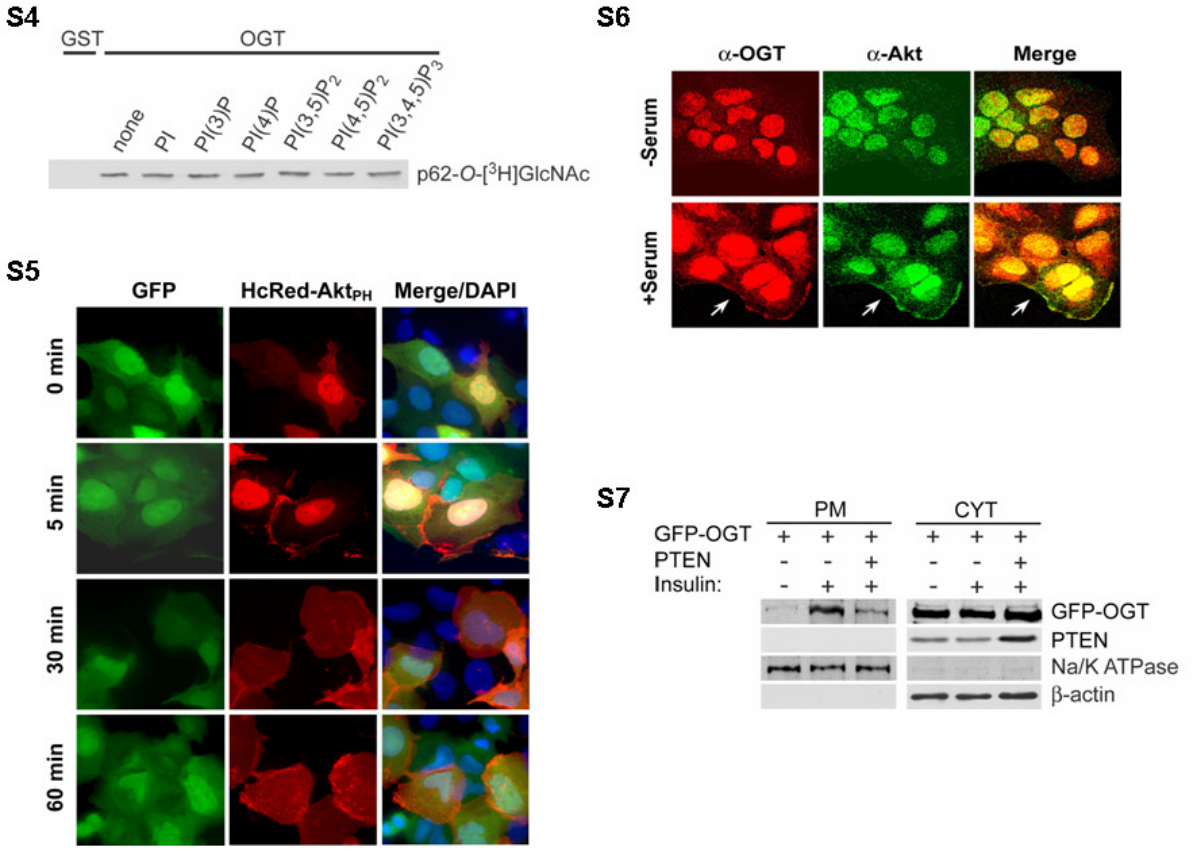
Supplementary Figures



Supplementary Figure S1. Binding of full-length OGT and deletion mutants to PIP strips (Echelon Biosciences).

Supplementary Figure S2. Binding of the OGT (919-1036) fragments with the indicated point mutations to PIP strips (Echelon Biosciences).

Supplementary Figure S3. Schematic representation of full-length OGT and various deletion mutants. TPR, tetratricopeptide repeats; CDI&II, catalytic domains I&II; PPO, PtdInsP-binding domain. KK indicates lysine 981 and lysine 982 in the PPO domain.

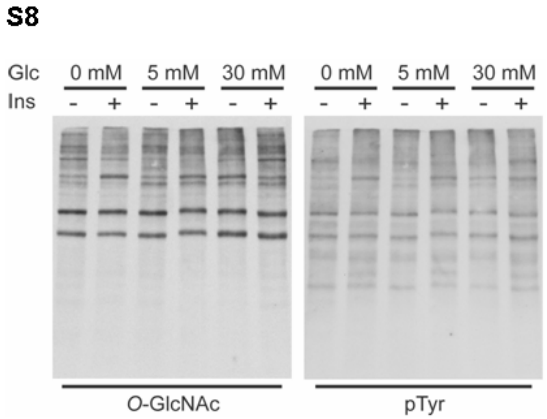


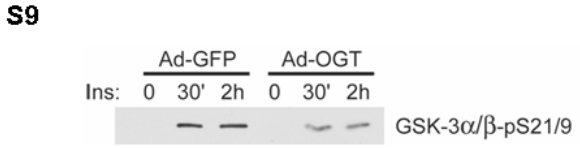
Supplementary Figure S4. The p62 protein substrate was incubated with GST-OGT beads and UDP-[^{3}H]GlcNAc in the OGT activity assay buffer in the presence of water soluble forms of phosphoinositide species (diC8) as indicated. The reactions were resolved with SDS-PAGE and subjected to autoradiography.

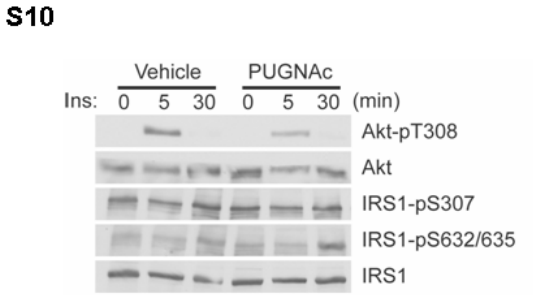
Supplementary Figure S5. Fluorescent images of Cos-7 cells co-transfected with the GFP and HcRed-Akt$_{PH}$ expression vectors followed by serum treatment for indicated periods of time.

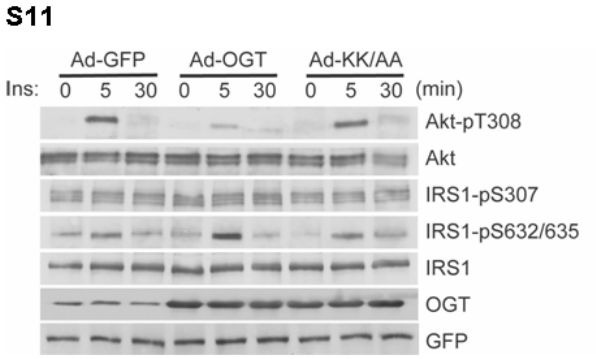
Supplementary Figure S6. Immunostaining of endogenous OGT and Akt in Cos-7 cells in the absence or presence of serum using α-OGT (Sigma) and α-Akt (Cell Signaling) antibodies. Arrows indicate protein co-localization at the plasma membrane.

Supplementary Figure S7. Immunoblot analysis of subcellular fractionations after 3T3-A14 cells were transfected with the GFP-OGT vector in the absence or presence of the PTEN expression vector and treated with 100 nM insulin. PM, plasma membrane; CYT, cytosol.







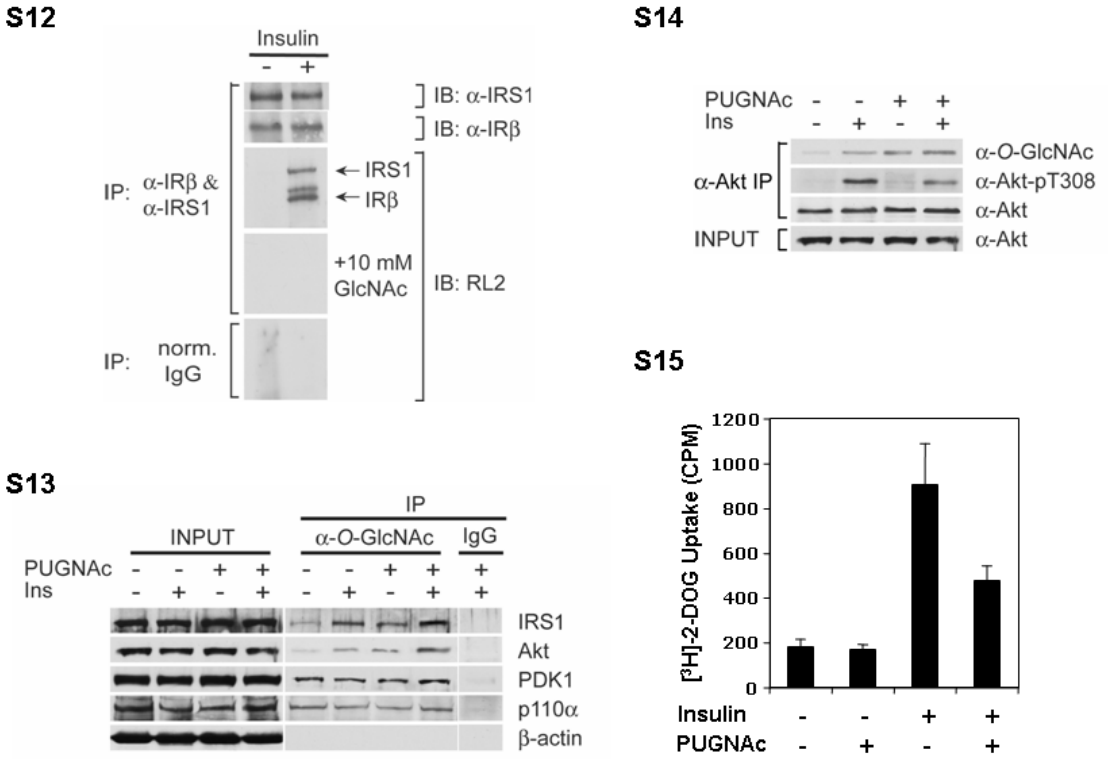


Supplementary Figure S8. Immunoblot analysis of 3T3-L1 whole-cell lysates with α-*O*-GlcNAc antibody (RL2) or α-phosphotyrosine antibody (4G10) after cells were incubated with indicated concentrations of glucose (Glc) for 16 h and then treated with 100 nM insulin (Ins) for 30 min.

Supplementary Figure S9. Akt kinase assay using 3T3-L1 cells infected with adenoviruses expressing GFP or OGT. Akt activity in phosphorylating GSK-3α/β crosstide was assessed after insulin treatment for indicated time periods.

Supplementary Figure S10. Immunoblot analysis of phosphorylation states and total amounts of the indicated proteins in Fao cells treated with PUGNAc or vehicle for 12 h followed by insulin stimulation for indicated time periods. Note that Ser307 of IRS1 was constantly phosphorylated in Fao cells.

Supplementary Figure S11. Immunoblot analysis of phosphorylation states and total amounts of the indicated proteins in Fao cells infected with adenovirus expressing GFP, the wildtype or KK/AA mutant of OGT and treated with insulin for indicated time periods. Note that Ser307 of IRS1 was constantly phosphorylated in Fao cells.



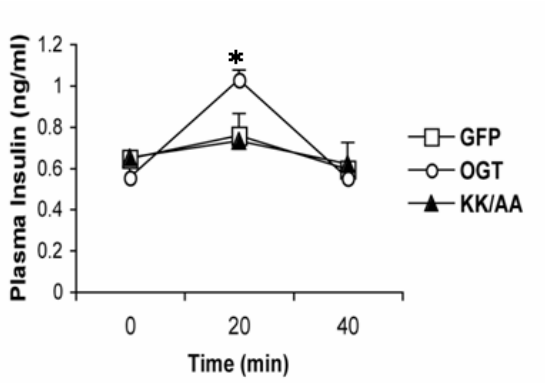
Supplementary Figure S12. 3T3-L1 adipocytes were treated with 100 nM insulin for 30 min. Whole-cell lysates were immunoprecipitated using either normal IgG or a mixture of α-IRβ and α-IRS1 antibodies, and then immuoblotted with α-IRβ, α-IRS1, or α-*O*-GlcNAc antibody (RL2) as indicated. Specificity of RL2 for *O*-GlcNAc moieties was confirmed by competition with 10 mM GlcNAc.

Supplementary Figure S13. Following combinatorial treatments with 100 μM PUGNAc for 16 h and then 100 nM insulin (Ins) for 30 min, 3T3-L1 whole-cell lysates were immunoprecipitated with either α-*O*-GlcNAc antibody (CTD110.6) or normal IgG and immuoblotted with antibodies against IRS1, Akt, PDK1, p110α, and β-actin.

Supplementary Figure S14. Following PUGNAc and/or insulin treatments, 3T3-L1 whole-cell lysates were immunoprecipitated with α-Akt and immuoblotted with antibodies against total Akt, phospho-Akt (Thr308) and *O*-GlcNAc (RL2).

Supplementary Figure S15. Effect of PUGNAc on glucose uptake in 3T3-L1 adipocytes. Cells were treated with 100 μM PUGNAc for 16 h, stimulated with insulin for 30 min, and then incubated with [^{3}H]-2-deoxyglucose (2-DOG) for 4 min. Non-specific uptake in the presence of 15 μM cytochalasin B was subtracted from all values.

S16



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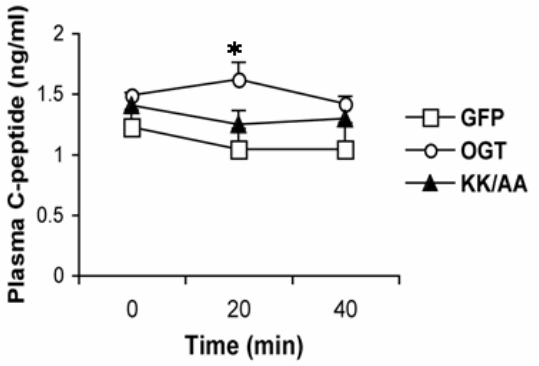
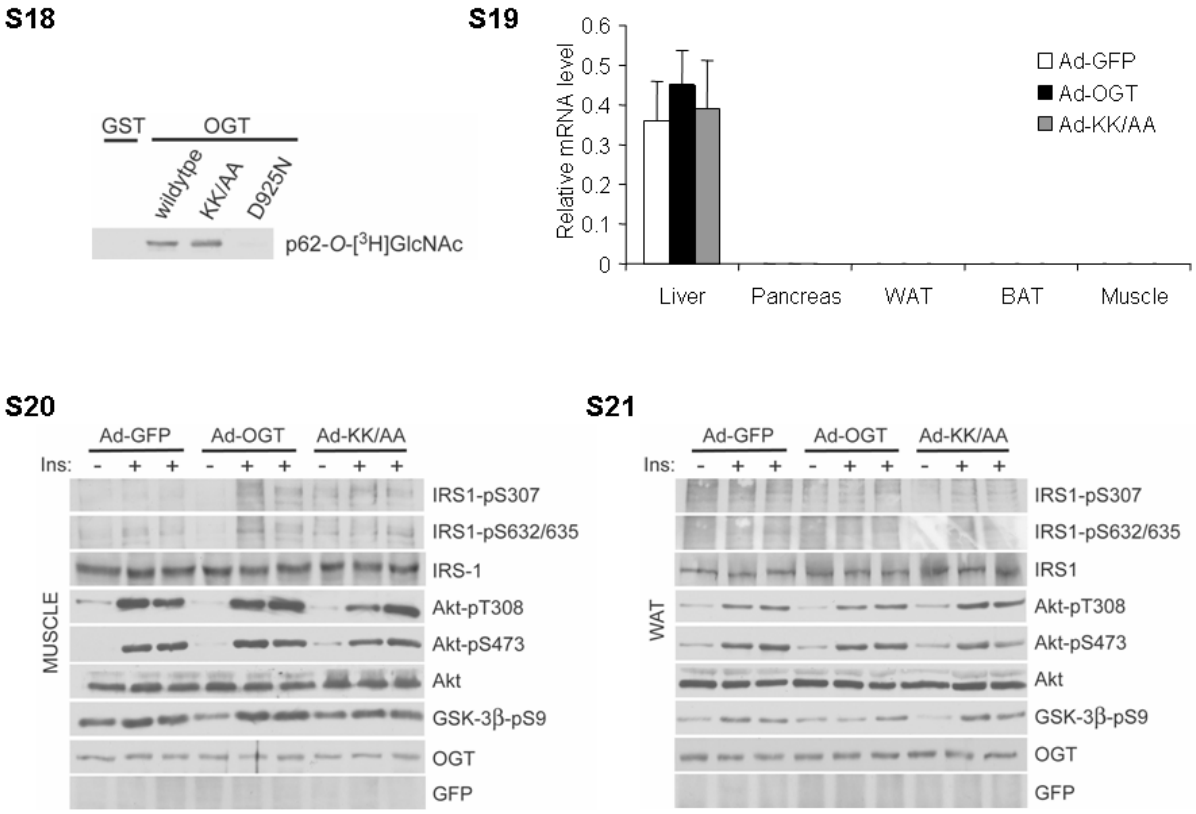


Supplementary Figure S16. Glucose-stimulated insulin release in 12-week-old C57BL/6J male mice infected with adenovirus expressing GFP, the wildtype or KK/AA mutant of OGT (n = 3). Glucose (1.5 g kg^{-1} body weight) was injected i.p. 6 h after food removal. Tail-vein blood was collected at 0, 20, and 40 min after glucose injection for measurement of plasma insulin levels. Asterisk, $P < 0.05$ versus GFP and KK/AA mice.

Supplementary Figure S17. Glucose-stimulated C-peptide release in mice infected with adenovirus expressing GFP, the wildtype or KK/AA mutant of OGT (n = 3). Glucose (1.5 g kg^{-1} body weight) was injected i.p. 6 h after food removal. Tail-vein blood was collected at 0, 20, and 40 min after glucose injection for measurement of plasma C-peptide levels. Asterisk, $P < 0.05$ versus GFP mice.



Supplementary Figure S18. OGT activity assay showing equal activity for wildtype OGT and the KK/AA mutant in catalyzing the transfer of the [^{3}H]GlcNAc moiety onto the p62 protein substrate. GST and the catalytically dead OGT (D925N) mutant serve as controls.

Supplementary Figure S19. Q-PCR analysis of GFP expression in indicated tissues from C57BL/6J mice infected with adenovirus expressing GFP, the wildtype or KK/AA mutant of OGT (n =4-6). Note that each recombinant adenovirus contains a cassette for the CMV promoter-driven GFP expression.

Supplementary Figure S20. Immunoblot analysis of muscle extracts from adenovirus-infected mice injected i.p. with insulin (3 U kg^{-1} body weight) or vehicle.

Supplementary Figure S21. Immunoblot analysis of white adipose tissue (WAT) extracts from adenovirus-infected mice injected i.p. with insulin (3 U kg^{-1} body weight) or vehicle.

Supplementary Movie Legends

Supplementary Movie 1. Serum treatment triggers OGT to translocate to the plasma membrane. Cos-7 cells were transfected with GFP-OGT and serum-starved overnight. Live fluorescence microscopy was performed, taking images every 30 seconds, while fetal bovine serum was added to a final concentration of 10%. As shown, in quiescent cells GFP-OGT is found diffusely throughout the cell, but upon serum stimulation rapidly translocates to the plasma membrane within 90 seconds.

Supplementary Movie 2. PI-3-kinase activity is necessary for plasma membrane translocation of OGT in response to serum. Cos-7 cells were transfected with GFP-OGT and serum-starved overnight. Live fluorescence microscopy was performed, taking images every 30 seconds. The cells were pre-treated with 100nM wortmannin for 10 minutes, and then stimulated with 10% fetal bovine serum. As shown, pre-treatment with the PI-3-kinase inhibitor wortmannin prevents GFP-OGT from translocating to the plasma membrane.

Supplementary Movie 3. PI-3-kinase activity is required to maintain OGT at the plasma membrane. Cos-7 cells were transfected with GFP-OGT and serum-starved overnight. Live fluorescence microscopy was performed, taking images every 90 seconds. At time=0 (frame 2) cells are stimulated with 10% fetal bovine serum, causing rapid translocation of GFP-OGT to the plasma membrane. At time=13:30 (frame 11) 100nM wortmannin is added. Within 3 minutes GFP-OGT dissociates from the plasma membrane and again is found diffusely throughout the cell, indicating that PI-3-kinase is required for GFP-OGT localization to the plasma membrane.

Supplementary Movie 4. Activated PI-3-kinase is sufficient to drive OGT to the plasma membrane. Cos-7 cells were co-transfected with GFP-OGT and constitutively active PI3K. Cells were serum-starved overnight and then live fluorescence microscopy performed. As shown, expression of constitutively active PI3K is sufficient to drive GFP-OGT to the plasma membrane. At time=0 (frame 2) cells are treated with 100nM wortmannin, which results in rapid dissociation of GFP-OGT from the plasma membrane, demonstrating that the localization at the plasma membrane is dependent on PI3K activity.

Supplementary Movie 5. Mutation of lysines 981 and 982 to alanines abolishes the ability of OGT to respond to serum. Cos-7 cells were co-transfected with the GFP-OGT (KK/AA) mutant (shown in green) and YFP-AKT-PH (a known reporter for PtdIns(3,4,5)P3 and PtdIns(3,4)P2, shown in red). Cells were serum-starved overnight and then live fluorescence microscopy performed. At time=1:12 (frame 2) cells were stimulated with 10% fetal bovine serum. As shown in red, YFP-AKT-PH rapidly responds by translocating to the plasma membrane, documenting the production of products of PI3K. However, the KK/AA mutant does not translocate, indicating an inability to bind to the products of PI3K.

Supplementary Movie 6. Mutation of lysines 981 and 982 to alanines abolishes the ability of OGT to respond to constitutively active PI-3-kinase. Cos-7 cells were co-transfected with GFP-OGT (KK/AA) and constitutively active PI3K. Cells were serum-starved overnight and then live fluorescence microscopy performed. As shown, the KK/AA mutant of OGT does not associate with the plasma membrane, and no change in localization is observed upon treatment with 100nM wortmannin (beginning at frame 2).

Supplementary Methods

OGT activity assay. Wildtype and mutant OGT and nucleoporin p62 proteins were expressed in bacteria and were purified by glutathione-affinity chromatography. The p62 protein served as the protein substrate. After purification, 100 ng GST-OGT fusion proteins were incubated with 0.5 μCi UDP-[^{3}H]GlcNAc (PerkinElmer) and 100 ng p62 in the OGT activity assay buffer (50 mM Tris-HCl, pH 7.5, 12.5 mM $MgCl_2$, 1 mM DTT) at room temperature for 90 min. Where indicated, the reactions also contained water soluble forms of phosphoinositide species (diC8) (Echelon Biosciences) at a final concentration of 50 μM. The reactions were resolved with SDS-PAGE, treated with Amplify (Amersham), and then subject to autoradiography.

Immunofluorescence. Cos-7 cells were seeded on the coverslips and serum starved for 16 h. After treated with 100 nM insulin or 10% FBS, cells were fixed in PBS containing 3.7% formaldehyde for 12 min at room temperature, permeablised with 0.2% Triton-X 100 for 5 min, washed with PBST (PBS + 0.05% Tween-20), and then blocked with PBSTB (PBST with 3% BSA) for 20 min. After blocking, cells were incubated with OGT (Sigma) and Akt (Cell Signaling) antibodies (1:250) in PBSTB at 4 °C overnight. Cells were washed three times with PBST then incubated with Alexa Fluor 568-conjugated donkey anti-mouse and Alexa fluor 488-conjugated donkey anti-rabbit secondary antibodies (1:500) (Molecular Probes) for 1.5 h, followed by four 10-min washes. For the nucleus staining, TO-PRO-3 (Molecular Probes) was added in PBST at a dilution of 1:10,000 for 10 min before the two final washes. Subsequently, cells were mounted on slides in GEL/MOUNT (Biomeda) and subject to fluorescent confocal microscopy.

Glucose uptake assay. Fully differentiated 3T3-L1 adipocytes were serum starved for 16 h in the presence or absence of 100 μM PUGNAc, treated with 100 nM insulin for 30 min at 37°C, then washed three times with KRH buffer (121 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 0.33 mM $CaCl_2$, 12 mM HEPES, pH 7.4). A cocktail containing 50 μCi [^{3}H]-2-deoxyglucose (PerkinElmer) and 2 mM 2-deoxyglucose in KRH was added. 15 μM cytochalasin B (Sigma) was included in control samples. After incubation at room temperature for 4 min, the reactions were terminated by washing three times with ice-cold KRH containing 25 mM glucose. Cells were lysed in 0.5 M NaOH containing 0.1% SDS. Incorporated radioactivity was measured by liquid scintillation counting.