Supporting Information

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SI Materials and Methods

Antibodies, Drugs, cDNA Constructs, and siRNA. Antibodies against phospho-Akt (Ser^{473} and Thr^{308}), Akt1, Akt2, phospho-Akt Substrate (PAS), phospho-FoxO1 (Ser^{256}), Akt, ATGL and FoxO1 were from Cell Signaling Technologies. Protein A/G PLUS agarose beads were purchased from Santa Cruz Biotechnology. Mouse and rabbit antiFlag epitope antibodies were from Sigma-Aldrich. Anti-TBC1D4 and anti-phosphoTBC1D4 (Thr^{642}) antibodies were from Upstate Biotech. Mouse antiHA epitope monoclonal antibody was from Covance. Antigreen fluorescent protein antibody was from Invitrogen. Fluorescent secondary antibodies were purchased from Jackson Immunolabs, Inc., and Invitrogen. PI3-kinase isoform specific inhibitors PI90 (p110α), p103 (p110α), TGX221 (p110β), SW14 (p110 δ & γ), SW30 (p110γ) were kindly provided by Kevan Shokat (University of California San Francisco) (1). Akt inhibitor (Akti1/2) was purchased from Calbiochem. The cDNA constructs encoding Flag-Akt1 and Flag-Akt2 were previously described (2). FoxO1-GFP construct was kindly provided by Domenico Accili (Columbia University) (3). The following targeting sequences were used to knockdown murine Akt1: CCCAGAACAATTAGATTAGATTCATGTAGA; Akt2: CACAGGACACAAGCATGGCCGTCAT; PTEN: TTTGCAGTATAGAGCGUGCAGAUAA; FoxO1: AGTTACGGAGGATTGAACCAGTATA; control: ATGTCACGGCGTACGTTCCTGTGTG. Stealth siRNAs were purchased from Invitrogen.

Cell Culture, Adipocyte Differentiation, and Electroporation. 3T3-L1 fibroblast were cultured, differentiated into adipocytes and electroporated as previously described (4, 5). All experiments were performed on day 5 or 6 after differentiation. Stably expressing Flag-tagged Akt1 and Akt2 3T3-L1 cells have been previously described (2). For transient knockdown of target genes, 3T3-L1 adipocytes were electroporated with specific siRNAs (4 μM), and experiments were performed 48 hr after electroporation or as noted.

Immunoblot Analyses and Immunoprecipitation. 3T3-L1 adipocytes were starved in serum-free-DMEM with 20 mM of sodium bicarbonate, 20 mM HEPES (pH7.2) (SF-DMEM) at 37 C in 5%CO2/air for 3–8 h prior to all experiments. After insulin treatments, 3T3-L1 adipocytes were washed with 150 mM NaCl, 20 mM HEPES, 1 mM CaCl2, 5 mM KCl, 1 mM MgCl2 (pH 7.2) (Media 1) and lysed in RIPA or Laemmli buffer. For immunoblot analyses cellular proteins were resolved in SDS/PAGE, transferred to nitrocellulose membranes, and probed with antibodies using protocols provided by the suppliers. Antibody binding was detected using enhanced chemiluminescence (Supersignal West Pico, Thermo Scientific). Densitometric analyses of the immunoblots were performed using a ChemiImager 4000 (Alpha-Innotech). Immunoprecipitation of Flag-tagged Akt1 or Akt2 was performed as described under in vitro Akt activity assay and in (2).

Glycerol Release. Control 3T3-L1 adipocytes or adipocytes exposed to chronic hyperinsulinemia (10 nM insulin for 16 h) were washed three times with serum-free media and incubated in serum-free media with 2% fatty acid free bovine serum albumin for 3 h. Glycerol content in the media was measured colorimetrically using a free glycerol assay kit from Biovison. Cells were then washed with PBS and lysed in 1% Triton X-100. The protein concentration was determined and used to normalize the glycerol release per sample. Experiments were carried out in duplicate.

In Vitro Akt Activity Assay. 3T3-L1 adipocytes expressing Flag-tagged Akt constructs were starved in serum-free-DMEM for 2–4 h. After insulin stimulation for 15 min, adipocytes were washed with Media 1 and lysed in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EDTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, protease inhibitors cocktail (Pierce). Cell lysates were centrifuged and supernatants incubated with mouse antiFlag M2 antibody (4 μg/mL) and Protein A/G-Sepharose beads overnight at 4 C. Flag-tagged Akt immunocomplexes were washed twice with lysis buffer and twice with kinase reaction buffer [25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na3VO4, 1 μg/mL leupeptin]. Immunocomplexes were next incubated in kinase buffer supplemented with ATP (0.2 mM) and GSK3β fusion protein (10 μg/mL, Cell Signaling Technologies) for 30 min at 30 C. The reaction was terminated by adding 2× Laemmli buffer. Samples were analyzed by Western blotting to asses GSK3β fusion protein phosphorylation using an anti-phosphoSer9-GSK3β antibody. In the same samples total Flag-Akt1 or Flag-Akt2 was determined by western blotting using antiFlag antibody.

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2. Gonzalez E, McGraw TE (2009) Insulin-modulated Akt subcellular localization determines Akt isoform-specific signaling. *Proc Natl Acad Sci USA* 106:7004–7009.
3. Frescas D, Valenti L, Accili D (2005) Nuclear trapping of the forkhead transcription factor FoxO1 via Sirt-dependent deacetylation promotes expression of glucogenetic genes. *J Biol Chem* 280:20589–20595.
4. Karylowski O, et al. (2004) GLUT4 is retained by an intracellular cycle of vesicle formation and fusion with endosomes. *Mol Biol Cell* 15:870–882.
5. Zeigerer A, et al. (2002) GLUT4 retention in adipocytes requires two intracellular insulin-regulated transport steps. *Mol Biol Cell* 13:2421–2435.

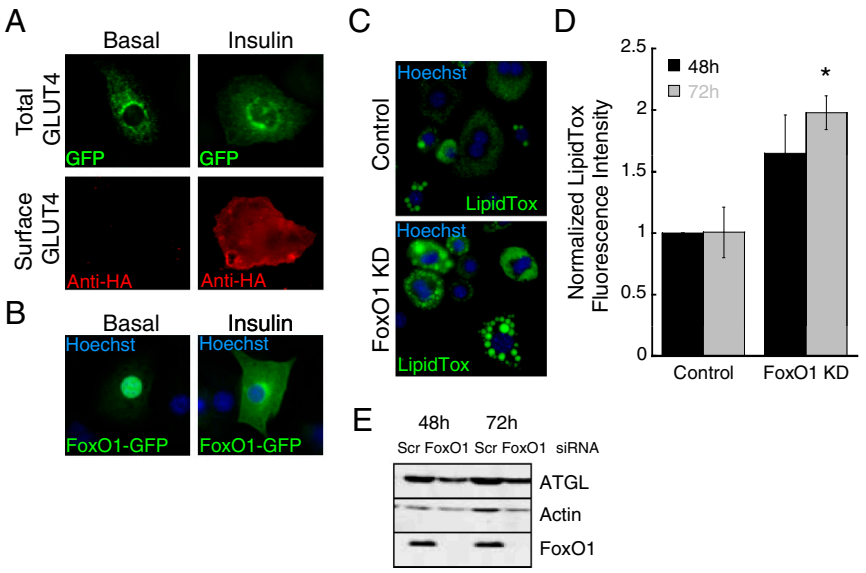

Fig. S1. Insulin regulates endogenous FoxO1 in adipocytes. (*A*) Micrographs of 3T3-L1 adipocytes expressing HA-GLUT4-GFP. Total HA-GLUT4-GFP was detected by GFP fluorescence and surface HA-GLUT4-GFP was measured by indirect immunofluorescence of the HA epitope (4, 5) Insulin: 1 nM Insulin for 30 min. (*B*) Micrographs of 3T3-L1 adipocytes expressing FoxO1-GFP (3). (*C*) Micrographs of control and FoxO1 KD 3T3-L1 adipocytes stained with LipidTox Green neutral lipid stain. Note that FoxO1 KD adipocytes displayed enhanced LipidTox neutral lipid staining. (*D*) Quantification of neutral lipid accumulation on 3T3-L1 adipocytes 48 h or 72 h after electroporation with control or FoxO1 specific siRNA. Neutral lipids were revealed using LipidTox neutral lipid stain and the amount of neutral lipid accumulation per cell quantified using quantitative fluorescence microscopy. Each point represents the mean ± SE of three experiments in which approximately 200 cells per experiment were quantified. (*E*) Immunoblot analyses of lysates from adipocytes electroporated with scrambled (Scr) or FoxO1 specific siRNA. Cell extracts were prepared 48 or 72 after electroporation.

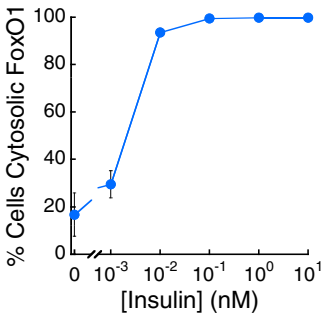

Fig. S2. Dose response for insulin-induced endogenous FoxO1 nuclear exclusion in 3T3-L1 adipocytes. Endogenous FoxO1 was detected by indirect immunofluorescence using an anti-FoxO1 antibody. Cells were scored for the presence of nuclear or cytosolic FoxO1 and results are expressed as the percentage of cells with cytosolic FoxO1. Each data point represents the mean ± SD from 2–3 experiments.

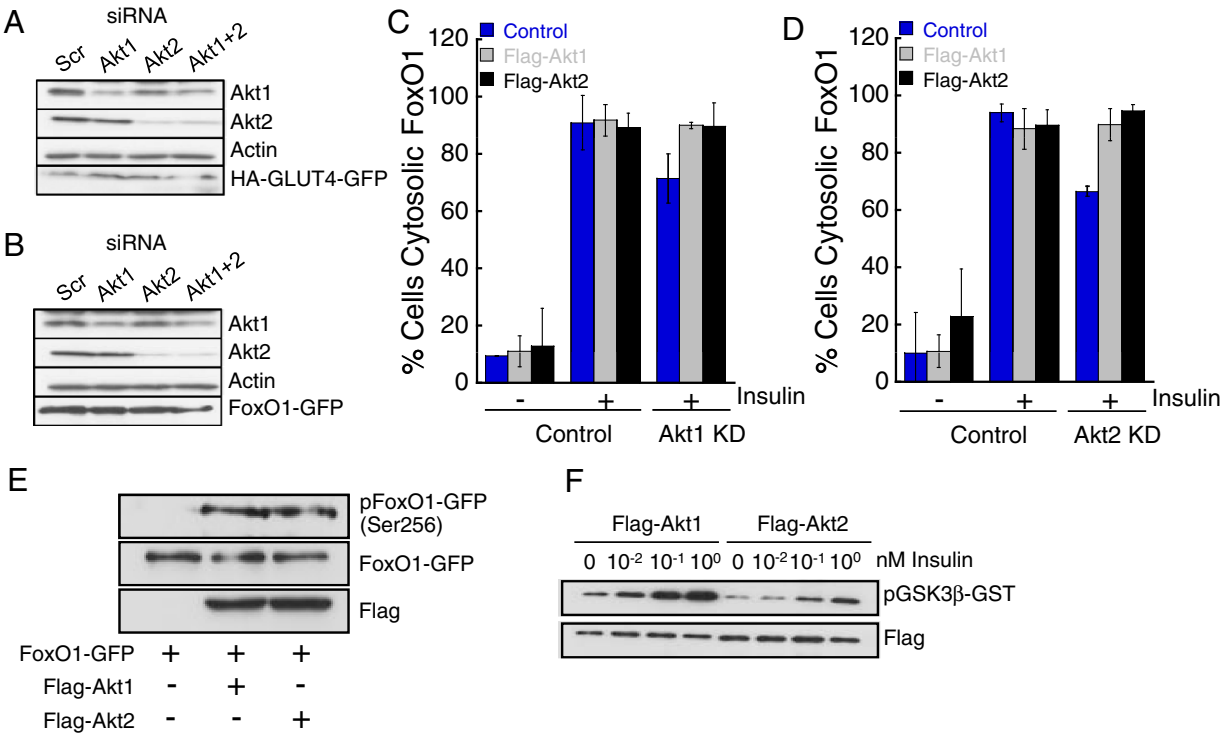

Fig. S3. Akt1 and Akt2 signaling regulates FoxO1 nuclear exclusion in adipocytes. Immunoblot analyses of Akt1 and Akt2 expression in 3T3-L1 adipocytes electroporated with control, Akt1, Akt2 and Akt1+Akt2 siRNA in combination with cDNAs encoding HA-GLUT4-GFP (*A*) or FoxO1-GFP (*B*). (*C*) Overexpression of Flag-Akt1 or Flag-Akt2 rescues Akt1 KD induced defect on insulin regulated FoxO1 nuclear exclusion. Adipocytes stably expressing Flag-Akt1 or Flag-Akt2 were generated via retroviral transduction which resulted in the stable expression of exogenous Flag-Akt1 and Flag-Akt2 at similar expression levels than endogenous Akt1 and Akt2 (2). Adipocytes expressing Flag-Akt1 or Flag-Akt2 were electroporated with control or Akt1 siRNA as noted, and the percentage of cells with cytosolic FoxO1 was measured in basal or 1nM insulin treated cells. (*D*) Overexpression of Flag-Akt1 or Flag-Akt2 rescues Akt2 KD induced defect on insulin regulated FoxO1 nuclear exclusion. Adipocytes stably expressing Flag-Akt1 or Flag-Akt2 as described in *C* were electroporated with control or Akt2 siRNA as noted and the percentage of cells with cytosolic FoxO1 was measured in basal or 1 nM insulin treated cells. Each data point represents the mean ± SD, $n = 2$. (*E*) Akt1 and Akt2 overexpression by electroporation leads to FoxO1 phosphorylation. Adipocytes were electroporated with cDNAs encoding FoxO1-GFP alone or in combination with Flag-Akt1 or Flag-Akt2. The phosphorylation state of FoxO1-GFP was measured by Western blot using a phospho-Ser256FoxO1 specific antibody. Flag-Akt1 and Flag-Akt2 expression was more than 10-fold higher than the endogenous kinases. (*F*) Dose response for insulin-induced Flag-Akt1 and Flag-Akt2 in vitro catalytic activity. Adipocytes stably expressing Flag-Akt1 or Flag-Akt2 were stimulated with insulin for 15 min as noted. Flag-tagged Akt1 or Akt2 were immunoprecipitated with an anti-Flag antibody and the in vitro kinase activity measured using a GSK3β fusion peptide as substrate as described in *SI Text*. The phosphorylated fusion protein was detected using an anti-phosphoGSK3β specific antibody. Total levels of Flag-Akt1 and Flag-Akt2 in the reaction sample were determined using an anti-Flag antibody. The data are representative of three independent experiments.