Supporting Information

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SI Methods

Antibodies and siRNA- Antibodies against phospho-Akt (Ser^{473} and Thr ^{308}), pGSK3α/β (Ser 21/9), Akt, Akt2, were from Cell Signaling Technologies (Beverly, MA). Antibodies against Akt1 and protein A/G PLUS agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse and rabbit anti-Flag epitope antibody was from Sigma-Aldrich. Anti-AS160 and anti-phosphoAS160 (Thr-642) antibodies were from Upstate Biotech. Mouse anti-HA epitope monoclonal antibody was purified from ascites (Covance, Berkley, CA) by using a protein G affinity column (Amersham Bioscience, Uppsala, Sweden). Fluorescent secondary antibodies were purchased from Jackson Immunolabs, Inc. (West Grove, Pennsylvania) and Invitrogen. The targeting sequences used to knockdown murine Akt isoforms were Akt1: CCCAGAACAATTAGATTCATGTAGA; Akt2: CACAGGACACAAGCATGGCCGTCAT; control: ATGTCACGGCGTACGTTCCTGTGTG. Stealth siRNAs were purchased from Invitrogen.

Molecular Cloning. Full length wild type mouse Akt1 and Akt2 cDNAs were subcloned into the vector pEGFP-N1 (BD Clontech Bioscience) by using the EcoRI/BamHI sites to obtain C terminus eGFP tagged Akt1 and Akt2. Full length wild-type mouse Akt1 and Akt2 cDNAs were subcloned into the vector Flag-CMV10 (Sigma) by using the sites HindIII/BamHI to obtain N terminus Flag tagged Akt1 and Akt2. The mutants E17K Akt1 and Akt2 were generated by site directed mutagenesis from the templates GFP or Flag tagged Akt1 and Akt2 cDNAs using the Quickchange system (Stratagene). The following Akt fragments were generated by PCR amplification from Akt1 and Akt2 cDNAs and subcloned into pEGFP-N1 vector: PHL-Akt1-GFP (Akt1, amino acid 1–149); PHL-Akt2-GFP (Akt2, amino acid 1–151); Akt12-GFP (Akt1 amino acid 1–149 fused to Akt2 amino acid 152–481); Akt21-GFP (Akt2 amino acid 1–151 fused to Akt1 amino acid 150–480). The chimeras Akt12 and Akt21 were also subcloned into the vector Flag-CMV10 to obtain N terminus Flag-tagged Akt12 and Akt21. N terminus Flag-tagged Akt1, Akt2, Akt12, Akt21 cDNAs were amplified from the FlagCMV10 backbone and subcloned into the retroviral vector pBABE-Hygro using the sites BamHI/EcoRI. All construct sequences were verified by automated DNA sequencing.

Cell Culture, Adipocyte Differentiation and Retroviral Infection and Electroporation. 3T3-L1 fibroblast were cultured, differentiated into adipocytes and electroporated as described in ref. 1. All experiments were performed on day 5 or 6 after differentiation. Akt constructs were subcloned into the retroviral vector pBABE-Hygro and used to create stably expressing 3T3-L1 cells. For transient Akt knockdown, 3T3-L1 adipocytes were electroporated with siRNA (4 μM), and experiments were performed 48 h after electroporation.

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 37C in 5%CO_2/air for at least 2h before 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 Laemmli buffer. Cells were harvested by scraping and cell lysates were sheared through a Q26G5/8 syringe. For immunoblot analyses cellular proteins were resolved in SDS/PAGE, transferred to nitrocellulose membranes, and probed with antibodies using protocols provided by the suppliers. For immunoprecipitation, 3T3-L1 adipocytes expressing Flag-tagged Akt1 or Akt2 were starved for 2 h followed by stimulation with 10 nM insulin. Cells were harvested by scrapping in 50 mM Tris-HCl, pH 7.4, NaCl 150 mM, 1% Igepal, 0.25% sodium deoxycholate, 1 mM EDTA, 2 mM sodium orthovanadate, 1 mM NaF and protease inhibitors mixture (Pierce) and centrifuged at 10,000 $\times$ g for 15 min at 4 °C. Aliquots of cell homogenates were incubated with M2 monoclonal antibody against the Flag epitope (4 μg/ml) for 1 h at 4 °C. Protein A/G-Sepharose beads were added to the supernatant, incubated overnight at 4 °C and washed extensively with lysis buffer. Bound immune complexes were eluted by boiling in Laemmli sample buffer, resolved by SDS/PAGE on 10% gels, and transferred onto nitrocellulose membranes. Immunoblots were probed with primary antibodies using protocols provided by the suppliers; antidoby biding was detected using enhanced chemiluminescence (Supersignal West Pico, Thermo Scientific). Densitometric analyses of the immunoblots were performed using a ChemiImager 4000 (Alpha-Innotech).

In Vitro Akt Activity Assay. 3T3-L1 adipocytes expressing Flag-tagged Akt constructs were starved in SF-DMEM for 2–4 h. After insulin stimulation, 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 mixture (Pierce). Cell lysates were centrifuged and supernatants incubated with mouse anti-Flag M2 antibody (4 μg/ml) and Protein A/G-Sepharose beads overnight at 4C. 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 2x 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 Akt, phospho-T308Akt and phospho-S473Akt were determined by Western blot analysis using specific antibodies.

Fluorescence Quantification. Fluorescence microscopy was performed using a DMIRB inverted microscope (Leica Microsystems, Deerfield, IL), with a cooled charge-coupled device camera (Princeton Instruments, Trenton, NJ). Images were collected with a 40 $\times$ 1.25 numerical aperture oil-immersion objective. MetaMorph software (Universal Imaging, West Chester, PA) was used for image processing and quantification as described in ref. 2. Confocal microscopy was performed using a Nikon TE2000 spinning disk confocal microscope equipped with a charge-coupled device camera (Hamamatsu). Images were collected with a 60X oil-immersion objective (N.A.1.49). Improvision Volocity acquisition and analysis software and MetaMorph software were used to collect and process the images and pictures were assembled using Adobe Illustrator. The colocalization of Akt and AS160–4P was determined using MetaMorph software. Images were threshold to account for total Akt and AS160 fluorescence. Four different confocal planes were analyzed per cell and the average Akt-AS160 colocalization values

were determined. Values shown are the average of $n = 7–20$ cells.

Total Internal Reflection Fluorescence (TIRF) Microscopy. For live cell TIRF microscopy studies, cells were allowed to equilibrate on the microscope stage equipped with a temperature controlled objective at 37 °C for 30 min. Videos were acquired at a rate of one frame per minute. To quantify the insulin-induced recruitment of Akt constructs to the vicinity of the PM, TIR fluorescence in each frame was quantified, background was subtracted and values were normalized to TIR fluorescence at time 0 and plotted as a function of time. In some cases epifluorescence and TIRF images of cells in basal conditions were acquired. The GFP fluorescence in the TIRF mode was divided by the GFP epifluorescence intensity, normalizing the TIRF fluorescence for the total GFP tagged-Akt construct expressed per cell. To measure Akt accumulation to the PM in adipocytes stably expressing Flag-Akt1 or Flag-Akt2 cells were stained with an anti-Flag antibody followed by secondary antibody conjugated to Alexa488. The anti-Flag fluorescence in the TIRF mode was divided by the anti-Flag epifluorescence intensity. Images were corrected for background fluorescence measured in cells that did not express Flag-Akt constructs.

Movies were generated using MetaMorph software and edited in Adobe After Effects software.

1. Zeigerer A, et al. (2002) GLUT4 retention in adipocytes requires two intracellular insulin-regulated transport steps. *Mol Biol Cell* 13:2421–2435.
2. Lampson MA, Schmoranzer J, Zeigerer A, Simon SM, McGraw TE (2001) Insulin-regulated release from the endosomal recycling compartment is regulated by budding of specialized vesicles. *Mol Biol Cell* 12(11):3489–3501.

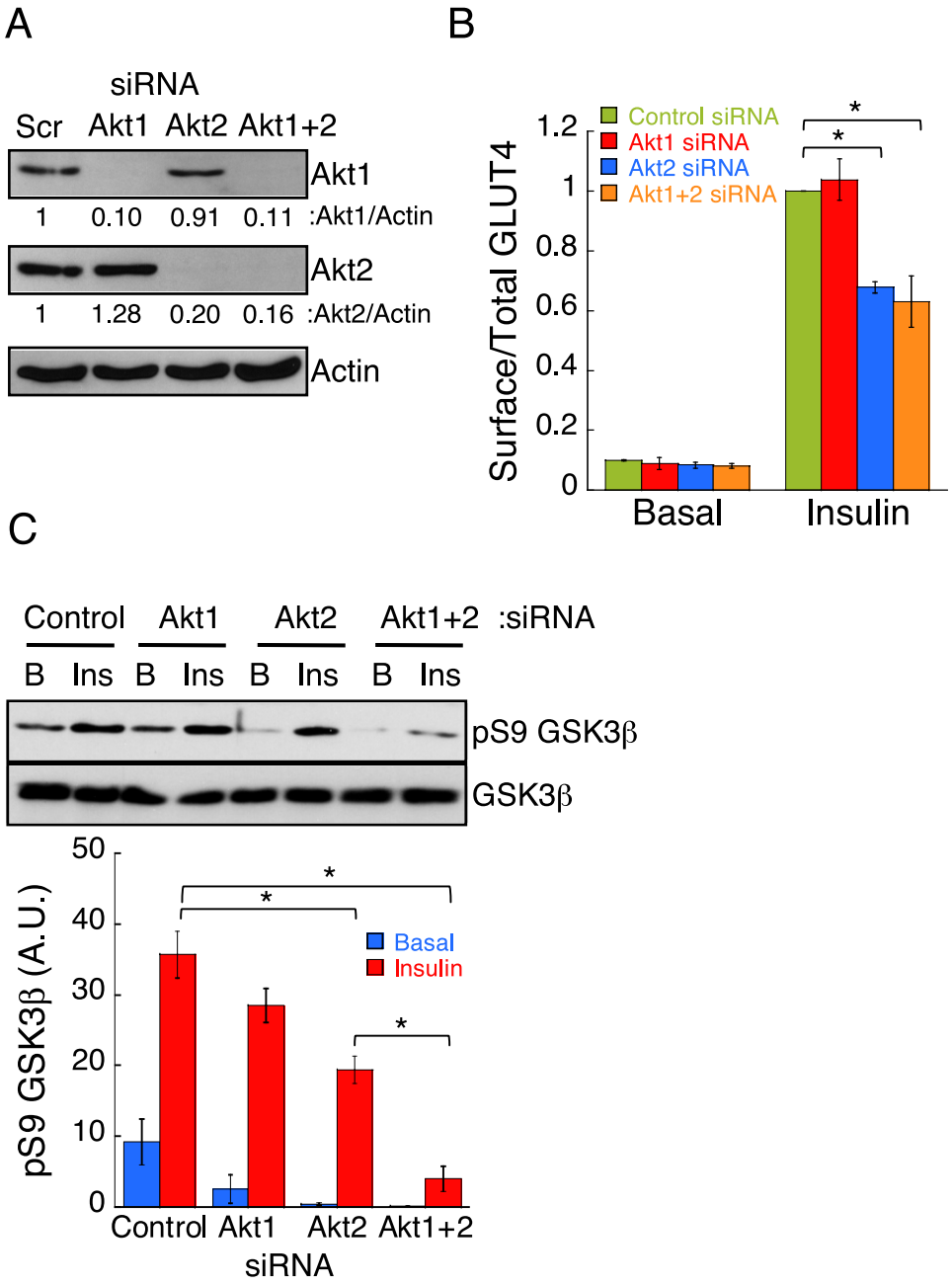


Fig. S1. Akt2 regulates insulin-induced GLUT4 translocation in 3T3-L1 adipocytes. (*A*) Adipocytes were electroporated with control, Akt1-, Akt2- or Akt1+Akt2-specific siRNAs and blotted for expression of Akt1, Akt2 and actin 48 h after electroporation. Annotated are the expression levels of Akt isoforms normalized to actin expression levels, average values from 3 independent measurements. (*B*) Surface-to-total distribution of HA-GLUT4-GFP in control, Akt1 or Akt2 siRNA electroporated adipocytes. The surface-to-total GLUT4 distribution was normalized to that of 1 nM insulin-stimulated control cells. Each bar is the mean ± SE, $n = 3–5$. *, $P < 0.01$ (ANOVA). (*C*) *Top*, immunoblot of GSK3β phosphorylation in control and Akt knockdown adipocytes. B, basal, and Ins, insulin, 10 nM for 15 min. *Bottom*, densitometric analyses of GSK3β phosphorylation. Each data point represents the mean ± SE of 3 independent experiments. *, $P < 0.05$ (ANOVA).

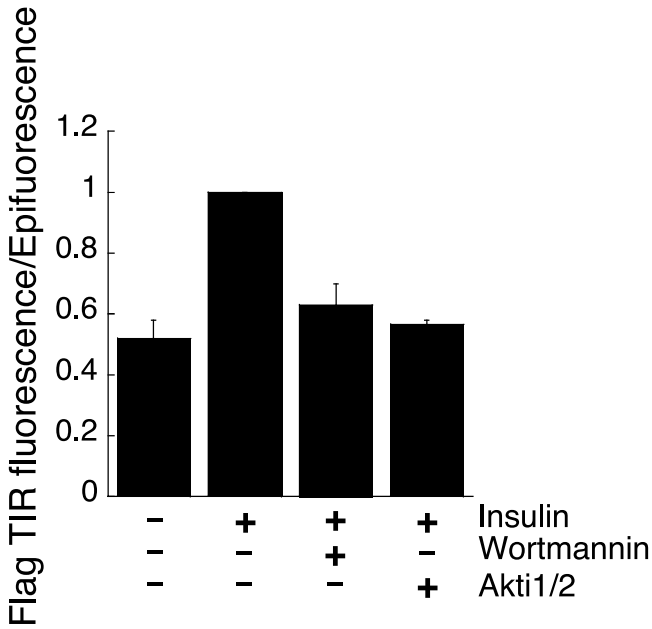


Fig. S2. Insulin-induced Flag-Akt2 redistribution to the plasma membrane is inhibited by wortmannin and the Akt inhibitor Akti1/2. 3T3-L1 adipocytes expressing Flag-tagged Akt2 were incubated with wortmannin 100 nM, the Akt inhibitor Akti1/2 1 μM or vehicle for 30 min followed by stimulation with 10 nM insulin for 30 min. Cells were fixed and stained with an anti-Flag epitope antibody. Total indirect immunofluorescence of the Flag epitope was measured in the epifluorescence mode and in the TIRF mode. The TIR fluorescence is normalized to the anti-Flag fluorescence in the epifluorescence mode. Each data point represents the mean $\pm$ SE from 2 independent experiments.

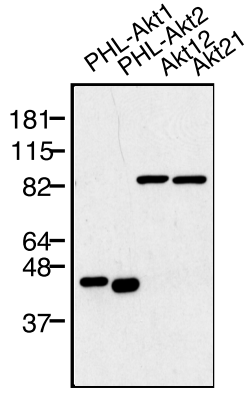


Fig. S3. Immunoblot analyses of cell extracts from 3T3-L1 adipocytes electroporated with PHL-Akt1-GFP, PHL-Akt2-GFP, Akt12-GFP and Akt21-GFP using an anti-GFP antibody.

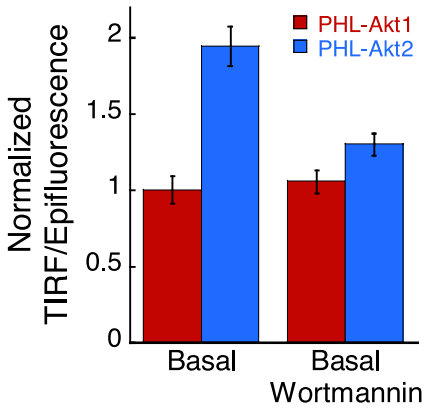
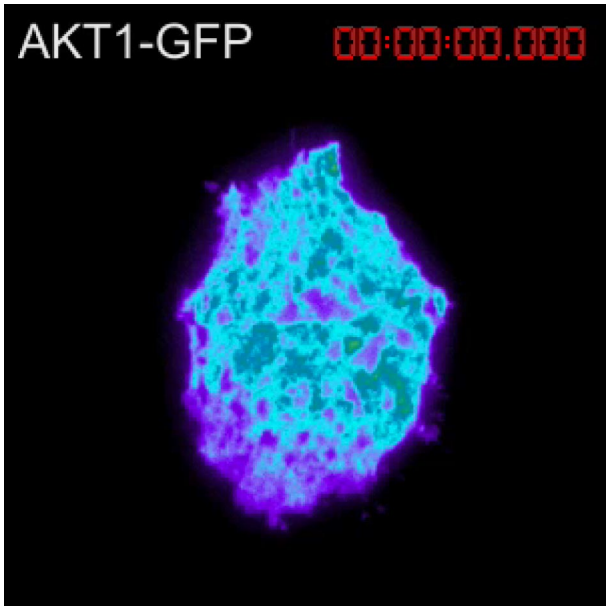
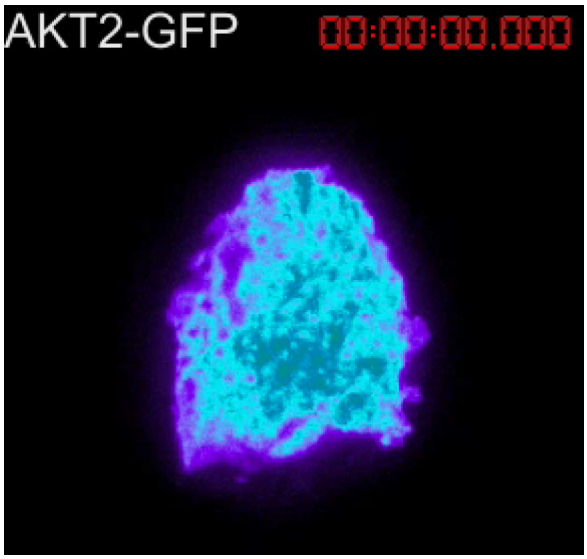


Fig. S4. Differences in PHL-Akt1 and PHL-Akt2 PM accumulation are inhibited by wortmannin. Quantification PHL-Akt1-GFP and PHL-Akt2-GFP TIRF versus epifluorescence in live adipocytes in basal conditions in the presence or absence of 100 nM wortmannin. The data are normalized to the PHL-Akt1-GFP TIRF/Epifluorescence ratio. Each data point represents the mean ± SE from 50 cells.



Movie S1. Video time-lapse TIRF microscopy of Akt1-GFP in 3T3-L1 adipocytes. The videos were acquired at a rate of one frame per minute. Insulin (10 nM) was added as noted. The movie is displayed at 5 frames/sec. Fluorescence intensity was normalized to that of the start frame of the image sequence.

Movie S1



Movie S2. Video time-lapse TIRF microscopy of Akt2-GFP in 3T3-L1 adipocytes. The videos were acquired at a rate of one frame per minute. Insulin (10 nM) was added as noted. The movie is displayed at 5 frames/sec. Fluorescence intensity was normalized to that of the start frame of the image sequence.

Movie S2