Supplementary Information

Supplemental Materials and Methods

Supplemental Materials and Methods

Animals: All experimental protocols involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Yale University School of Medicine prior to study initiation. Male Sprague-Dawley rats weighing 160 g were obtained from Charles River Laboratories. Rats received free access to food and water and were housed with 12 h light/dark cycles at 23°C.

Antisense Oligonucleotide (ASO) Knockdown and Diets: 2'-O-methoxyethyl chimeric antisense oligonucleotides were synthesized and screened as described (1). Rats received either control nonspecific ASO (5′- CCTTCCCTGAAGGTTCCTCC-3′) or ASO targeting PKCε (5′-CCTTCCCTGAAGGTTCCTCC-3′) by intraperitoneal injection at a dose of 75 mg kg^{-1} wk^{-1} for 4 weeks as described previously (2). Seven days before sacrifice, jugular venous and carotid artery catheters were placed. After 4 days of recovery, control rats were maintained on regular chow (Harlan TD2018: 18% fat, 58% carbohydrate, 24% protein) while study rats were switched to a high-fat safflower oil-based diet (Diets: 59% fat, 26% carbohydrate, 15% protein) supplemented with 6% *w/v* sucrose water for 3 days (2-4).

Animal Phenotyping: After 3 days of high-fat feeding, rats were fasted 6 h (0700-1300h). Jugular venous blood samples (2 ml) were collected, and used to determine basal plasma glucose (YSI Biochemistry Analyzer, Yellow Springs Instruments), insulin (radioimmunoassay, Millipore), and NEFA (NEFA-HR, Wako). Rats were anesthetized with pentobarbital sodium injection (150 mg kg^{-1}), and livers were taken within 1 minute, snap-frozen with liquid N_2–cooled aluminum tongs, and stored at –80°C for subsequent analysis.

The DAG extraction and analysis were performed as previously described (5).(5). DAG was extracted from cytosolic/lipid droplet and membrane-associated subcellular fractions and measured by LC/MS/MS. Total DAG content is expressed as the sum of individual species. Liver triglyceride was extracted by the method of Bligh and Dyer (6) and measured with Triglyceride-SL reagent (Sekisui).

Cell Lines and Reagents: S6K1-/-S6K2-/- MEFs (DKO) were generously provided by Dr. Carol Mercer and Dr. Sara Kozma. McArdle-7777 rat hepatoma cells were obtained from American Type Culture Collection (ATCC). Both lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, Life technologies) supplemented with 10% fetal bovine serum (FBS, Life Technologies). Cells were maintained at 37 °C with 5% CO2.

Lentiviral Transfection: Lentiviral particles were generated in HEK 293-T cells (ATCC) by co-transfecting 3rd generation lentiviral packaging plasmids (Addgene 12251, 12253, 12259) with WT PKCε (Addgene 10795), A159E PKCε (generated from 10795 by site-directed mutagenesis), or Myristolated PKCε (Myr-10797). After plating, cells were transfected with lentiviral particles containing WT, A159E, or Myr PKCε. After 48hrs, media was removed and replaced with serum-free DMEM with or without 1μM trametinib (Selleckchem) for 12hrs. Cells were treated with either DMSO or 1 μM Phorbol 12-myristate 13-acetate (PMA-Sigma) for 30min.

Western Blotting: Ground livers were lysed in buffer containing 10mM Tris-HCL pH 8.0, 1mM EDTA, 0.5mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140mM NaCl, protease inhibitor cocktail (cOmplete, Roche), and phosphatase inhibitor (Phosphatase Inhibitor

Cocktail 1, Sigma), sonicated, normalized using a Bradford Assay (Bio-Rad), and resolved by SDS-PAGE electrophoresis. Proteins were transferred to PVDF membranes using semi-dry transfer cells (Bio-Rad), and blocked in 5% milk.

Cell cultures were washed 1x with PBS, lysed in buffer containing 1% Triton x-100, 50mM Tris-HCl pH 7.4, 150mM NaCl, 5% Glycerol, 2.5mM EDTA, 2.5mM EGTA, 1mM DTT, protease and phosphatase inhibitors (cOmplete, Roche; Phosphatase Inhibitor Cocktail 1, Sigma), spun at 21,000g for 10 min at 4°C, mixed with SDS-PAGE loading buffer and resolved by electrophoresis.

PKCε activation/knockdown was determined as described previously (7), where lysates were spun at 100,000g for 1hr to separate membrane and cytosolic fractions, which were mixed with Laemmli buffer, resolved by SDS-PAGE, and transferred to PVDF membranes as described. Membranes were blotted for PKCε (Millipore) and translocation expressed as the membrane to cytosol ratio.

Target	**Manufacturer**	**Cat. #**	**Dilution**
Actin	Abcam	ab6276	1:10000
AKT pS473	Cell Signaling Technologies	4060	1:1000
GAPDH	Cell Signaling Technologies	5174	1:1000
IRS1 pS302	Cell Signaling Technologies	2384	1:1000
PKCε	BD Transduction Laboratories	610086	1:1000
PRAS40 pT246	Cell Signaling Technologies	13175	1:1000
RPS6 pS235/236	Cell Signaling Technologies	4858	1:1000
TSC2 pT1466	Cell Signaling Technologies	3617	1:1000

Phosphoproteomics: Phosphoproteomic analysis of protein extracts was performed as reported earlier (8) with the following changes. Dimethyl labeling of peptides was performed prior to phosphopeptide enrichment with TiO2. Furthermore, we introduced an additional ERLIC fractionation step to further separate TiO2 enriched phosphopeptides. In our hands, this

combination of phosphopeptide enrichment by TiO2 and fractionation by ERLIC provides superior performance compared with ERLIC fractionation alone (9).

Lysis and protein extraction: 50 mg of ground livers from each animal selected was lysed using 1mL Cytobuster (Millipore) supplemented with protease inhibitor (cOmplete, Roche), phosphatase inhibitor (Phosphatase Inhibitor Cocktail 1, Sigma), 14.3 mM β-mercaptoethanol, 50mM NaF, and 1mM $NaVO_4$, incubated for 45 minutes on ice, and sonicated (30s on 30s off for 4 cycles at 55 amps) on a pedestal sonicator. Protein was precipitated using methanol and chloroform as described (10), washed 1x with methanol, and dried under vacuum.

Digestion: An aliquot of 5 mg protein was reconstituted in 50 µLsample solubilization buffer consisting of 2.5 % acid labile detergent (ALS-110, Protea) 50mM Tris-HCL pH=8.5, 5mM EDTA, and 50 mM DTT. Protein was extracted by sonication on ice performed on an S-4000 sonicator (Misonix) using the following sonication program: 30s on, 30s off performed for 4 cycles at 55 amps). Samples were then diluted with ice cold 166.6 mM Tris-HCL pH=835 to reduce the detergent concentration to 0.83% and the solution was incubated for 35 min at 55°C in a heat block. Free cysteines were alkylated with freshly prepared iodoacetamide supplemented at a final concentration of 38.4 mM. The alkylation proceeded for 30 min at room temperature in the dark and excess iodoacetamide was quenched with DTT. Protein was digested for 4 h at 37°C with LysC (Wako) using an enzyme to protein ratio of 1:100.

The digest was diluted with a solution of 117.4 mM Tris-HCL pH 8.5 to obtain a detergent concentration of 0.05 % and the digest was supplemented with 1 M $CaCl_2$ solution to obtain a final CaCl2 concentration of 4 mM (after addition of trypsin). Digestion with sequencing grade

modified trypsin (Promega) was performed with an enzyme to protein ratio of 1:50 for 16 hrs at 37°C. To stop the digest, 20 % TFA was added resulting in a final sample pH of 2-3. Acid labile detergent was allowed to cleave for 15 min at room temperature and peptides were desalted with SepPak Classic SPE cartridges (Waters) following instruction provided. The desalted peptides were lyophilized and stored at -80°C until further use.

Dimethyl Labeling: Lyophilized peptides were reconstituted in 50% ACN .1 % TFA and concentration was estimated by A280 using a Nanodrop 2000 (Thermo). 16mg of peptides were labeled using reductive dimethylation as described (11) using $NaCH_3CN$ and CH_2O (light-8mg) or CD_2O (medium-8mg) in *100mM* MES buffer pH 5.5 (12), mixed 1:1, and desalted on SepPak Classic SPE cartridges. Samples were labeled in technical duplicate, but with switched heavy/light labels for a total of 4 samples. *Dimethyl Labeling:* Lyophilized peptides were reconstituted in 50% ACN .1 % TFA and concentration was estimated by A280 using a Nanodrop 2000 (Thermo). 16mg of peptides were labeled using reductive dimethylation as described (11) using $NaCH_3CN$ and CH_2O (light-8mg) or CD_2O (medium-8mg) in *100mM* MES buffer pH 5.5 (12), mixed 1:1, and desalted on SepPak Classic SPE cartridges. Samples were labeled in technical duplicate, but with switched heavy/light labels for a total of 4 samples. *Dimethyl Labeling:* Lyophilized peptides were reconstituted in 50% ACN .1 % TFA and concentration was estimated by A280 using a Nanodrop 2000 (Thermo). 16mg of peptides were labeled using reductive dimethylation as described (11) using $NaCH_3CN$ and CH_2O (light-8mg) or CD_2O (medium-8mg) in *100mM* MES buffer pH 5.5 (12), mixed 1:1, and desalted on SepPak Classic SPE cartridges. Samples were labeled in technical duplicate, but with switched heavy/light labels for a total of 4 samples. *Dimethyl Labeling:* Lyophilized peptides were reconstituted in 50% ACN .1 % TFA and concentration was estimated

by A280 using a Nanodrop 2000 (Thermo). 16mg of peptides were labeled using reductive dimethylation as described (11) using $NaCH_3CN$ and CH_2O (light-8mg) or CD_2O (medium-8mg) in *100mM* MES buffer pH 5.5 (12), mixed 1:1, and desalted on SepPak Classic SPE cartridges. Samples were labeled in technical duplicate, but with switched heavy/light labels for a total of 4 samples.

TiO_2 Enrichment: Phosphopeptide enrichment was performed by resuspending 8mg peptides in 50% ACN 2M lactic acid and iteratively incubating with Titansphere TiO_2 beads (GL Sciences) with a 10:1 TiO_2 to peptide ratio 3 times, eluting peptides from the beads 1x with 0.2M Sodium Phosphate pH 7.8, 2x with 5% NH_4OH, and 1x with 5% pyrrolidine and combining all elutions into 70% formic acid (13).(13).(13). Enriched phosphopeptides were desalted with C18 Microspin Columns (The Nest Group). Phosphopeptides were reconstituted in 85% ACN .1% formic acid, quantified, and separated on an PolyWAX LP column (150 x 1.0 mm, 5µm particle size, 300 Å pore size, PolyLC) using an 70min non-linear gradient from 85% ACN .1% formic acid to 30% ACN .1% formic acid (adapted from (14)) and 32 fractions were collected (2 min fractions for 30 min, 2-5 min fractions from 60-70min). Fractions were dried using vacuum centrifugation, and reconstituted for ERLIC.

Offline ERLIC Chromatography: TiO_2 enriched samples were reconstituted in 50% ACN 0.1% formic acid, and concentration was measured by A280 (Nanodrop, Thermo). Samples were further diluted to 85% ACN 0.1% formic acid. 50µg of peptides were injected onto a PolyWAX LP 150x1.0mm, 5µm, 300 Å resin column (PolyLC) and separated on a 70 min non-linear gradient (adapted from Hao et al. (14)) from 85% ACN 0.1% formic acid to 30% ACN 0.1% formic acid

at a flow rate of 50µl/min. 30 2-min fractions were collected across the entire gradient, with the addition of 2 5-min fractions at the end. Each sample was separated in triplicate on the ERLIC column for a total of 150ug. Fractions were dried in a rotary evaporator (Thermo) and reconstituted for LC-MS.

LC-MS/MS: Samples were separated using a NanoAquity (waters) HPLC with a vented split on a 30mm 150µM ID trap column with a Kasil frit packed with Magic C18AQ 3µM 200Å resin (Bruker) and a 20cm 75µM ID self-packed Pico-frit column (New Objective) packed with 1.9µM 120Å reprosil-Pur C18-AQ resin (Dr. Maisch) on a non-linear 120min gradient from 5% ACN 0.1% formic acid to 95% ACN 0.1% formic acid and analyzed with a LTQ Orbitrap Velos (Thermo) using a Top 10 method. ERLIC samples were run in duplicate, with the first ERLIC fraction of each set of 3 run separately, while the 2nd and 3rd fraction of each set were pooled for a single run. In addition, for each sample 2µg of the pre-enriched peptides and 2µg of the total (unfractionated) enriched phosphopeptides were run using a non-linear 200min gradient from 5% ACN 0.1% formic acid to 95% ACN 0.1% formic acid.

Bioinformatics: Data was searched using Maxquant version 1.5.1.2 (15) with Acetyl (N-Term), Deamidation (NQ), Oxidation (M), and Phospho(STY) as variable modifications and Carbamidomethyl (C) as a fixed modification with up to 3 missed cleavages, 5 AA minimum length, and 1% FDR against the Uniprot Rat database (Downloaded Dec 11, 2013). The pre-enriched, total enriched, and ERLIC fractionated peptides were combined into 1 search for each sample. Searches were analyzed with Perseus version 1.5.0.31. This analysis included removed of

contaminant and reverse protein hits, normalization of ratios (correction for H/L channels in label switched samples), log2 transformation, and averaging across technical (label-switch) replicates.

2-D enrichment analysis was performed by plotting the fold change in both comparisons against each other, and imposing a 2-fold change cutoff to separate phosphopeptides into designated categories (I-IX). Only peptides that had valid ratios in both comparisons were considered for further analysis. The pLogo tool (plogo.uconn.edu) (16) was used to perform motif analysis. Sequences were aligned to the central phosphorylated residue with +/- 15 amino acids on either site (where possible), duplicate peptide sequences were removed, and sequences were submitted to the tool using the Rat Protein Database as background. The pLogo tool fixes S as the central amino acid and calculates the probability of amino acid representation surrounding this site relative to all possible sites. Amino acids taller than the red line are considered significant.

Kinase Substrate Validation:

Candidate Kinase Substrate Selection: Of the 203 phosphosites observed in Categories II and III, we selected the 52 peptide sequences that had -3R for this library. We also selected the 72 peptides out of the 269 observed in Category VI that had +1P as a control group. In Category V we selected 50 peptides that had 0 fold change in both comparisons, and that had no elements of either the PKC motif (-3R) or the +1P motif surrounding the central phosphorylated residue to serve as additional controls.

Substrate Peptide Design: Predicted kinase substrate phosphosites were encoded as peptides at least 21 amino acids long. The predicted phosphorylatable Ser or Thr residue was positioned in the middle of the peptide (position 0) flanked on the N- and C-termini by the 10 amino acids

occurring in the native sequence of the corresponding rat protein. Ser at position 0 was encoded by an amber (TAG) codon, allowing incorporation of either Ser or phosphoserine, as described below. Thr at position 0 was encoded by a threonine sense codon. Gene fragments that did not contain any Lys or Arg codons in the N-terminal (positions -10 to -1) and/or C-terminal (positions +1 to +10) regions of the encoded peptides were extended to encompass the nearest native tryptic site found within the parent protein. Gene sequences were codon optimized for expression in K12 *E. coli* using IDT's codon optimization tool (https://www.idtdna.com/CodonOpt) and contained a 5' SfoI site and a 3' TAA stop codon followed by a HindIII site. These genes were synthesized as 400-750 base pair concatamers by Genewiz.

Cloning of Library Members: Gene libraries were inserted into a modified pCRT7/NT-TOPO vector(17), but with tetR and pLtetO elements replaced with the araC gene and a multiple cloning site for arabinose-based protein induction.(17), but with tetR and pLtetO elements replaced with the araC gene and a multiple cloning site for arabinose-based protein induction.(17), but with tetR and pLtetO elements replaced with the araC gene and a multiple cloning site for arabinose-based protein induction. A cassette was introduced in the multiple cloning site encoding GST, a human rhinovirus protease 3C cleavage site, SfoI and HindIII restriction sites, and a TAA stop codon. This plasmid and sequence information is available from Addgene as plasmid #734456. Genes inserted between the SfoI and HindIII sites can therefore be expressed as a fusion protein with an N-terminal cleavable GST tag. The plasmid and synthetic DNA were digested with SfoI and HindIII, and ligation was performed with mixed insert populations. Plasmid libraries were separately generated for each group of predicted kinase substrates (i.e. predicted CDK, PKC, and negative control substrates were each encoded in separate libraries). Ligation reactions were

transformed into ElectroMAX™ DH10B™ *E. coli* cells (Invitrogen) and transformation mixtures were plated in serial dilutions in triplicate to obtain countable colonies. <500 cfu are necessary for a 95% probability that 100% of library members are represented; approximately 100,000 cfu in the -3R predicted substrate library and 1,000,000 cfu in the +1P and negative control libraries were obtained. Plasmids harvested from these cells were then introduced into genomically recoded C321.ΔA.ΔserB *E. coli* including either a plasmid containing the serine tRNA amber suppressor supD or SepOTSλ, allowing Ser or phosphoserine incorporation at amber codons, respectively.(18, 19)

Gene Expression and Purification: Electroporated cells were transferred to 100 mL LB + 50 ug/ul ampicillin + 25 ug/ul kanamycin and incubated shaking at 30°C overnight. Cultures were back in the morning into 2L of LB + 50 ug/mL ampicillin + 25 ug/mL kanamycin with an OD=0.15 and then incubated at 30°C until OD=0.6-0.8, after which cultures were induced by adding 1 mL 20% arabinose (0.04% final concentration) to each culture and incubated at 30°C for three hours. Cells were pelleted by centrifugation at 4000g for 1 min, transferred to 50mL conical tubes, and pelleted by centrifugation at 4000g for 20 min. Supernatant was removed pellets were stored at -80°C.

Lysis was achieved by adding lysis buffer (1 X Stock buffer [50 mM Tris/HCl, pH 7.5, 500 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 10% glycerol], 1 mM DTT, 1 mg/mL lysozyme, 50 mM NaF, 1 mM $NaVO_4$, 1 protease inhibitor tablet [cOmplete, Roche]), incubated on ice for 30min, and sonicated (1.5 minutes total, pulse: 10 sec, off: 40 sec). Lysates were spun down lysate at 22000g for 15 minutes at 4°C.

GST purification was performed by equilibrating 2mL Hi-Cap GST resin (Qiagen) with lysis buffer 3x, then incubating lysates with the resin for 90min on a rotisserie shaker at 4°C. After

lysate was removed, the resin was washed with 5mL wash buffer (1X Stock buffer [50 mM Tris/HCl, pH 7.5, 500 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 10% glycerol], 1 mM DTT, 50 mM NaF, 1 mM $NaVO_4$), and transferred to a 5mL gravity column. After mobile phase was drained, an additional 5mL of wash buffer was allowed to pass through the column. Proteins were eluted with 3x1 mL elution buffer (1X Stock buffer [50 mM Tris/HCl, pH 7.5, 500 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 10% glycerol], 1 mM DTT, 50 mM NaF, 1 mM $NaVO_4$, 50 mM reduced glutathione, pH 8).

Eluates were buffer exchanged using a 10 kDa cutoff spin column (Amicon) according to manufacturer's recommendations into 1X storage buffer (50 mM Tris, pH 7.4, 150 NaCl, 20% glycerol) three times. Storage buffer was added up to 500 ul total volume, after which GST was cleaved by addition of 10ul PreScission protease (GE Healthcare) and incubated overnight at 4°C on rotisserie shaker.

Kinase substrate peptides were purified from GST by using a 30 kDa cutoff spin column (Amicon) according to manufacturer's protocol. The flow through (containing the substrate peptides) was concentrated on a 3 kDa cutoff spin column (Amicon) to a final volume of 100 ul. Protein concentration was determined by BCA assay (Pierce).

*In V*itro *Kinase Reactions:* PKCε and its lipid activator were acquired from Millipore. PKCε was used at 0.5 ng/µl final concentration (25ng/50µl reaction). Kinase reactions were performed in 50µl containing 5µl Kinase Assay Buffer I (Signal Chem), 10µg of purified substrate peptide library, 5µl of 10s 250 uM ATP mix (50 uM ATP final concentration), diluted kinase, 5µl of lipid activator, and ddH_2O to 50µl. Reactions were incubated for 30 min at 30°C.

Sample Workup: Samples were desalted using C18 Ultramicrospin columns (The Nest Group) according to manufacturer's protocol, and dried on a rotary evaporator (Thermo).

Samples were reconstituted in ALS-110 (Protea) and digested with Trypsin (Promega) as described previously (20).(20) Briefly, peptides were solubilized with solubilization buffer (50 mM Tris buffer pH=8.5, 5 mM EDTA, 50 mM DTT and 2.5 % ALS-110), reduced for 35 min at 55°C, and alkylated with iodoacetamide. Samples were diluted to an ALS-110 concentration of <0.1% and digested with 3ug of trypsin (Promega) for 16 hours at 37°C. Digests were desalted with C18 Ultramicrospin columns (The Nest Group) and dried on a rotary evaporator (Thermo).

Peptides were reconstituted in 3:8 70% formic acid: 0.1% TFA; $1/5^{th}$ of the sample was placed in an HPLC vial and diluted to 7µl with 3:8:1 70% formic acid: 0.1% TFA:50 mM sodium phosphate pH 7.4 for Mass Spectrometry. The remaining sample was dried, and enriched using a TiO_2 stage tip.(21, 22) Briefly, tips were prepared by placing 0.6mm diameter punches of Empore C18 (3M) discs into a standard 200µl pipette tip. TiO_2 (Titanshpere, GL Science) was activated by washing with 50% ACN with 0.5% TFA 3 times, and 400µg (in a slurry) was loaded into the tip. Peptides were resuspended in 50% ACN 0.5% TFA, loaded into the tip, and spun through. Tips were washed 2x with 50% ACN 0.5% TFA, and 1x with 80% ACN 0.1% formic acid. Peptides were eluted with 1% NH_4OH followed by 80% ACN 0.1% formic acid into a tube containing 70% formic acid. Eluates were dried on a rotary evaporator (Thermo), and reconstituted in 3:8:1 70% formic acid: 0.1% TFA:50 mM sodium phosphate pH 7.4 for Mass Spectrometry.

LC-MS/MS: Samples were analyzed as described above, except that peptides were separated on a non-linear 90min gradient from 5% ACN 0.1% formic acid to 95% ACN 0.1% formic acid.

Bioinformatics: Data was searched as described above against the EcoCyc *E. coli* database (v17) and a custom database containing all 204 GST-tagged kinase substrate peptides. Results for each kinase assay from both the unenriched and enriched Mass Spec runs were combined and analyzed manually in Excel.

siRNA Screen: All screening was performed at and in collaboration with the Yale Center for Molecular Discovery

Cell Lines and Reagents: Lipofectamine RNAiMAX and poly-D-lysine were purchased from ThermoFisher Scientific and Sigma Aldrich, respectively. Insulin (Sigma) was purchased as a dry powder, diluted in water to 10 mg/mL stock concentration, and stored at 4°C. Dharmacon siGENOME rat siRNAs were obtained from GE Healthcare as SMART pools of four siRNAs per gene. All siRNAs were purchased lyophilized and diluted in 1x siRNA buffer (ThermoFisher Scientific).

Transfection, Serum Starvation and Insulin Stimulation: 384-well cell culture microplates (Greiner Bio-One International, catalog number 781091) were coated with 0.1 mg/ml poly-D-lysine for 1 hour, washed with Phosphate Buffered Saline (PBS), and allowed to air-dry prior to cell seeding. Cells were plated at 1,000 cells per well, grown for 24 hours, and subjected to forward transfection with 20 nM siRNA using lipofectamine RNAiMAX according to manufacturer's protocol. 48-hours post-transfection, cells were washed with PBS, and media was changed to serum-free

DMEM. Cells were serum-starved for 12 hours, and then stimulated with 1 nM or 10 nM insulin for 30 minutes. Following stimulation, cells were fixed and subjected to immunofluorescence.

Immunofluorescence: Cells were fixed with 4% paraformaldehyde (PFA, Electron Microscopy Sciences) in PBS at room temperature for 20 min, permeabilized with 0.5% Triton X100 in PBS at room temperature for 5 minutes, and blocked with 2% Bovine Serum Albumin (BSA) in PBS at room temperature for 1 hour. Primary antibodies (AKT pS473, Cell Signaling 4060P) were diluted 1:1000 in 2% BSA in PBS and used to stain cells for 2 hours at room temperature. The secondary antibodies used were Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes). Cells were co-stained with the nucleic acid dye Hoechst 33342 to visualize the nuclei.

Imaging and image analysis: Cells were imaged using the InCell 2200 Imaging System (GE Corporation). Automated image analysis protocol for the quantification of pS473 AKT signal intensity was developed using the InCell Analyzer software (GE Corporation). Nuclei were segmented based on the nuclear HOECHST staining channel, and cytoplasm was identified based on pS473 AKT fluorescence channel. Mean cytoplasmic pS473 AKT intensity was quantified for all cells. Intensity threshold was defined based on cell-level distributions of the mean pS473 AKT intensity in unstimulated RISC-free negative control samples and RISC-free samples treated with 10 nM insulin. Cells with pS473 AKT intensity above the selected threshold were defined as positive cells; percent of positive cells per well was quantified as a main assay readout.

Screening Data analysis: Each screening plate contained sixteen negative control wells (RISC-free siRNA) and sixteen positive control wells (INSR siRNA).To evaluate the robustness of the

screen, Z' factors were calculated from the mean signals of the positive and negative controls (μc+ and μc–) and their standard deviations (σc+ and σc–) for each plate using the formula Z' = 1 – [3(σc+ + σc–)/|μc- – μc+|]. Signal to background ratio (S/B) was calculated as a ratio of the mean intensity of the negative control over mean intensity of the positive control (μc- / μc+). Primary screening data was analyzed using the commercial software and database package ActvityBase (IDBS). The effect of each library siRNA for the inhibitor screen (10nM insulin) was calculated as the normalized percent effect using the following formula: percent effect = [(sample - μc_)/(μc+ - μc_)]*100. For the activator screen (1nM insulin), hits were determined by having greater than 150% of the RISC-free control siRNA signal. Histograms of normalized data for the entire screening population were plotted using the JMP software (SAS).

Supplemental Figures:

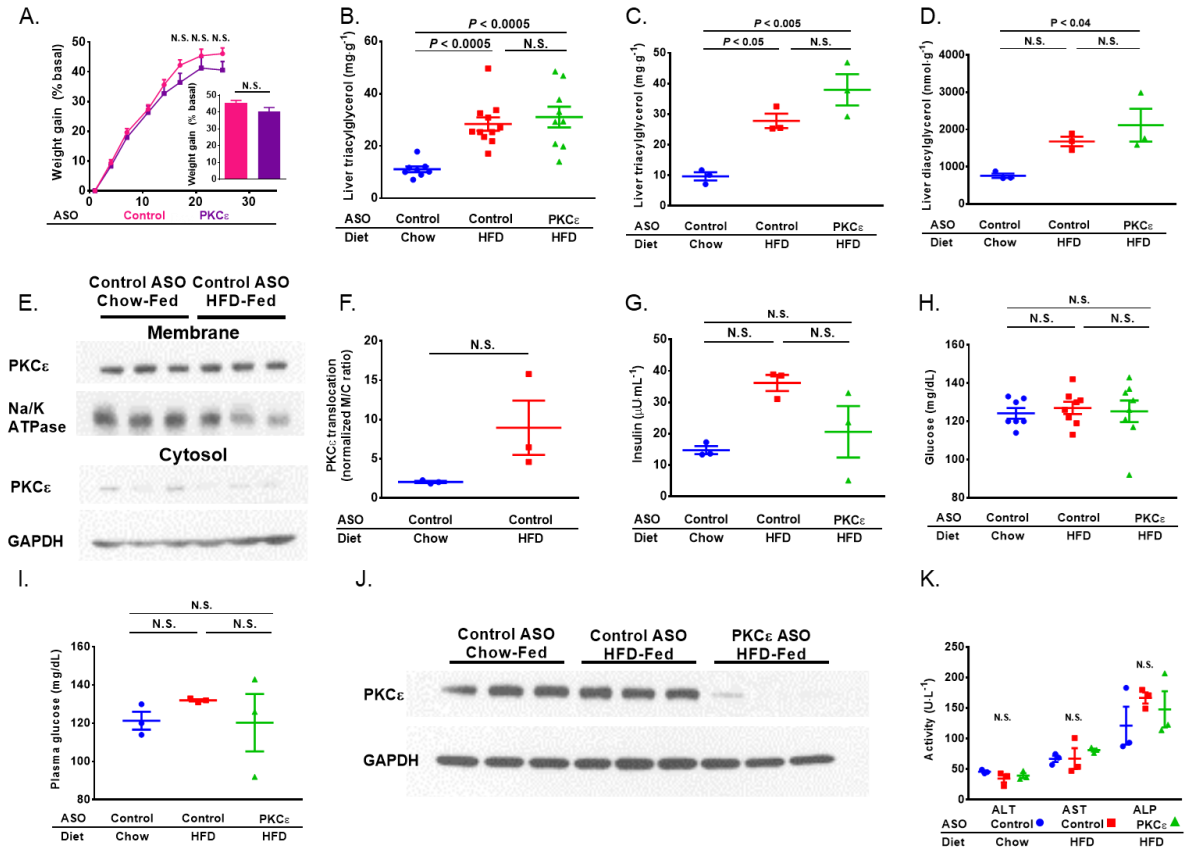


Figure S1: Phenotyping of Animal Model. (A) Percent weight gain over the 4 week course of ASO treatment; total weight gain as a percent of basal (inset). (B) Liver triglycerol for all rats (B) and rats selected for proteomics (C). Liver diacylglycerol for rats selected for proteomics (D). Western blot showing PKCε activation as measured by membrane translocation (E). Quantification of PKCε translocation in rats selected for proteomics (F). Basal plasma insulin for rats selected for proteomics (G). Basal plasma glucose for all rats (H) and rats selected for proteomics (I). Western blot showing >90% knockdown of PKCε at the protein level (J). Plasma liver enzymes as a measure of liver function (K).

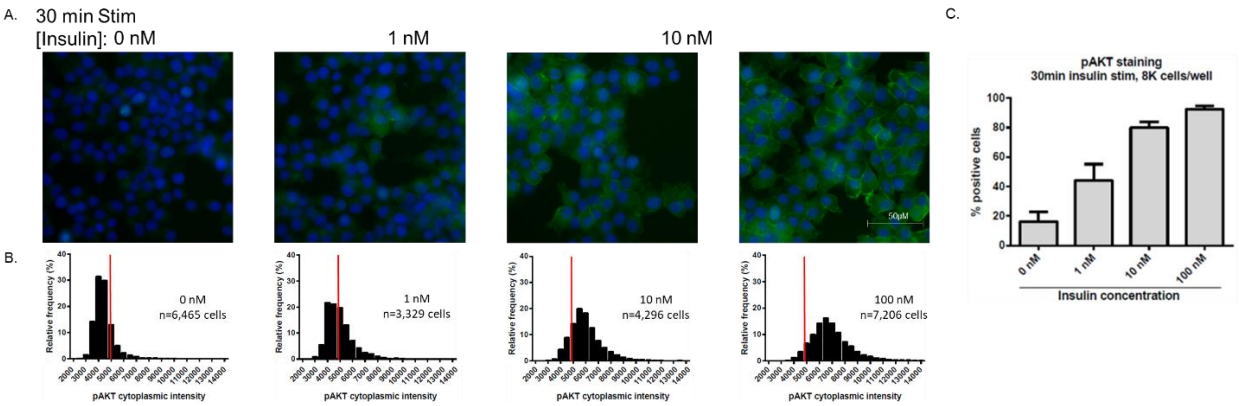
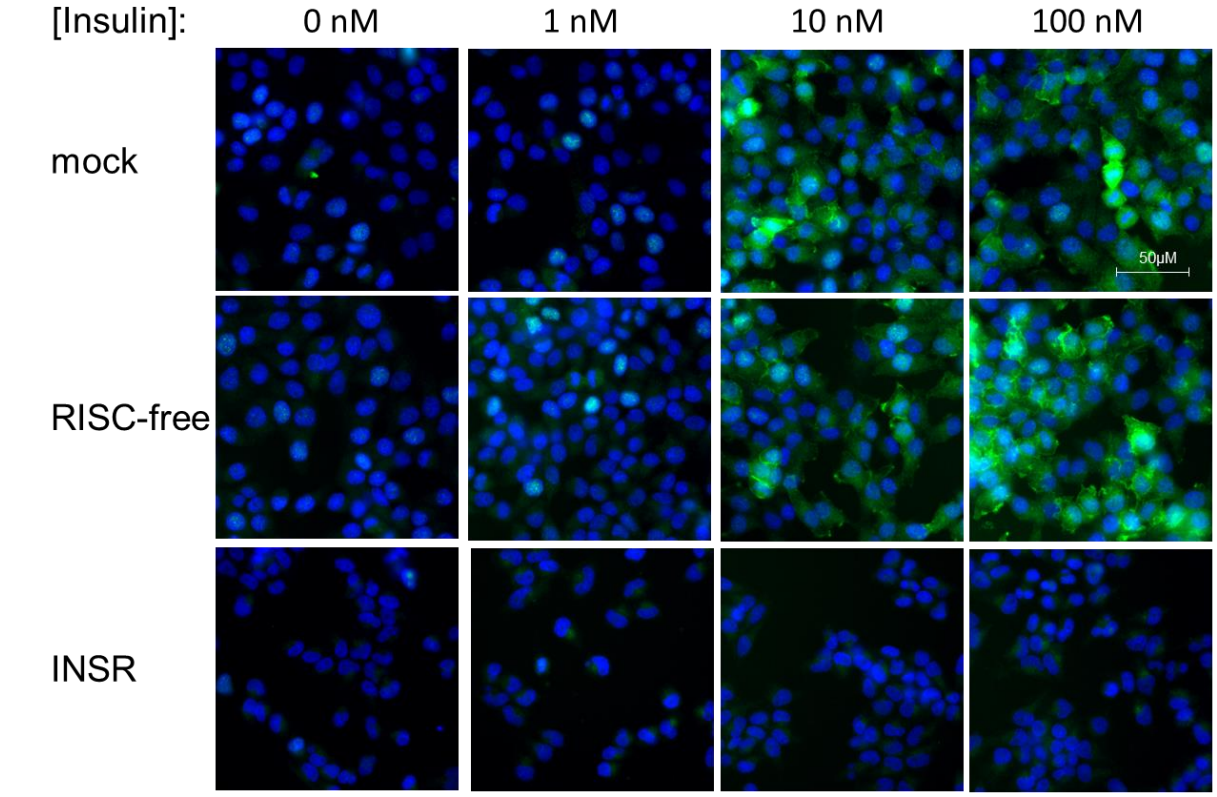


Figure S2: Assay Development: Insulin Dose Response. McCardle cells have a dose-dependent AKT pS473 response to insulin at 0, 1, 10, and 100 nM insulin (A). Signal quantification is performed by binning relative intensity of AKT pS473 staining in each cell in a histogram (B), then setting a positive signal cutoff (5000 relative intensity units in this case). The percentage of positive cells is then plotted as a population measure of signal strength (C).

A. 30 min Stim



B.

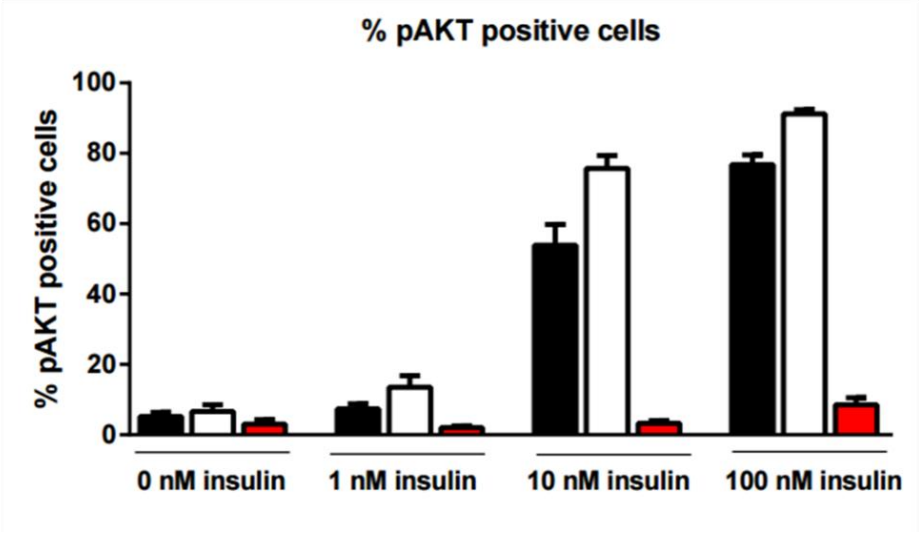
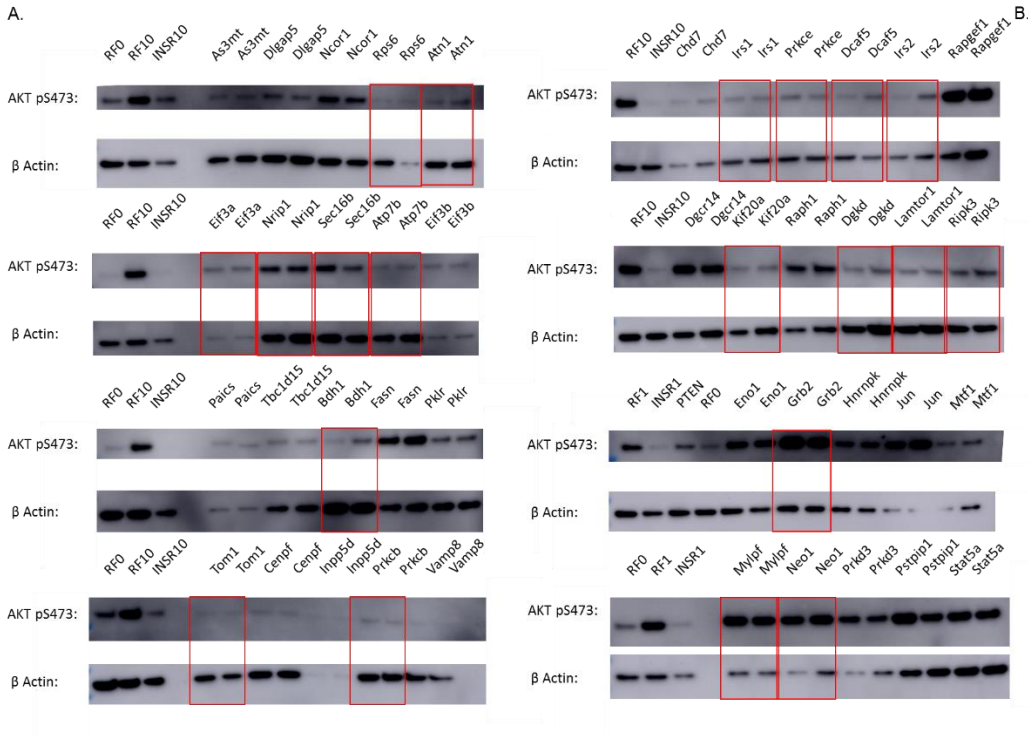


Figure S3: Dose response of control siRNAs. Images showing AKT pS473 staining for mock transfection, RISC-free siRNA, and siRNA directed against the INSR (A). Quantification of the percentage of positive cells in A (B), showing a significant reduction in staining in the INSR-transfected cells. Black bars represent mock transfected cells, white bars represent RISC-free siRNA transfected cells, and red bars represent INSR siRNA transfected cells.



Rep	Average	SEM	n
As3mt	63.89	28.72	6
Dlgap5	63.93	32.86	6
Ncor1	55.01	22.64	6
Rps6	**87.23**	**22.03**	**4**
Atn1	**100.83**	**14.97**	**6**
Eif3a	**85.00**	**6.80**	**4**
Nrip1	**72.53**	**8.66**	**6**
Sec16b	**68.76**	**8.90**	**5**
Atp7b	**80.51**	**12.02**	**5**
Eif3b	38.45	7.55	4
Paics	-25.70	52.31	6
Tbc1d15	50.03	28.41	6
Bdh1	**97.78**	**11.61**	**4**
Fasn	-8.59	48.92	6
Pklr	44.87	9.46	6
Tom1	**84.68**	**10.58**	**6**
Cenpf	40.57	29.21	6
Inpp5d	-7.40	46.01	6
Prkcb	**94.80**	**11.13**	**4**
Vamp8	40.19	29.85	6
Chd7	**78.05**	**4.02**	**3**
Irs1	**85.87**	**5.10**	**5**
Prkce	**89.04**	**3.98**	**4**
Dcaf5	**76.13**	**9.12**	**4**
Irs2	**57.43**	**8.97**	**4**
Rapgef1	15.98	49.21	4
Dgcr14	54.39	22.76	6
Kif20a	**71.54**	**6.73**	**6**
Raph1	35.36	35.08	4
Dgkd	**98.51**	**5.35**	**4**
Lamtor1	**100.31**	**1.71**	**4**
Ripk3	**57.99**	**6.42**	**5**
Eno1	123.16	34.78	4
Grb2	**201.07**	**74.56**	**4**
Hnrnpk	131.62	21.36	4
Jun	141.44	6.62	2
Mtf1	108.92	9.25	3
Mylpf	**290.69**	**92.91**	**5**
Neo1	**267.34**	**65.25**	**4**
Prkd3	174.49	96.99	3
Pstpip1	154.92	50.41	6
Stat5a	160.68	45.13	6

Figure S4: Validation of screen hits by western blot. McCardle cells were transfected with selected siRNAs in a larger 96 well format, and insulin signaling strength was measured by western blot for AKTpS473 (A). Western validation results are summarized in table B, with validated genes in bold red (n represents the number of valid, independently transfected wells that were analyzed by western blot and included in this analysis). Signal across blots was controlled by using the controls RF0 (RISC-Free siRNA, 0nM insulin), RF10 (RISC-Free siRNA, 10nM Insulin), and INSR10 (INSR siRNA, 10nM Insulin). (B) Table showing the results of replicate blots, proteins highlighted in red were considered replicated.

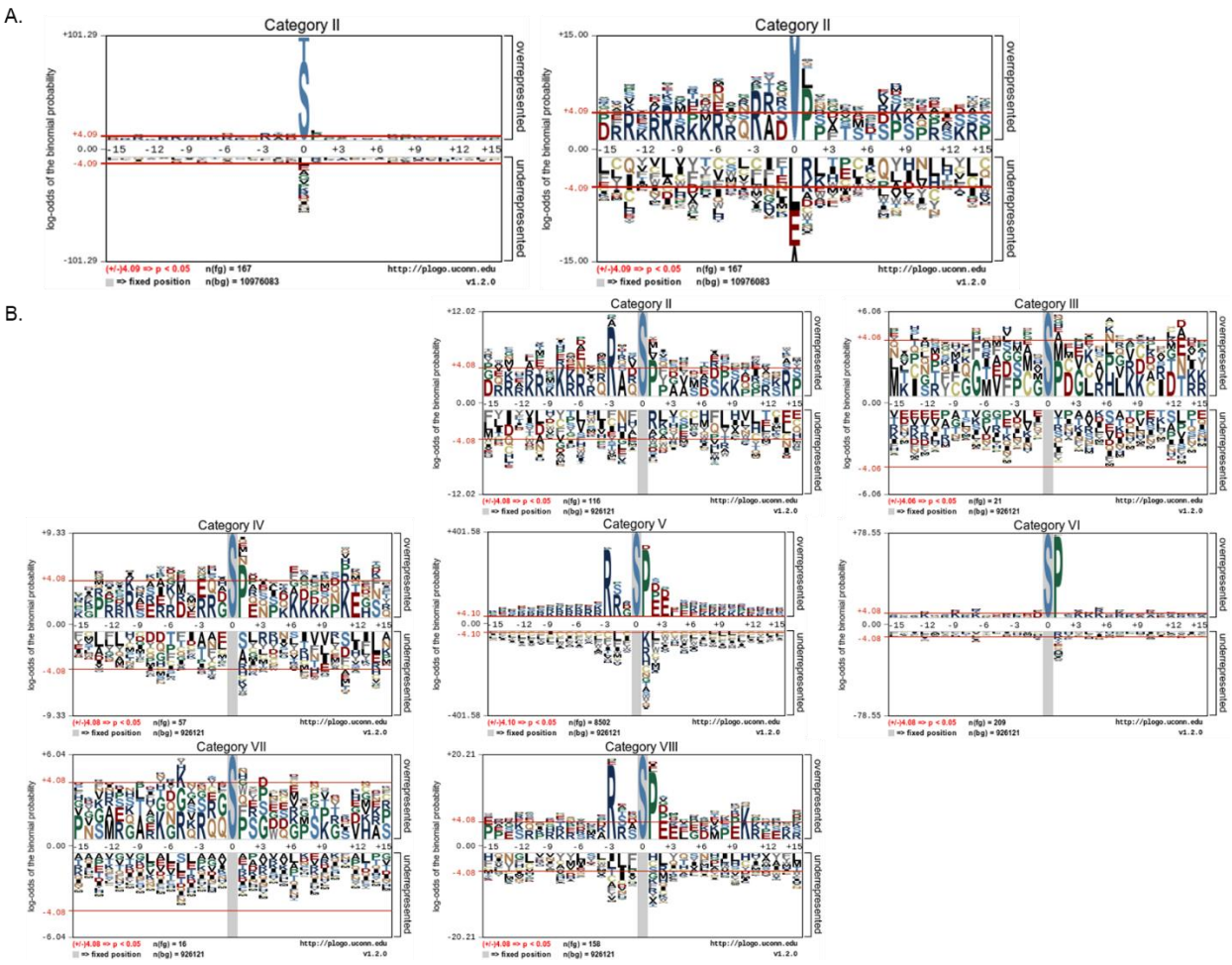
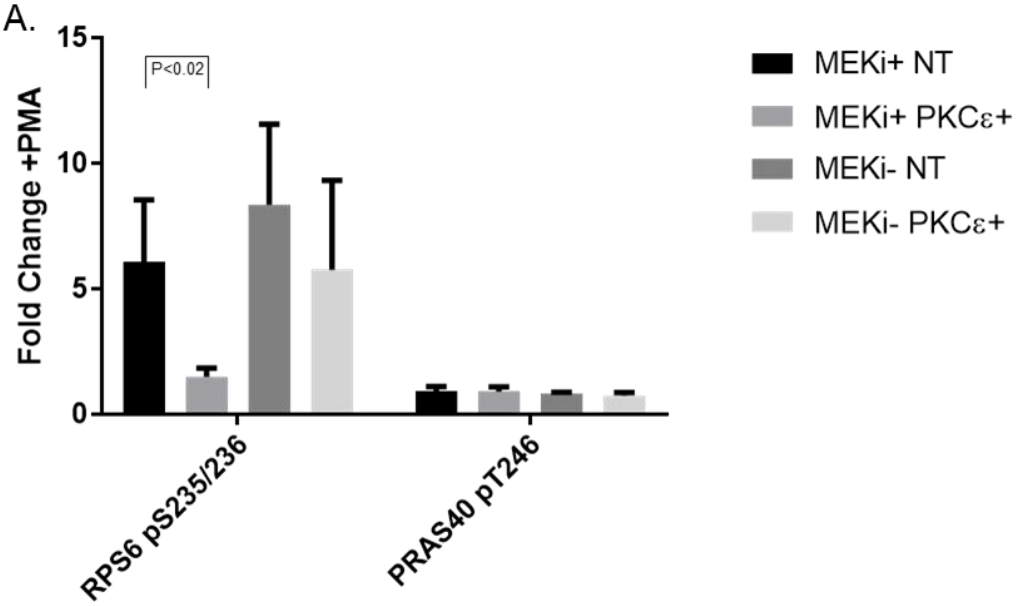


Figure S5: Motif preferences analysis using the pLogo tool (16). (A) Motif preferences from Category II with both serine and threonine residues (zoom on right). (B) Motif preferences for each category II-VIII. Phosphosites from Figure 1E were used to generated motif preferences with +/- 15 amino acids on either side.

A.


15
10
5
0
Fold Change +PMA
P<0.02
RPS6 pS235/236
PRAS40 pT246
MEKi+ NT
MEKi+ PKCε+
MEKi- NT
MEKi- PKCε+

B.

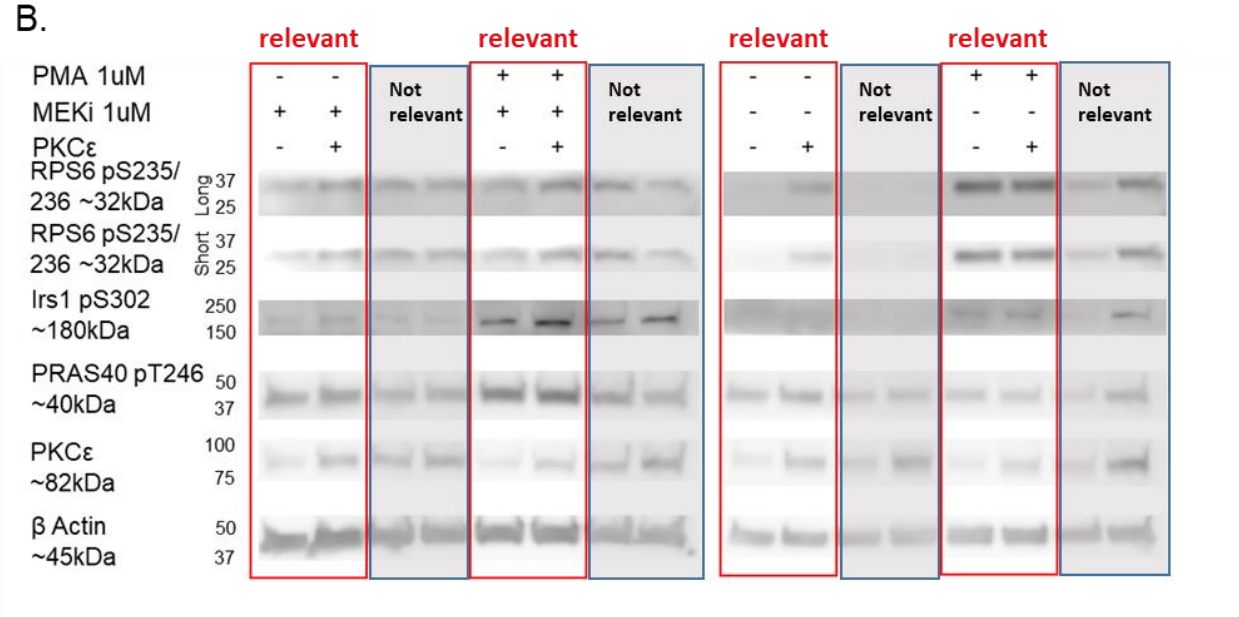

relevant
relevant
relevant
relevant
Not relevant
Not relevant
Not relevant
Not relevant
PMA 1uM
MEKi 1uM
PKCε
RPS6 pS235/236 ~32kDa
Long
Short
Irs1 pS302 ~180kDa
PRAS40 pT246 ~40kDa
PKCε ~82kDa
β Actin ~45kDa
37
25
250
150
50
100
75

Figure S6: (A) Fold-change in phosphorylation with PMA treatment. Phosphorylation of RPS6 S235/236 and PRAS40 T246 was measured in p70S6K1/p70S6K2 DKO cells with (PKCε+) or without (NT) PKCε transfection, treated with (MEKi+) or without (MEKi-) 1μM MEKi, followed by treatment with or without 1μM PMA. Fold change was calculated by dividing the PMA+ band by the PMA- band within MEKi and PKCε treatment groups. n =3, P values for 2-way ANOVA with Tukey's multiple comparisons test are as indicated. (B) Western blots for Fig. 4a with relevant lanes indicated, and short/long exposures indicated for RPS6 pS235/236.

Supplemental Tables:

siRNA Target	Category	Inclusion Criteria	ave % viability (relative to RF)	ave % effect (relative to RF and INSR) 10 nM	ave % control (relative to RF) 1 nM	% viability (relative to RF) 10 nM replicate 1	% viability (relative to RF) 10 nM replicate 2	% viability (relative to RF) 10 nM replicate 3	% viability (relative to RF) 1 nM replicate 1	% viability (relative to RF) 1 nM replicate 2	% viability (relative to RF) 1 nM replicate 3	% effect (relative to RF and INSR) 10 nM replicate 1	% effect (relative to RF and INSR) 10 nM replicate 2	% effect (relative to RF and INSR) 10 nM replicate 3	% control (relative to RF) 1 nM replicate 1	% control (relative to RF) 1 nM replicate 2	% control (relative to RF) 1 nM replicate 3
Abcg2	VI	O	1.19	55.19	49.67	2.01	0.90	1.04	0.49	2.43	0.27	26.12	67.75	71.71	0.00	43.53	105.48
Abcg5	VII	R	63.60	-14.12	37.01	53.46	25.24	121.39	61.25	46.84	73.41	-42.09	-0.90	0.65	38.87	31.40	40.76
Acaca	III,VI	R	49.77	65.19	9.94	75.92	29.54	63.25	32.06	51.27	46.58	41.79	81.36	72.40	7.46	8.56	13.80
Acacb	III,VI	R	26.46	60.58	42.52	9.36	4.80	40.65	9.70	16.05	78.22	46.40	80.08	55.27	34.54	43.53	49.49
Acbd5	VIII	O	8.44	22.77	51.59	2.23	2.64	7.55	3.64	7.41	27.15	34.47	18.41	15.42	55.85	39.46	59.45
Acly	VI	I	9.56	4.86	42.48	5.30	2.20	10.47	3.68	9.39	26.34	21.02	5.68	-12.10	25.99	37.19	64.27
Acox2	IV	R	39.17	60.02	29.17	34.11	14.07	34.14	4.50	52.18	95.98	70.98	45.80	63.29	0.00	40.32	47.17
Agl	II	I	27.30	61.64	88.58	13.88	15.15	29.34	10.00	24.04	71.38	29.94	83.36	71.61	114.81	100.70	50.22
Ajuba	IV	F	61.37	68.83	53.31	87.74	38.77	62.66	49.65	59.13	70.26	55.51	76.89	74.08	35.90	48.09	75.94
Ampd2	VI	O	53.77	38.81	66.87	57.53	15.60	57.19	34.70	73.95	83.64	23.21	55.15	38.08	28.62	78.22	93.76
Anln1	VI	F	55.96	45.60	34.32	57.53	26.67	96.66	27.21	52.20	75.49	16.02	68.96	51.81	43.09	28.17	31.69
Apoa4	III	R	42.64	60.25	24.96	46.93	15.60	76.95	18.29	48.82	49.27	52.97	61.53	66.25	18.32	13.65	42.91
As3mt	VI	F	10.59	91.31	34.76	10.03	5.47	15.50	13.56	4.30	14.69	86.24	96.22	91.47	14.99	5.67	83.61
Ascl1	VII	R	14.44	61.36	38.67	1.62	2.51	6.99	1.17	5.61	68.77	47.43	92.93	43.73	20.52	37.65	57.85
Aspscr1	VII	R	53.78	40.99	68.42	51.78	22.28	74.37	26.02	52.40	95.85	31.31	70.67	20.97	19.77	63.88	121.60
Atn1	VI	R	49.02	95.78	11.07	76.48	18.83	54.91	43.82	69.90	30.15	97.06	100.39	89.88	1.91	11.39	19.90
Atp7b	II	F	68.83	72.07	30.35	79.82	54.50	74.82	24.04	127.32	52.47	74.35	72.56	69.29	7.47	42.76	40.83
Bcl6	IV	O	51.36	42.28	50.81	45.09	24.29	52.98	49.61	75.45	60.73	25.64	53.93	47.26	28.70	38.23	85.49
Bdh1	VIII	F	61.68	75.80	54.34	70.68	17.26	81.02	51.13	74.49	75.51	71.37	84.88	71.16	44.46	53.66	64.88
Bet1l	II	O	48.27	-27.08	133.91	85.06	26.13	57.16	37.23	16.05	68.00	-65.30	-3.92	-12.02	146.84	148.82	106.08
Cald1	VI	F	76.93	-12.40	88.99	65.05	48.01	178.72	53.70	40.65	75.45	-55.06	15.99	1.89	146.81	74.35	45.82
Cdkn1a	VI	O	17.84	9.20	66.51	12.99	11.65	31.30	1.82	12.18	37.08	-13.37	35.62	5.36	59.13	67.39	73.00
Cenpf	VI	F	62.80	76.91	30.19	48.72	64.86	98.76	48.04	61.35	55.04	77.52	78.86	74.34	25.90	28.60	36.07
Chd7	IV	F	1.90	93.83	43.33	2.12	1.70	1.40	0.80	0.58	4.78	104.34	96.61	80.53	29.99	0.00	100.01
Cps1	II,VI,VIII	O	32.89	-41.99	98.39	12.15	9.91	51.68	38.91	14.38	70.32	-70.56	-15.95	-39.45	103.61	122.59	68.96
Dcaf5	IV	F	16.27	88.60	22.09	13.49	15.19	18.19	8.82	13.36	28.57	99.68	76.50	89.61	0.00	12.77	53.49
Deptor	II	O	130.32	34.44	22.68	196.93	100.85	160.84	55.26	153.05	114.99	28.51	36.48	38.34	1.08	15.29	51.68
Dgcr14	VI	F	53.37	72.36	27.49	95.32	23.13	70.35	48.00	56.08	27.35	59.58	79.34	78.17	1.99	34.77	45.69
Dgkd	II,VI	R	42.74	71.42	19.31	49.39	14.88	53.00	28.36	60.93	49.90	53.75	82.54	77.96	4.22	7.47	46.23
Dis3l2	II	F	4.83	15.40	131.51	4.52	5.38	3.37	1.39	3.78	10.52	0.76	19.89	25.56	94.60	141.69	158.23
Dlgap5	VI	F	8.83	72.22	18.58	8.42	4.62	7.44	3.40	5.50	23.63	49.90	85.37	81.39	3.52	2.96	49.27
Dsp	VI,VII	F	110.41	61.00	32.53	137.74	97.98	158.53	84.12	102.80	81.29	62.82	60.28	59.91	13.23	10.91	73.45
Dus3l	VII	R	53.21	13.83	44.50	16.33	40.79	113.08	46.99	35.60	66.49	1.55	4.92	35.02	35.89	50.67	46.94
Eif3a	VI	O	2.81	106.02	15.84	2.01	0.81	6.54	0.37	1.20	5.96	104.05	106.92	107.08	0.00	6.76	40.76
Eif3b	VI	O	35.30	102.45	7.50	40.91	21.74	32.71	24.59	35.00	56.85	101.70	99.92	105.72	1.46	3.95	17.09
Eif4e	IV	I	55.04	31.79	43.58	81.77	53.38	64.65	25.98	52.56	51.91	15.36	31.29	48.73	8.29	28.75	93.70
Eif4h	II	O	6.24	30.91	55.26	2.51	2.91	13.11	0.12	8.82	9.95	56.18	40.64	-4.10	0.00	74.65	91.13
Elf4	VIII	O	16.22	43.02	64.65	16.39	20.98	20.52	19.05	12.13	8.28	30.55	42.76	55.76	67.84	38.17	87.95
Eno1	II	O	23.39	-4.11	152.13	27.26	9.68	33.69	6.57	34.24	28.91	-42.01	-6.10	35.79	129.35	144.99	182.07
Fasn	III,VI	O	60.29	70.31	44.50	70.85	70.64	37.23	47.24	59.66	76.10	69.10	69.15	72.68	8.87	37.18	87.47
Fxyd1	II	F	5.42	-2.41	54.12	6.08	4.62	7.89	3.95	4.12	5.89	-19.08	19.46	-7.62	78.78	53.27	30.31
Gab2	XIII	R	12.10	63.77	45.52	5.02	9.77	31.11	1.60	5.97	19.14	60.63	60.81	69.88	22.49	28.60	85.46
Gapdh	II	O	15.29	13.46	33.92	16.00	22.41	18.56	11.74	12.71	10.34	25.13	75.07	90.19	28.53	17.26	55.97
Gck	II	O	131.76	-34.81	115.33	231.22	130.57	151.01	81.91	103.32	92.51	-73.78	-10.86	-19.79	175.41	128.42	42.15
Grb14	VI	O	26.92	37.41	35.67	22.74	17.39	44.10	16.53	32.97	27.76	17.28	56.45	38.49	19.54	41.40	46.05
Grb2	VI	I	43.01	-51.30	183.72	42.64	54.06	52.61	12.73	32.35	63.67	-82.92	-19.73	-51.26	161.71	232.84	156.60
Gtse1	VI	F	57.40	34.41	28.48	68.95	41.64	72.96	17.35	80.61	62.88	21.00	41.15	41.09	15.17	36.18	34.08
Hn1	VI	F	27.01	34.07	105.87	31.66	17.75	31.11	12.48	25.04	44.03	3.42	48.24	50.56	74.78	125.87	116.95
Hnrnpc	VI	F	11.19	45.73	99.50	9.70	8.70	12.35	2.54	18.63	15.23	32.45	66.54	38.18	61.31	100.29	136.90
Hnrnpk	VI	F	41.68	-17.79	140.24	47.05	24.74	41.58	49.45	51.11	36.13	-32.13	-0.69	-20.54	182.43	150.53	87.76
Idh3B	VI	O	24.38	57.52	55.63	46.60	12.37	50.08	7.53	11.98	17.74	39.38	65.30	67.89	41.32	84.12	41.47
Inpp5d	VIII	I	1.07	92.41	13.84	1.51	1.26	0.90	0.88	0.93	0.93	94.78	74.28	108.16	0.00	26.07	15.44
Irs1	II	I	32.04	81.82	37.99	25.47	33.44	56.29	8.98	31.66	36.40	74.54	86.27	84.65	30.64	22.33	61.02
Irs2	IV	I	111.15	39.24	28.72	154.12	163.42	134.33	75.10	67.56	72.38	90.34	94.96	94.41	10.36	26.58	49.24
Jun	VI	O	52.85	-27.36	199.77	40.97	44.02	84.14	29.42	49.29	69.24	-52.61	-3.02	-26.46	217.16	171.11	211.04
Kif20a	VI	F	115.75	72.62	46.04	103.57	146.53	143.12	48.12	119.52	133.67	74.67	67.90	75.28	31.58	22.50	84.04
Kif4a	VI	F	38.31	33.56	77.74	33.39	33.44	50.20	12.56	42.52	57.75	31.33	25.19	44.16	69.53	74.52	89.16
Lamtor1	II	O	43.37	83.11	54.06	15.16	23.04	46.72	8.88	88.09	78.33	78.68	86.85	83.80	4.04	36.25	121.90
Limk1	VIII	O	142.13	52.00	71.69	265.27	142.81	129.81	109.10	114.69	91.11	45.53	49.06	61.41	51.43	37.97	125.66
Lnpep	VIII	O	70.36	31.02	48.39	93.98	22.01	53.90	25.20	149.24	77.82	13.46	35.66	43.92	31.33	71.15	42.68
Lpar3	VII	R	33.88	48.25	55.36	42.98	32.27	58.76	22.16	13.18	33.97	58.41	38.20	48.13	34.56	23.43	108.08
Lrp1	VIII	O	28.64	69.57	37.93	15.50	18.96	60.44	4.73	6.70	65.49	72.86	64.33	71.54	22.78	25.46	65.54
Map7d1	VI	F	85.24	50.94	37.21	80.10	74.05	154.35	71.28	49.58	82.06	41.01	44.25	67.56	49.85	24.26	37.51
Mapk1	II	I	50.92	28.20	92.53	57.08	54.33	66.20	14.95	34.39	78.56	15.09	22.47	47.03	133.60	52.44	91.54
Mastl	VI	F	26.37	21.29	70.10	13.66	12.60	50.53	8.16	23.37	49.88	13.10	23.76	27.02	60.09	66.74	83.48
Mcm2	VI	F	38.70	-3.34	91.32	23.58	28.82	42.31	16.45	72.06	49.00	-14.98	19.01	-14.06	125.11	80.05	68.80
Mcm3	VI	F	41.90	34.58	26.27	60.31	55.98	27.82	16.84	23.26	67.21	15.90	44.83	43.00	22.74	31.43	24.65
Me1	VI	O	17.88	21.64	86.50	12.37	16.63	12.10	11.31	8.01	46.87	26.57	5.93	32.42	104.69	72.99	81.82
Mnt	III	F	75.18	11.67	73.45	99.89	70.64	85.71	42.49	76.36	75.96	-17.76	25.24	27.52	59.69	41.07	119.59
Mtf1	VIII	F	19.88	-3.49	205.39	6.41	10.80	12.72	6.20	25.51	57.62	-22.82	20.25	-7.91	274.06	150.95	191.14
Mylpf	IV	F	83.57	39.91	136.07	100.28	67.73	127.46	61.89	103.05	41.01	28.30	44.54	46.90	180.95	110.22	117.04
Ncor1	VII	R	31.36	80.76	38.97	8.70	19.32	67.86	15.14	14.02	63.10	91.64	79.66	71.00	18.18	35.92	62.82
Neo1	VII	R	54.90	-44.08	270.47	39.69	46.53	88.04	38.11	18.19	98.87	-72.49	-15.08	-44.68	403.67	185.83	221.90
Nrip1	VIII	O	15.13	73.75	46.39	11.76	7.13	15.58	7.77	7.12	41.41	78.28	88.04	54.95	13.85	42.20	83.12
Nucks1	VI	F	116.60	47.21	32.32	165.44	160.20	127.18	58.10	80.72	107.94	28.26	46.61	66.76	15.44	49.62	31.89
Ociad1	VI	F	49.11	-15.19	107.47	93.42	51.14	50.81	14.73	38.69	45.86	-38.17	-3.03	-4.36	104.78	118.43	99.20
Paics	VI	F	67.28	78.09	15.47	101.28	86.64	70.35	25.20	43.12	77.07	80.17	66.06	88.04	4.27	20.92	21.22
Pde5a	VIII	O	17.81	55.93	78.03	16.28	26.98	14.74	17.02	10.06	21.78	47.85	63.98	55.96	84.35	93.66	56.08
Per2	VI	O	123.61	58.40	83.02	93.65	123.89	149.80	112.15	143.72	118.44	48.66	47.03	79.51	72.75	86.83	89.48
Phkb	II	I	16.44	6.25	23.78	4.12	6.36	13.45	1.72	30.37	42.63	-17.66	42.56	-6.14	0.00	22.74	48.58
Phtf1	VIII	O	24.43	69.30	78.04	8.86	12.37	64.21	1.41	19.81	39.92	73.07	58.67	76.16	84.75	46.34	103.04
Pik3r5	VIII	I	71.45	-23.95	132.08	41.58	79.52	142.08	79.66	30.14	55.70	-38.13	-11.12	-22.59	194.78	124.26	77.19
Pklr	III,VI	O	120.93	79.10	33.35	127.26	119.14	129.79	117.04	109.11	123.25	85.92	69.64	81.74	19.53	34.85	45.66
Plin2	VI	O	38.59	19.73	119.99	17.95	60.60	42.95	25.94	34.19	49.88	11.91	27.93	19.36	128.67	118.33	112.98
Plin5	VI	O	73.91	43.91	30.73	62.49	91.84	103.99	61.87	93.97	29.32	38.11	38.49	55.13	26.49	29.40	36.30
Ppp1r3c	II	I	66.40	-20.51	126.92	73.47	61.05	51.77	42.96	82.86	86.28	-52.16	-0.05	-9.32	118.91	99.53	162.33
Prkaa2	II	O	95.78	-19.94	62.82	158.53	71.27	105.56	50.20	118.41	70.73	-63.58	-1.13	4.90	31.22	69.71	87.54
Prkc1	II	O	60.13	28.44	59.99	58.64	39.00	56.63	36.70	80.81	89.01	25.42	20.79	39.10	21.84	54.69	103.43
Prkcb	VIII	O	71.57	72.68	38.39	104.29	116.18	51.49	55.65	81.86	19.95	81.88	71.66	64.50	16.98	45.56	52.62
Prkcd	VIII	O	34.24	35.94	50.24	28.71	65.93	21.11	20.40	30.88	38.43	43.86	28.82	35.14	41.06	58.94	50.73
Prkce	II	O	40.71	76.72	16.05	47.94	16.81	80.46	26.96	28.83	43.24	77.70	75.59	76.89	26.18	12.40	9.58
Prkd3	II	O	68.97	-18.33	145.39	24.80	90.18	123.19	72.16	46.37	57.14	-46.20	-8.32	-0.46	228.15	141.39	66.62
Prr11	VI	F	32.62	55.98	72.55	14.38	34.38	62.86	21.77	28.52	33.81	64.57	44.11	59.26	90.14	60.12	67.39
Pstpip1	VIII	F	45.69	3.31	183.73	22.24	47.78	71.56	22.93	32.30	77.32	-23.98	27.81	6.11	249.36	200.22	101.60
Ptpn21	VIII	O	69.54	-18.54	62.95	59.64	65.26	97.84	39.48	60.84	94.15	-18.49	2.01	-39.15	7.88	80.26	100.73
Ptpn6	VIII	O	43.04	59.07	45.34	14.21	48.01	52.08	25.76	81.12	37.08	78.97	46.46	51.78	33.44	40.16	62.41
Ptprc	VIII	O	10.46	13.62	23.45	9.42	10.40	13.25	4.15	10.22	15.32	43.21	5.63	-8.00	17.29	30.22	22.84
Rapgef1	IV	I	30.27	81.34	49.75	7.08	10.53	28.16	27.21	62.40	46.26	92.26	73.03	78.72	32.98	44.66	71.63
Raph1	VIII	F	67.88	87.83	24.10	61.20	69.70	101.04	73.18	67.28	34.91	94.47	91.22	77.79	15.70	25.72	30.89
Peak1	VI	F	37.09	51.22	28.50	24.69	21.43	36.89	16.20	57.93	65.38	64.83	48.47	40.35	10.34	47.97	27.20
Ripk3	VIII	O	53.92	81.36	38.69	36.01	71.40	84.17	44.99	36.13	50.85	80.15	80.69	83.25	37.76	24.51	53.80
Rps6	III	I,R	16.95	108.17	4.31	14.66	20.17	26.31	18.33	9.02	13.20	104.28	103.73	116.50	3.92	0.90	8.11
Sec16b	II	O	38.28	81.91	33.84	23.63	34.16	63.81	32.39	48.02	27.65	77.48	80.71	87.55	23.27	21.15	57.09
Sh2b2	VIII	I	63.04	61.77	50.21	34.23	67.82	81.72	62.60	63.67	68.18	37.49	63.60	84.23	51.79	45.43	53.42
Shroom3	II	F	104.10	47.43	42.66	93.14	130.75	154.52	97.18	90.23	58.79	51.57	38.18	52.54	36.32	30.08	61.59
Sirt2	VI	O	23.54	30.57	83.33	6.80	14.34	26.90	6.12	34.02	53.06	-7.01	42.05	56.68	62.58	93.87	93.55
Slc2a2	III,IV	O	49.98	41.73	46.68	30.71	39.18	56.74	48.92	80.57	43.78	10.33	86.01	28.84	41.58	64.84	33.60
Sorbs2	II	O	57.97	62.85	32.54	29.26	45.63	59.60	77.15	101.47	34.73	61.90	61.91	64.75	28.22	27.86	41.53
Speg	VI	F	87.40	-3.75	74.08	57.13	107.44	105.64	98.87	105.21	50.12	-18.44	4.58	2.61	68.36	114.14	39.75
Sptbn1	II	F	44.64	67.84	56.15	8.58	50.65	47.95	39.08	91.12	30.44	74.48	60.25	68.79	53.27	60.99	54.19
Stat2	IV	O	17.58	67.67	30.03	12.43	10.94	28.24	16.31	10.42	27.17	81.76	75.89	45.35	45.49	7.80	36.80
Stat5a	VI	O	28.85	-31.38	210.96	15.55	31.47	36.81	43.13	22.55	23.61	-58.46	-14.14	-21.52	331.79	179.78	121.32
Stk10	VIII	O	16.22	-42.43	83.41	6.97	21.56	30.74	10.15	7.55	20.36	-74.75	-13.04	-39.49	108.47	95.82	45.96
Stmn1	VI	F	3.56	63.16	42.51	1.17	1.52	9.35	2.13	2.80	4.38	80.99	91.56	16.92	22.49	72.41	32.62
Sumo1	VII	R	35.74	61.60	35.34	13.27	22.73	48.96	22.42	61.13	45.93	57.41	63.40	64.00	18.14	30.97	56.91
Tbc1d15	VI	O	56.06	78.22	45.44	27.48	58.00	51.49	46.01	98.29	55.09	85.23	81.39	68.04	15.60	24.14	96.58
Tbc1d2	IV	O	25.25	64.00	28.54	2.12	7.17	27.15	13.79	83.26	18.03	35.79	66.12	90.09	32.97	22.93	29.71
Tcf20	IV	F	33.86	21.48	55.48	22.69	29.22	46.63	6.49	59.86	38.30	28.87	19.42	16.14	40.58	72.34	53.52
Timeless	VI	O	24.07	34.51	92.41	22.24	23.17	29.93	22.48	29.70	16.93	39.81	49.09	14.62	133.03	93.57	50.63
Tom1	VI	F	31.12	92.82	18.34	19.90	20.44	46.52	15.81	68.88	15.19	99.52	88.31	90.64	7.57	24.89	22.57
Tpx2	VI	F	2.44	38.88	66.91	1.06	1.66	2.84	1.88	1.56	5.62	4.14	64.58	47.91	120.77	46.92	33.04
Tsc2	II	I	12.58	50.42	70.23	10.03	22.81	17.77	10.82	6.83	7.18	48.38	59.98	42.91	84.02	68.95	57.72
Vamp8	IV	O	21.60	76.40	14.95	21.24	9.82	28.86	36.23	13.56	19.88	72.80	75.33	81.06	23.77	4.19	16.88
Wfs1	VI	O	71.56	18.12	82.24	55.52	120.84	91.72	58.51	29.61	73.14	2.58	19.13	32.66	104.89	47.20	94.61
Xkr5	VI	F	20.70	15.97	79.08	6.19	10.49	23.13	10.82	33.59	39.99	12.13	25.47	10.32	68.54	76.91	91.79

Table S1: Functional siRNA Screen results. Phosphoproteins were selected based on several criteria: phosphoproteins in "rescue" categories (i.e. Categores III and VII from figure 1e) are designated (R); phosphoproteins that are known or putative members of the insulin signaling pathway are designated (I); phosphoproteins belonging to other pathways of interest are designated (O); and phosphoproteins chosen for their fold change are designated (F). Averages are given for % viability (compared to the RISC-free control siRNA), % effect (with the RISC-free siRNA staining intensity set to 0% effect and INSR siRNA staining set to 100% effect, 10nM insulin) and %control (intensity compared to RISC-free siRNA, 1nM insuliln), followed by individual values for the 3 replicates of the 10nM and 1nM insulin stimulation screens.

Peptide Substrate Display Library Replicate Results

Gene	Phosphosite	Peptide Observed in ? Replicates	Peptide Observed in ? Replicates EN	>3 Reps?	On Target?	Off Target?
Caskin2	S855	4	4	TRUE	x	
Dennd4a	S1151	4	4	TRUE	x	
Ethe1	S14	4	4	TRUE	x	
Myl9	S20	3	4	TRUE	x	
Prkce	S729	0	4	TRUE	x	
Aff4	T821	4	4	TRUE	x	
Ppp1r12c	T561	4	4	TRUE	x	
Shroom3	S897	4	4	TRUE	x	
Shank3	S891	4	4	TRUE	x	
Akt1s1	T247	0	4	TRUE	x	
Ppp1r12b	T644	4	4	TRUE	x	
Tsc2	T1466	4	4	TRUE	x	
Irs1	S265	0	4	TRUE	x	
Irs1	S302	3	4	TRUE	x	
Phkb	S27	4	4	TRUE	x	
P33monox	S236	2	3	TRUE	x	
Rps6	S235	4	4	TRUE	x	
Srrm1	S427	0	4	TRUE		x
Dkc1	S444	0	4	TRUE		x
Dock7	S904	4	4	TRUE		x
Krt18	S19	0	4	TRUE		x
Hspa12b	S280	4	3	TRUE		x
Lmnb1	T25	2	3	TRUE		x
Mios	S768	4	4	TRUE		x
Fbrsl1	S181	1	4	TRUE		x
Nucks1	T179	4	4	TRUE		x
Scrib	S1516	0	2	FALSE	x	
Deptor	S237	2	0	FALSE	x	

Table S2: PKCε peptide substrate library replicate results. The peptide substrate library was incubated with PKCε *in vitro* in 4 independent experiments, followed by trypsin digestion. Peptides were analyzed by LC-MS/MS without (Peptides Observed in ? Replicates) or with TiO_2 enrichment (Peptides Observed in ? Replicates EN). Identified substrates were considered valid if they reproduced in at least three of four experiments in either the unenriched or enriched samples.

Supplemental Data Files:

Data File S1: Study phenotyping values. Control ASO-Chow cohort in red; Control ASO-HFD cohort in green; PKCε ASO-HFD cohort in purple. Animals selected for proteomics in darker shading.

Data File S2: Phosphoproteomics Data. LC-MS/MS data was searched using Maxquant v1.5.1.2 and analyzed using Perseus. Data represent filtering of contaminants, averaging of replicates, and annotation with Category (from Fig. 1e) and known kinase-substrate relationships from Phosphositeplus.

Data File S3: Significantly Changed Phosphopeptides. Phosphopeptides that had greater than a two-fold change in at least one experiment based on the 2D-Enrichment Analysis (Fig. 1e). This table only includes phosphopeptides that had quantitative information (ratios) in both comparisons, and does not include phosphopeptides in Category V.

Data File S4: siRNA SMART Pools. siRNA SMART Pools used in Fig. 2 and Table S2; these pools contain 4 siRNAs to each target gene, and 3 of 4 siRNAs are guaranteed to knockdown the intended target.

Data File S5: PKCε Peptide Substrate Library. The peptide substrate library consists of an N-terminal tag with a flexible linker and Precision Protease cleavage site that is fused to GST. The target peptide includes the central phosphorylatable residue, with 10 amino acids on either site from the native protein context if that included the native trypsin cleavage site, or extending the amino acids until the next native trypsin cleavage site to fully mimic the native tryptic peptide sequence.

Supplemental References:

1. Geary RS, *et al.* (2001) Pharmacokinetic properties of 2'-O-(2-methoxyethyl)-modified oligonucleotide analogs in rats. *The Journal of pharmacology and experimental therapeutics* 296(3):890-897.
2. Samuel VT, *et al.* (2007) Inhibition of protein kinase Cepsilon prevents hepatic insulin resistance in nonalcoholic fatty liver disease. *The Journal of clinical investigation* 117(3):739-745.
3. Kraegen EW, *et al.* (1991) Development of muscle insulin resistance after liver insulin resistance in high-fat-fed rats. *Diabetes* 40(11):1397-1403.
4. Samuel VT, *et al.* (2004) Mechanism of hepatic insulin resistance in non-alcoholic fatty liver disease. *The Journal of biological chemistry* 279(31):32345-32353.
5. Cantley JL, *et al.* (2013) CGI-58 knockdown sequesters diacylglycerols in lipid droplets/ER-preventing diacylglycerol-mediated hepatic insulin resistance. *Proceedings of the National Academy of Sciences of the United States of America* 110(5):1869-1874.
6. Bligh EG & Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology* 37(8):911-917.
7. Qu X, Seale JP, & Donnelly R (1999) Tissue and isoform-selective activation of protein kinase C in insulin-resistant obese Zucker rats - effects of feeding. *The Journal of endocrinology* 162(2):207-214.
8. Kettenbach AN, Sano H, Keller SR, Lienhard GE, & Gerber SA (2014) SPECHT - Single-stage phosphopeptide enrichment and stable-isotope chemical tagging: Quantitative phosphoproteomics of insulin action in muscle. *Journal of proteomics* 114C:48-60.
9. Alpert AJ (2008) Electrostatic repulsion hydrophilic interaction chromatography for isocratic separation of charged solutes and selective isolation of phosphopeptides. *Analytical chemistry* 80(1):62-76.
10. Wessel D & Flugge UI (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Analytical biochemistry* 138(1):141-143.
11. Boersema PJ, Raijmakers R, Lemeer S, Mohammed S, & Heck AJ (2009) Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. *Nature protocols* 4(4):484-494.
12. Wilson-Grady JT, Haas W, & Gygi SP (2013) Quantitative comparison of the fasted and re-fed mouse liver phosphoproteomes using lower pH reductive dimethylation. *Methods* 61(3):277-286.
13. Kettenbach AN & Gerber SA (2011) Rapid and reproducible single-stage phosphopeptide enrichment of complex peptide mixtures: application to general and phosphotyrosine-specific phosphoproteomics experiments. *Analytical chemistry* 83(20):7635-7644.
14. Hao P, Ren Y, Dutta B, & Sze SK (2013) Comparative evaluation of electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) and high-pH reversed phase (Hp-RP) chromatography in profiling of rat kidney proteome. *Journal of proteomics* 82:254-262.
15. Cox J & Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nature biotechnology* 26(12):1367-1372.
16. O'Shea JP, *et al.* (2013) pLogo: a probabilistic approach to visualizing sequence motifs. *Nat Methods*.
17. Heinemann IU, *et al.* (2012) Enhanced phosphoserine insertion during Escherichia coli protein synthesis via partial UAG codon reassignment and release factor 1 deletion. *FEBS letters*.
18. Lajoie MJ, *et al.* (2013) Genomically Recoded Organisms Expand Biological Functions. *Science* 342(6156):357-360.
19. Pirman NL, *et al.* (2015) A flexible codon in genomically recoded Escherichia coli permits programmable protein phosphorylation. *Nature communications* 6:8130.
20. Amiram M, *et al.* (2015) Evolution of translation machinery in recoded bacteria enables multi-site incorporation of nonstandard amino acids. *Nature biotechnology* 33(12):1272-1279.
21. Rappsilber J, Ishihama Y, & Mann M (2003) Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Analytical chemistry* 75(3):663-670.
22. Rappsilber J, Mann M, & Ishihama Y (2007) Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nature protocols* 2(8):1896-1906.