Supplementary Material

Akt-dependent Skp2 mRNA translation is required for exiting contact inhibition, oncogenesis, and adipogenesis

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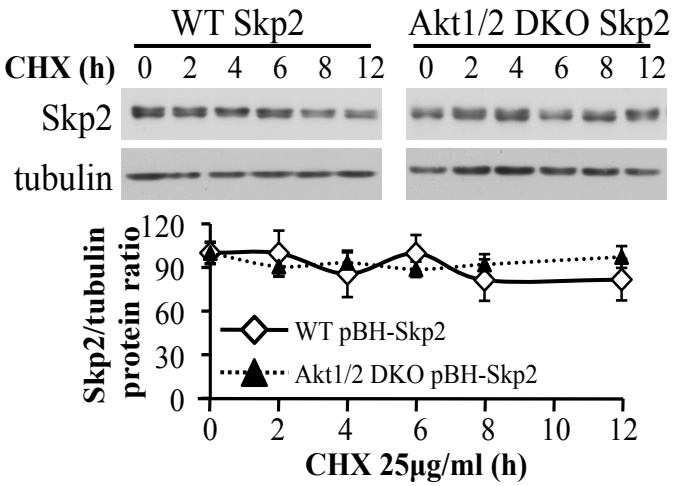
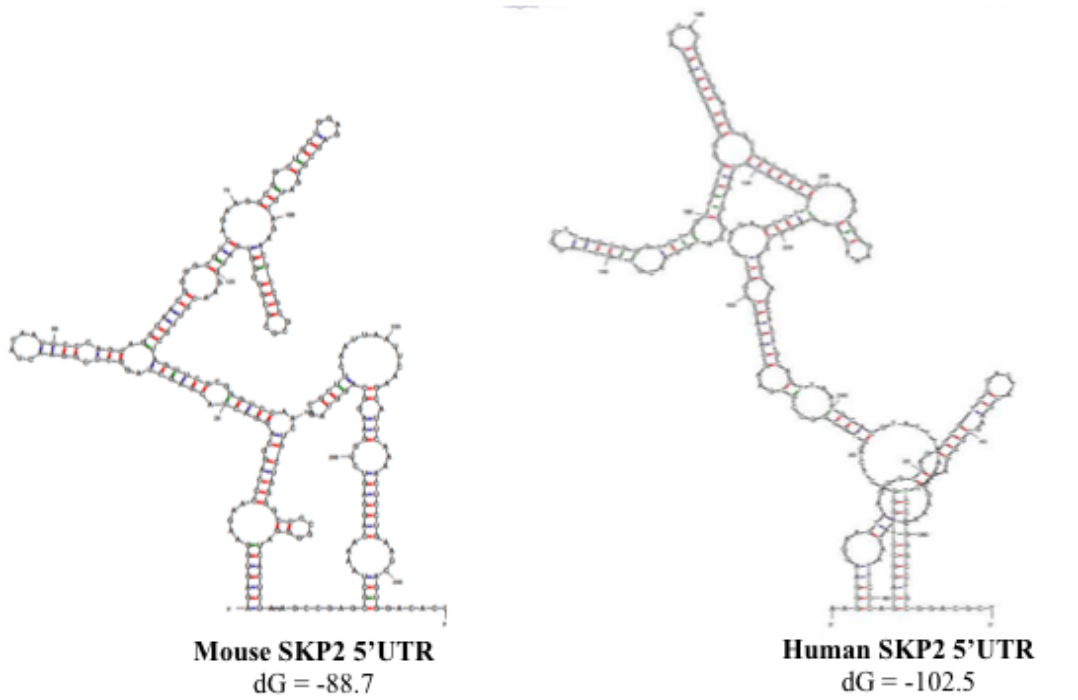
Supplementary Figures

Supplementary Fig. 1



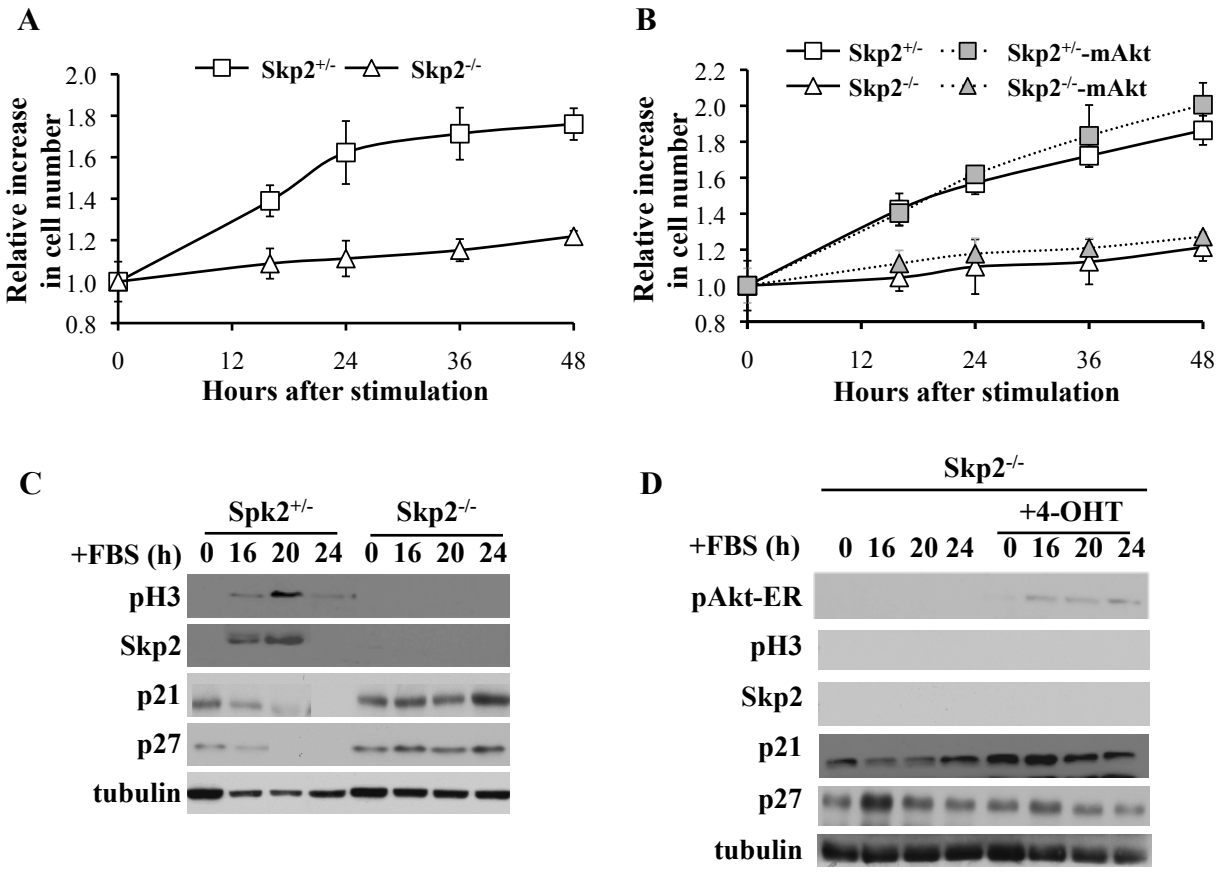
Supplementary figure 1: WT (LT) and Akt1/2 DKO (LT) MEFs expressing ectopic Skp2 were incubated for 0, 2, 4, 6, 8 and 12h with cycloheximide (CHX; 25 µg/ml) and cell lysates were prepared and subjected to immunoblotting with anti-Skp2 and tubulin antibodies. Quantification of at least 3 experiments was performed and expressed as Skp2 over tubulin protein ratio. Results represent the average ±SE of 3 independent experiments.

Supplementary figure 2



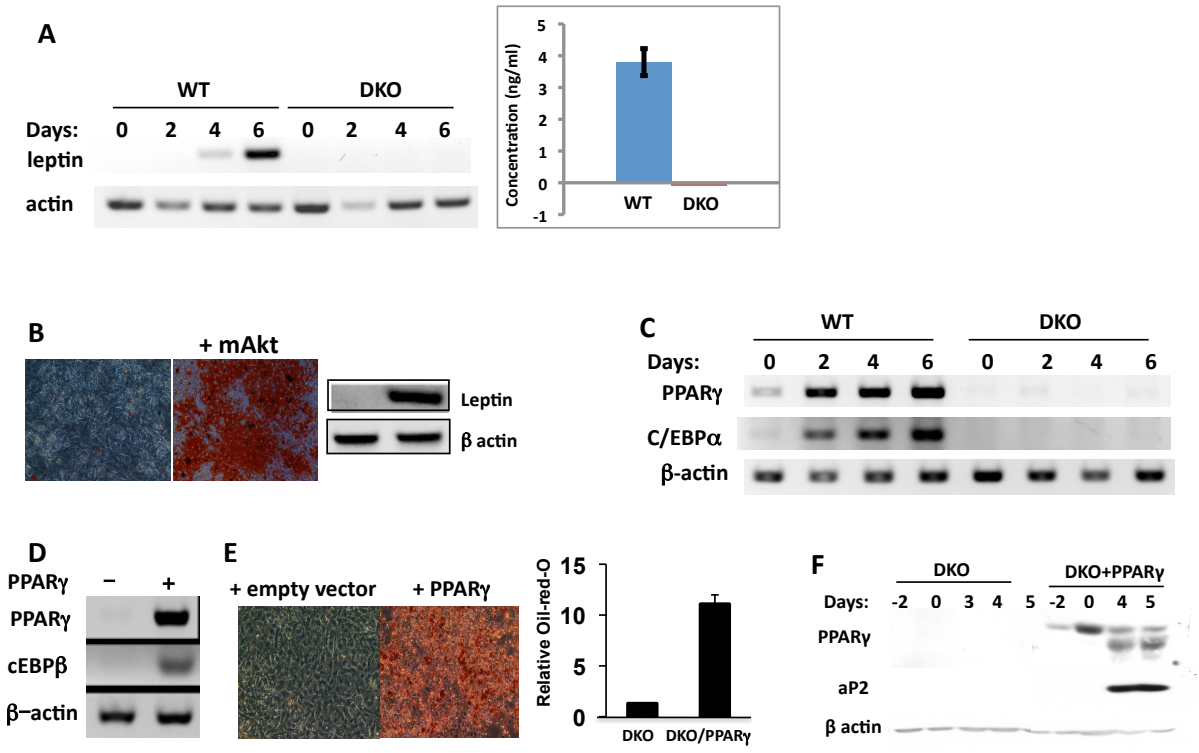
Supplementary figure 2: The 5'UTR structures of Skp2 mRNA. The 5'UTR free energy of mouse and human Skp2 mRNAs were determined and calculated using the mFOLD program (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form2.3).

Supplementary figure 3



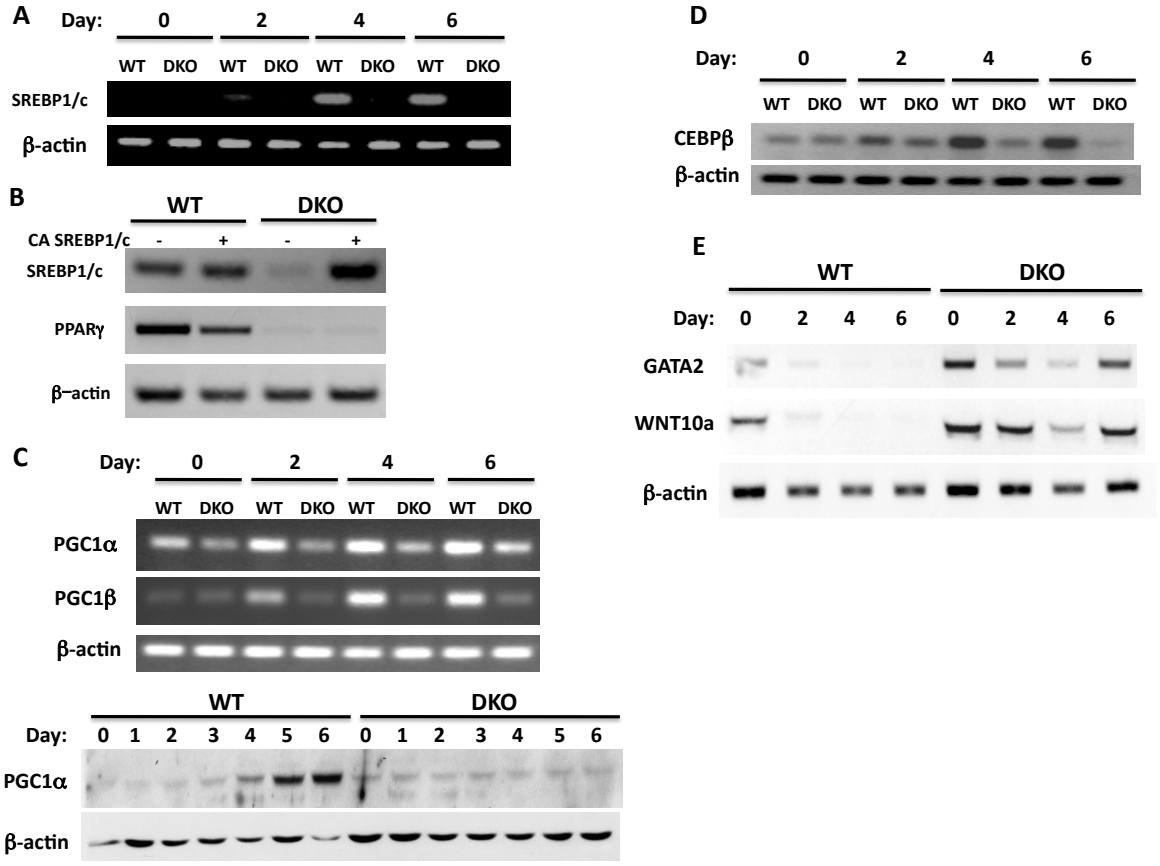
Supplementary figure 3: Activated Akt cannot promote exit from contact inhibition in the absence of Skp2. A. Skp2$^{+/-}$ and Skp2$^{-/-}$ MEFs were grown to confluence. Medium was replaced with medium containing 20% FBS, and cells were counted at time 0, 16, 24, 36 and 48h post addition of 20% FBS. Results represent the average ±SE of 3 independent experiments. **B.** Skp2$^{+/-}$ and Skp2$^{-/-}$ MEFs expressing ectopic mAkt-ER were grown to confluence and then incubated with 4OHT (300nM) for 48h. After this treatment, medium was replaced with medium containing 20% FBS, and cells were counted at time 0, 16, 24, 36 and 48h post-medium replacement. Results represent the average ±SE of 3 independent experiments. **C, D.** Cell lysates from cells treated as in A and B were prepared at 0, 12, 16, 20 and 24h post FBS stimulation and subjected to immunoblotting with anti-phospho-H3, p21, p27, phospho-Akt, Akt, Skp2, and tubulin antibodies.

Supplementary figure 4



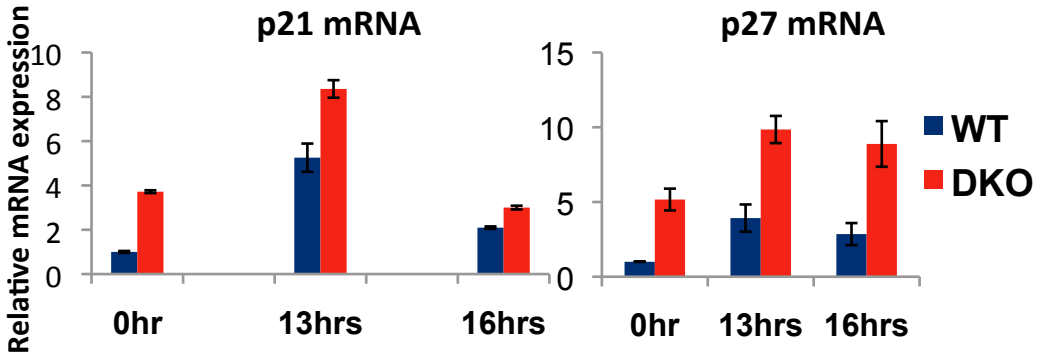
Supplementary figure 4. Akt1 and Akt2 deficiency impairs adipogenesis of preadipocytes due to the failure to induce PPARγ and cEBPα. A. Differentiation of WT and Akt1/2 DKO preadipocytes as assessed by leptin mRNA expression using semi-quantitative RT-PCR (left panel) and by measuring leptin secretion using ELISA (right panel). The average +/- SE of 3 different experiments is shown. **B.** Ectopic expression of myristolated Akt (mAkt) in Akt1/2 DKO preadipocytes was sufficient to induce adipogenesis as measured by Oil-red-O staining and leptin mRNA expression. **C.** WT preadipocytes progressively induce PPARγ and cEBPα expression, as measured by semi-quantitative RT-PCR, during differentiation, but Akt1/2 DKO preadipocytes failed to induce PPARγ and cEBPα expression during a period of 6 days after induction of differentiation. **D.** Ectopic expression of PPARγ in Akt1/2 DKO preadipocytes induces cEBPα. **E.** Ectopic expression of PPARγ in Akt1/2 DKO preadipocytes is sufficient to restore adipocyte differentiation as measured by Oil-red-O staining and quantification. The average +/-SE of 3 different independent experiments is shown. **F.** Ectopic expression of PPARγ in Akt1/2 DKO preadipocytes is sufficient to restore the expression of aP2. Immunoblot shows expression of PPARγ, aP2, and β–actin.

Supplementary figure 5



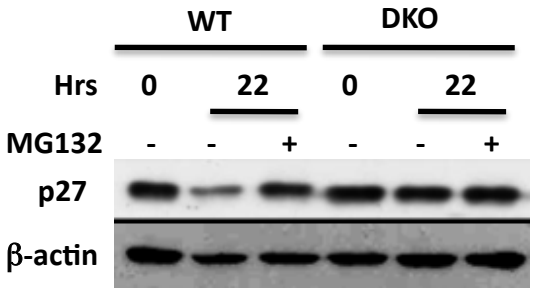
Supplementary figure 5: Akt1/2 DKO preadipocytes fail to induce positive transcriptional regulators repress negative transcriptional regulators of adipogenesis. A. Akt1/2 DKO preadipocytes fail to induce SREBP1/c following induction of differentiation. Induction of SREBP1c mRNA was measured by semi-quantitative RT-PCR, 0, 2, 4, and 6 days after induction of differentiation. **B.** Ectopic expression of activated SREBP1c in Akt1/2 DKO preadipocytes fails to induce PPARγ expression. **C.** The induction of PGC1α and PGC1β during differentiation is impaired in Akt1/2 DKO preadipocytes. Upper panel- assessment of PGC1α and PGC1β mRNA by semi-quantitative RT-PCR. Bottom panel- assessment of PGC1α protein expression by immunoblotting. **D.** Akt1/2 DKO preadipocytes fail to induce cEBPβ mRNA as measured by semi-quantitative RT-PCR, following induction of differentiation. **E.** Akt1/2 DKO preadipocytes fail to repress the expression of the repressors of adipogenesis, GATA2 and Wnt10a, as measured by semi-quantitative RT-PCR, following induction of differentiation.

Supplementary figure 6



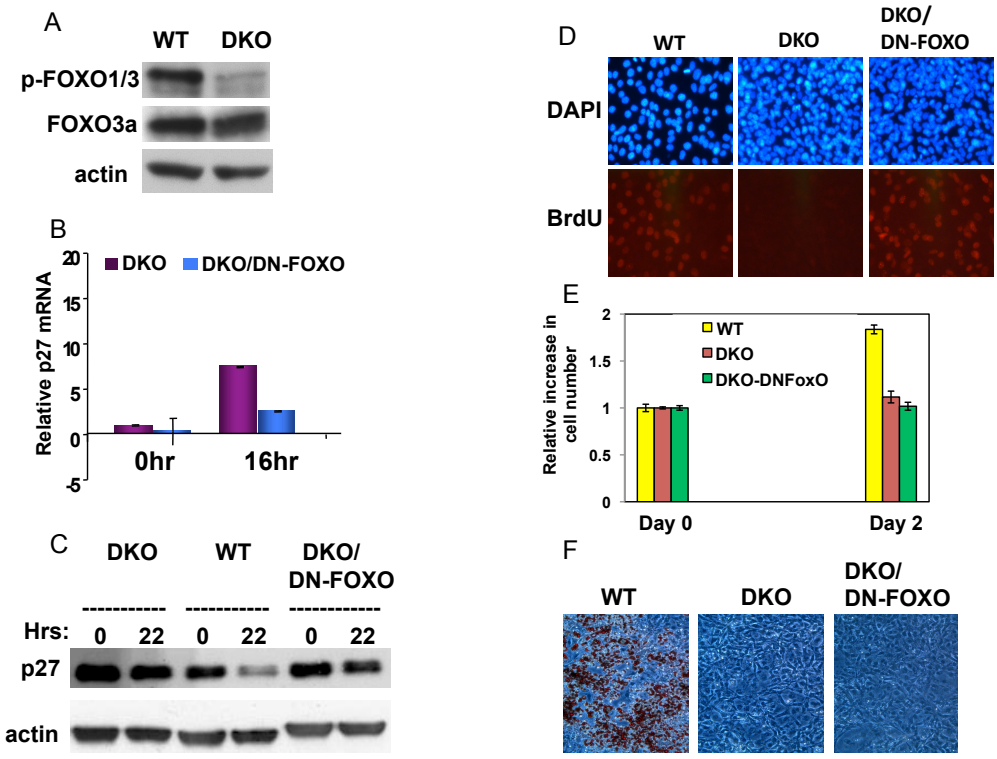
Supplementary figure 6: p21 and p27 mRNA levels, as measured by quantitative RT-PCR, are elevated in both WT and Akt1/2 DKO preadipocytes, immediately after induction of differentiation. p21 and p27 mRNAs are elevated to a higher extent in Akt1/2 DKO preadipocytes. Results represent the average +/- SE of 3 independent experiments.

Supplementary figure 7



Supplementary figure 7. p27 protein stability is downregulated in WT but not in Akt1/2 DKO preadipocytes immediately after induction of differentiation. Twenty two hr after induction of differentiation, the cells were treated (+) or untreated (-) with the proteasome inhibitor MG132 for three hours , and protein extracts were subjected to immunoblotting using anti-p27 antibodies.

Supplementary figure 8



Supplementary figure 8: Stable expression of dominant-negative (DN) FOXO1 in Akt1/2 DKO preadipocytes accelerates entry into S phase but is not sufficient to promote MCE and adipocyte differentiation. A. The phosphorylation of FOXO1 and FOXO3 is impaired in Akt1/2 DKO preadipocytes. Cell extracts from WT and Akt1/2 DKO preadipocytes were subjected to immunoblotting using anti-phospho FOXO1 (Thr24)/FOXO3a (Thr32), anti-FOXO3a, and anti-actin antibodies (Cell Signaling Technology). **B.** Stable expression of DN-FOXO1 in Akt1/2 DKO preadipocytes reduced p27 mRNA levels. RNA was isolated from either Akt1/2 DKO preadipocytes or Akt1/2 DKO preadipocytes stably expressing DN-FOXO1 before (0 h) and 16 h after induction of differentiation and was subjected to q-PCR analysis. **C.** Expression of DN-FOXO1 in Akt1/2 DKO preadipocytes was not sufficient to markedly reduce p27 protein levels following induction of differentiation. Cell extracts were isolated from WT, Akt1/2 DKO preadipocytes or Akt1/2 DKO preadipocytes stably expressing DN-FOXO1 before (0 h) and 22 h after induction of differentiation and were subjected immunoblotting with anti-p27 antibodies. **D.** Expression of DN-FOXO1 in Akt1/2 DKO preadipocytes accelerates entry into S-phase post induction of differentiation. Twelve hours after induction of differentiation cells were exposed to BrdU for 1 h, fixed and stained with DAPI and anti-BrdU antibody. **E.** DN-FOXO1 cannot promote cell division of Akt1/2 DKO preadipocytes following induction of differentiation.

WT, Akt1/2 DKO, and Akt1/2 DKO expression DN-FOXO1 preadipocytes were induced to differentiate. Cell numbers were counted before and 48 h after induction of differentiation. **F.** DN-FOXO1 fails to promote differentiation of Akt1/2 DKO preadipocytes. WT, Akt1/2 DKO, and Akt1/2 DKO expression DN-FOXO1 preadipocytes were subjected to differentiation, and stained with Oil-Red-O at 6 days post-differentiation.

Supplementary materials and methods

Antibodies and reagents: Antibodies against phospho-Akt (Ser473), Akt1, Akt2 panAkt, phospho-S6K1 (Thr389), S6K1, 4E-BP1, 4E-BP2, phospho-CDK1 (Thr161), aP2, FoxO1, FoxO3, and phospho-Foxo1/3 were purchased from Cell Signaling Technology (Beverly, MA). Skp2 antibody was purchased from Abcam. p27 and phospho-H3 antibodies were purchased from BD Biosciences. CDK1, p21. PGC1α, and CDK2 antibodies were purchased from Santa Cruz. β actin and tubulin antibodies were purchased from Sigma. mTORC1 inhibitor, rapamycin, cycloheximide and MG132 were purchased from Sigma.

Preadipocytes isolation and immortalization: Inter scapular brown adipose tissue was isolated from day 18.5 embryos and subjected to collagenase digestion (1 mg/ml of collagenase in isolation buffer containing 0.123 M NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 5 mM glucose, 100 mM Hepes, 1% Pen/Strep and 4% BSA) for 40 minutes. The digested tissue was filtered through a 100-µm nylon filter (PGC Scientific) and centrifuged at 1500 rpm for 5 min. The pellet consisting of precursor cells was washed re suspended in 2 ml of culture medium (Dulbecco's modified Eagle medium (DMEM) containing 25 mM glucose, 20% FBS, 20 mM Hepes, 100 units/ml penicillin/streptomycin), seeded on 3cm plates. After reaching 50% confluence, the cells were infected with pBabe-puro retorivirus expressing SV40 large T. Following infection, the cells were selected with 2ug/ml of puromycin for a week to obtain stable immortalized preadipocyte cell lines. The cells were subsequently grown in DMEM supplemented with 10% FBS, penicillin and streptomycin.

BrdU incorporation: Cells were grown to confluence in each well of a 6-well dish. Two days after confluency was reached, cells were stimulated to reenter the cell cycle by addition of 20% FBS in DMEM. Two-hours prior to each time point, cells were pulse labeled with 3 mg/ml BrdU for 2 hrs, and subsequently fixed with 70% ice-cold ethanol for 20 minutes at -20°C. Cells were permeabilized, and DNA was denatured for 1 hr with 2 M HCl containing 0.5% Triton X-100,

followed by neutralization with 0.1 M sodium borate and rinsing with PBS. Cells were then incubated overnight with mouse anti-BrdU (Dako; diluted 1:200). Cells were rinsed with PBS and then incubated for 2 hr with FITC-conjugated anti-mouse IgG (Vector; 1:200) and prior to counting were counter-stained with DAPI. Three experiments, in triplicate, were performed for each cell line.

Primers for RT-PCR:

For quantitative RT-PCR

β-actin: 5'-AGA GGG AAA TCG TGC GTG AC -3'and 5'-CAA TAG TGA TGA CCT GGC CGT -3'; p21: 5'- CGG TGG AAC TTT GAC TTC GT -3' and 5'- GAA GTA CTG GGC CTC TTG TCC -3'; p27: 5'- CAG CTT GCC CGA GTT CTA CT -3' and 5'- AGA GTT TGC CTG AGA CCC AA -3'; skp2: 5'- AAC TGC GCC TAT TTC ACC AC -3' and 5'- CAG AGT CAG TCG GCA CTT GA -3'. PPARγ: 5'- GCT GTT ATG GGT GAA ACT CTG -3' and 5'- ATA AGG TGG AGA TGC AGG TTC -3'

For Semi-quantitative RT-PCR

WNT10b 5'- ATG GAA GGG TAG TGG TGA GCA AGA -3' and 5'- CTG ACG TTC CAT GGC ATT TGC ACT -3'; GATA2 5'- AGG ATG GCG TCA AGT ACC AAG TGT -3' and 5'- TTC CTT CTT CAT GGT CAG TGG CCT -3'; PPARγ: 5'- AGT CCT TCC CGC TGA CCA AAG C -3' and 5'- CCT CGA TGG GCT TCA CGT TCA G -3'; C/EBPα: 5'- GGC GGG AAC GCA ACA ACA TC -3' and 5'- TGC CCA TGG CCT TGA CCA AG -3'; C/EBPβ: 5'- AGC GGC AGC CTG TCC ACG TC -3' and 5'- TTC CGC AGG GTG CTG AGC TCT C -3'; LEPTIN: 5'- TCC TGT GGC TTT GGT CCT ATC TGT -3' and 5'- TGA GGA CCT GTT GAT AGA CTG CCA -3'; PGC1α: 5'- AGC ACT CAG AAC CAT GCA GCA AAC -3' and 5'- ACT GCG GTT GTG TAT GGG ACT TCT -3'; PGC1β: 5'- TGA CAG CTT ACT GAA AGA GGC CCA -3' and 5'- CGG ACG GAA GCA GCT TTG TTC AAT -3';

Polysomes fractionation and analysis: In brief, 16h post FBS stimulation, cells were treated with 0.1 mg/ml cycloheximide (CHX) in PBS for 3 min at 37°C, washed twice with ice-cold PBS/CHX and collected in hypotonic lysis buffer (5 mM Tris-HCL pH 7.5, 2.5 mM MgCl2, 1.5 mM KCl, 0.1 mg/ml CHX, 2 mM DTT, 0.5% Triton X-100, 0.5% sodium deoxycholate, 400 U/ml RNaseOUT (Invitrogen)). After lysis, cellular debris was removed by centrifugation at 13,000xg for 5 min at 4°C. Lysates were loaded on a 10-ml continuous sucrose gradient (10% to 40% sucrose in 20mM HEPES-KOH (pH 7.6), 100 mM KCl, 5mM $MgCl_2$) and centrifuged in a Beckman SW41-

Ti rotor at 35,000 rpm for 2 hours at 4°C. Gradients were fractionated and the optical density continuously recorded at 254nm. RNA from each fraction was isolated using Trizol (Invitrogen) and treated with DNase (Promega).

mRNA analysis: Total cellular RNA was extracted using Trizol reagent (Invitrogen), reverse transcribed using SuperScript III reverse transcriptase (Invitrogen) to produce first strand cDNA. Semi Quantitative PCR was performed using primers specific to the cDNA of interest using a thermocycler. Relative levels of mRNA were standardized to actin. Real-time PCR was performed on an iQ5 Real-Time PCR Detection System (Bio-Rad) using iQ^{TM} SYBR Green Supermix (Bio-Rad) and following the manufacturer's protocols. Primer sequences used in this study are described in supplementary information: