Cell Metabolism, Volume *4*

Supplemental Data

SREBP Activity Is Regulated by mTORC1 and Contributes to Akt-Dependent Cell Growth

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Supplemental Experimental Procedures

Material

Antibodies for SREBP-1 (2A4) and SREBP2 (1C6) were from BD Biosciences. FASN, ACLY, mTOR/FRAP1, P-Akt (S473, T308 or T450), P-GSK3α/β, P-ACLY (S454) and P-S6 (S240/S244 or S235/236) antibodies were from Cell Signalling and raptor (NB100-766) and rictor (NB100-612) antibodies from Novus. HRP-conjugated anti-GAPDH was from Abcam and β-actin from Sigma. 4-hydroxy-tamoxifen, 25-hydroxycholesterol, cholesterol, SB-216763, 2-deoxy-D-glucose, and insulin were from Sigma. ALLN, rapamycin and AICAR were from Calbiochem. The ACLY inhibitor SB204990 (Pearce et al., 1998) was kindly provided by GlaxoSmithKline. FASN(-150/-43) was kindly provided by T.Osborne (UC Irvine, Irvine). pCDNA3-AU1-TOR was a gift from Dr. Chiang (Burnham Institute for Medical Research, La Jolla). pCDNA3-mycSREBP1a(1-490) wt and T426A/S430A were kindly provided by J. Ericsson (LICR, Uppsala).

Primers for semiquantitative PCR (qPCR)

The following primers were used to detect human transcripts:
fatty acid synthase, forward 5' GAAACTGCAGGAGCTGTC 3'
reverse 5' CACGGAGTTGAGCCGCAT 3'
human GAPDH forward 5' ACAGCCTCAAGATCATCAGCAA 3'
reverse 5' ATGGCATGGACTGTGGTCATG 3'.

The following primers were used to detect fly transcripts:
fatty acid synthase, forward 5' CCCCAGGAGGTGAACTCTATCA 3'
reverse 5' GACTTGACCGATCCGATCAAC 3'
dSREBP, forward 5' GGCAGTTTGTCGCCTGATG 3'

reverse 5’ CAGACTCCTGTCCAAGAGCTGTT 3’
actin, forward 5’ CACCCTGAAGTACCCCATTGAGCAC 3’
reverse 5’ CAGACGCAGGATGGCATGGGGAAGG 3’.

RNA intereference:

Human siRNA oligonucleotides (all from Dharmacon):

Non-Targeting siRNA 001210-01
Non-targeting siRNA 001210-03
Human FRAP1 003008
Human Raptor 1:1 mix of 004107-01 and 004107-03
Human Rictor 1:1 mix of 016984-01 and 016984-02
Human SREBF1 006891
Human SREBF2 009549

Drosophila RNAi sequences:

Primers contained 5’T7 RNA polymerase-binding sites preceded by a GAA overhang and followed by sense (forward) or antisense (reverse) sequence.
dAkt, forward 5’ GACCGTTTGTTCTTCAGCGGCG 3’
reverse 5’ TCCGGAATCGTGTGTAGGGGC 3’
Dp110, forward 5’ CGCGCTCGAAGAAATCGTCC 3’
reverse 5’ TCAGAAAGTGTAAGCACCGG 3’
dfatty acid synthase, forward 5’ CATTCCCGGTAACTTGCACT 3’
reverse 5’ GTTTTCGAAGCTCTTGTCCG 3’
dPTEN, forward 5’ CATGCCCAGCATTACAAA 3’
reverse 5’ TATATATTTGTTAACTGT 3’
dSREBP, forward 5’ GCTACAATTGTCCCCAGCAACAGCCG 3’
reverse 5’ GCCCAGCTGAAGTAAATCCTTCACC 3’
dTSC2, forward 5’ AATGTGCTGACAGCCTTCCT 3’
reverse 5’ GGCACACTCGACTCCAGATGA 3’
gfp, forward 5’ CGACGGCCAGTGAATTGTAATACGACTC 3’
reverse 5’ TACGCCAAGCTCATAATACGACTCACTAT 3’

Fly genotypes:

daughterless-GAL4

engrailed-GAL4 UAS-GFP/Cyo

MS1096-GAL4

wgSp1/Cyo; P(w+ Dpp-GAL4)/TM6b

yw; P(w+, UAS-Dp110WT)

yw; P(w+, UAS-Dp110[KD])

w; UAS-GFP

UAS-Dp110[KD]/SM6a-TM6b; dpp-GAL4/SM6a-TM6b

dSREBP$^{RNAi\text{-}S1}$: *yw; UAS-dSREBPRNAi[8]*

dSREBP$^{RNAi\text{-}S2}$: *yw; UAS-dSREBPRNAi[22]; UAS-dSREBPRNAi[10]*

dSREBP$^{RNAi\text{-}S3}$: *yw; UAS-dSREBPRNAi[18]; UAS-dSREBPRNAi[15]*

8237: *P(UAS-HLH106.Exel)1; yw*

8240: *yw; P(UAS-HLH106.Ndel)3*

8241: *yw; P(UAS-HLH106.NTdel)3*

8243: *P(UAS-HLH106.Cdel)1; yw*

Luciferase detection

U2OS-mycAkt-ER cells were transfected using Effectene (Qiagen) according to manufacturer's instructions. Firefly and renilla luciferase were detected using the Dual-Luciferase Detection kit (Promega) and all experiments were repeated at least twice.

Supplemental Figures

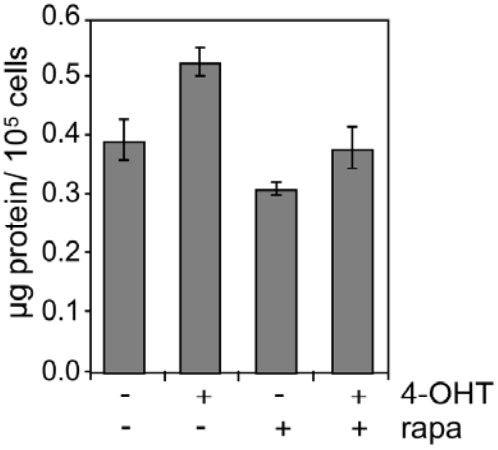

Figure S1: Activation of Akt induces an increase in intracellular protein concentration

RPE myrAkt-ER cells were stimulated with 100 nM 4-OHT or solvent in the presence or absence of 50 nM rapamycin as indicated for 48 hours in 1% LPDS. Total protein concentrations were determined and normalized to cell number. Error bars represent SD.

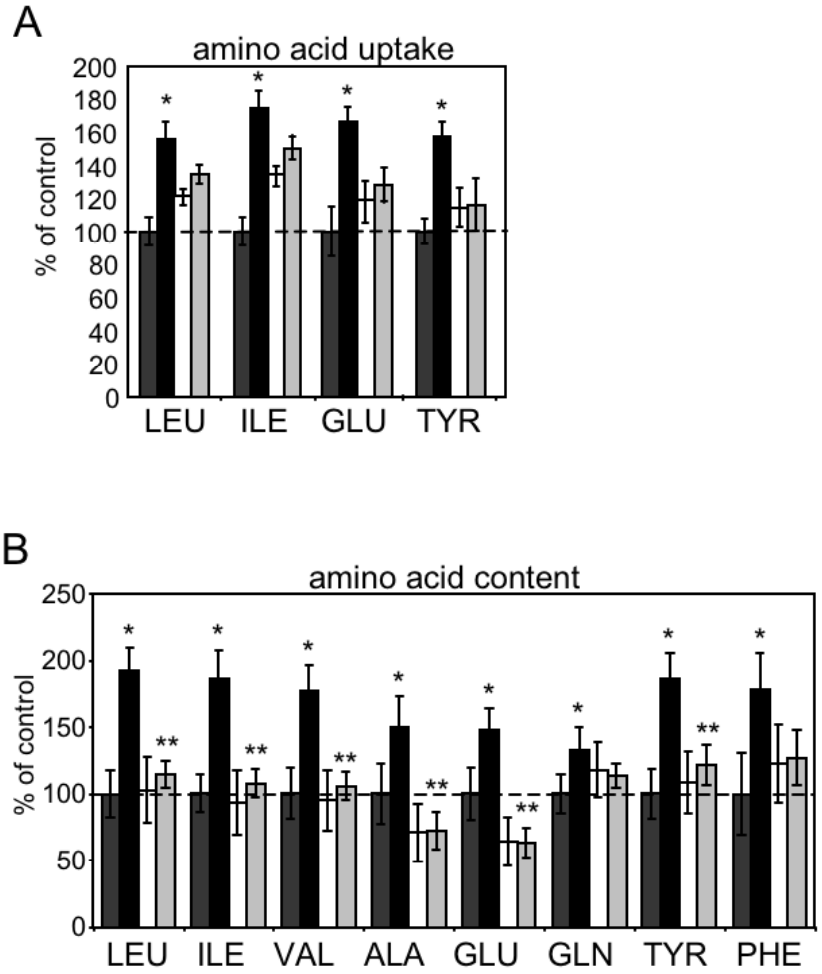

Figure S2: Activation of Akt induces an increase in amino acid uptake and intracellular amino acid concentrations

RPE myrAkt-ER cells were treated with ethanol (dark grey bars), 4-OHT (black bars), rapamycin (open bars) or rapamycin and 4-OHT (light grey bars) for 48 hours in medium containing 1% LPDS. **A)** Amino acid uptake was determined by measuring metabolite concentrations in culture supernatant using NMR spectroscopy. Metabolite levels were compared to medium incubated without cells and are normalised to cell number. **B)** Intracellular metabolite levels were determined using NMR spectroscopy. (*) $p \leq 0.05$ ethanol vs. 4-OHT; (**) $p \leq 0.05$ 4-OHT vs. 4-OHT plus rapamycin. (LEU = leucine, ILE = isoleucine, VAL = valine, ALA = alanine, GLU = glutamate, GLN = glutamine, TYR = tyrosine, PHE = phenylalanine).

Error bars represent SEM.

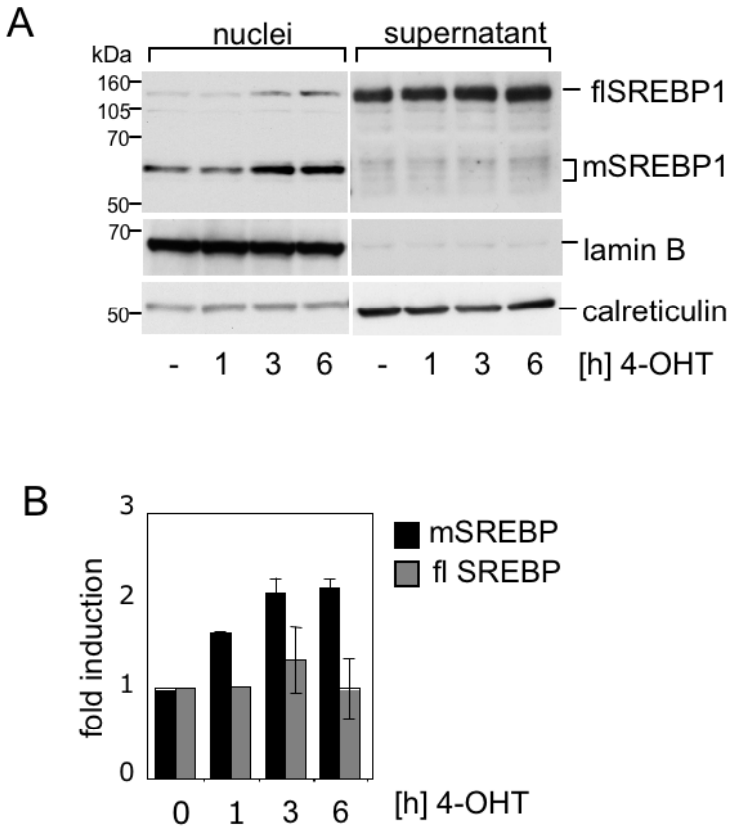

Figure S3: Accumulation of mature SREBP1 in the nucleus in response to Akt activation precedes induction of full length SREBP1

A) RPE myrAkt-ER cells were cultured in medium containing 1% LPDS and treated with 100 nM 4-OHT for the indicated times. Nuclear lysates and membrane-containing supernatants were used to detect mature and full length SREBP1, respectively. FASN was detected in supernatants. Calreticulin and lamin B are shown as loading controls. **B)** Quantitative representation of the results of two independent experiments performed as in (A). Error bars represent SD.

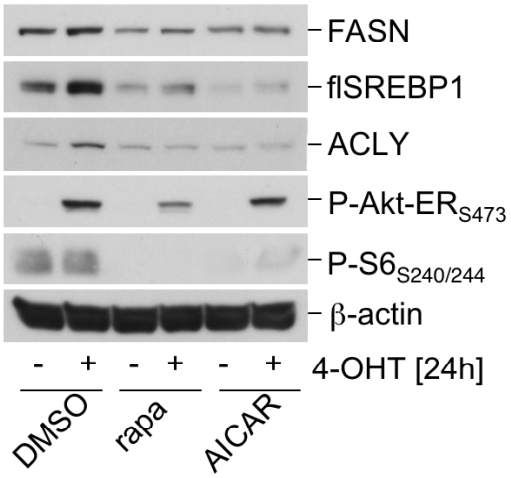

Figure S4: Activation of AMPK prevents induction of FASN and ACLY in response to Akt activation

RPE-myrAkt-ER cells were grown in medium with 1% LPDS and treated with 100 nM 4-OHT or solvent for 24 hours. Whole cell lysates were used to determine expression of FASN, flSREBP and ACLY. Activity of mTORC1 was determined by detecting phosphorylation of S6.

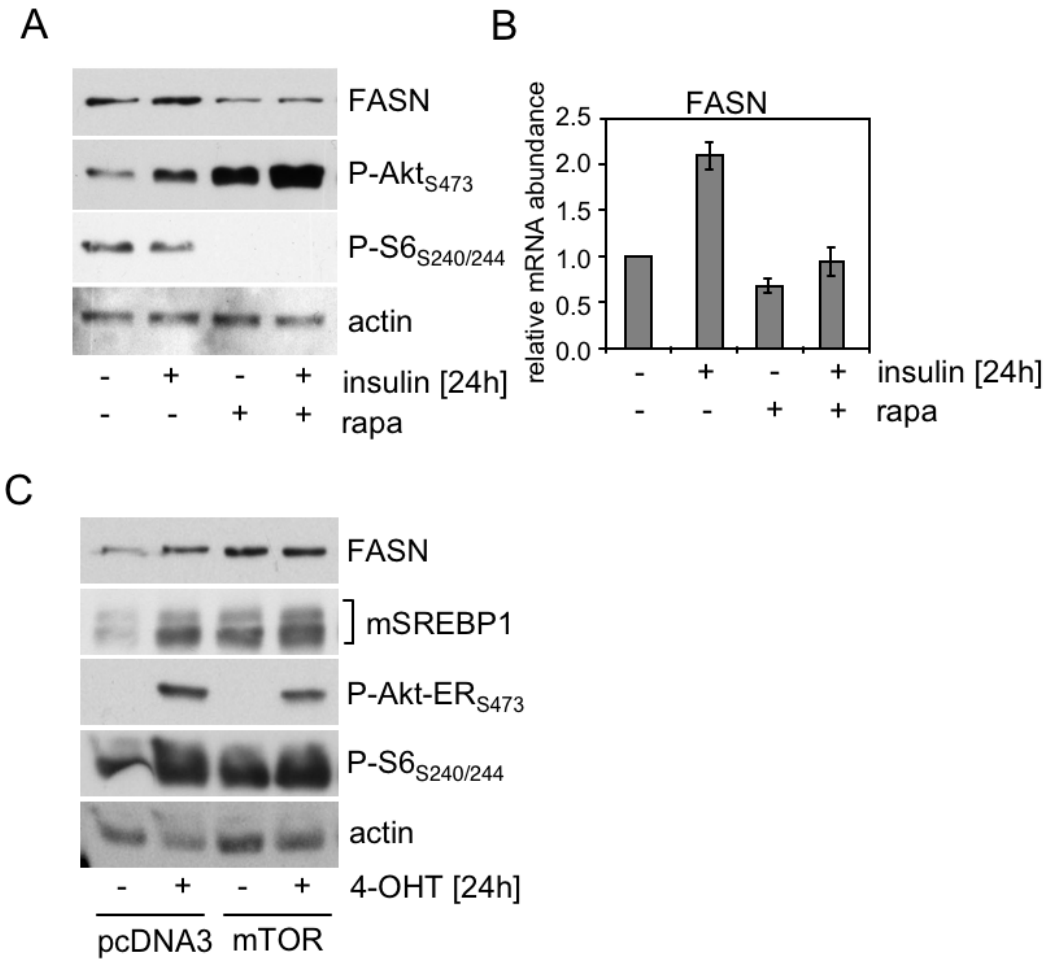

Figure S5: Activation of fatty acid synthase in response to insulin requires mTORC1 activity and overexpression of mTOR can activate FASN expression in the absence of Akt activation

A) RPE cells were treated with 50 nM rapamycin for 30 mins and subsequently induced with 1 µg/ml insulin for 24 hours. Whole cell lysates were analysed for expression of FAS. **B)** RNA from cells treated as in (A) was analysed by qPCR for expression of FASN. Values are normalised to GAPDH expression levels. Error bars represent SD. **C)** U2OS myrAkt-ER cells were transfected with 0.4 µg expression construct for rat mTOR or empty vector, as indicated. 24 hours post-transfection, cells were treated with 100 nM 4-OHT in medium containing 0.5% FCS for 24 hours. Whole cell lysates were analysed for expression of FASN and mature SREBP1.

Activation of Akt or the myrAkt-ER fusion protein was detected using a phosphospecific antibody. Inhibition of mTOR was detected using a phosphospecific S6 antibody. Actin is shown as loading control.

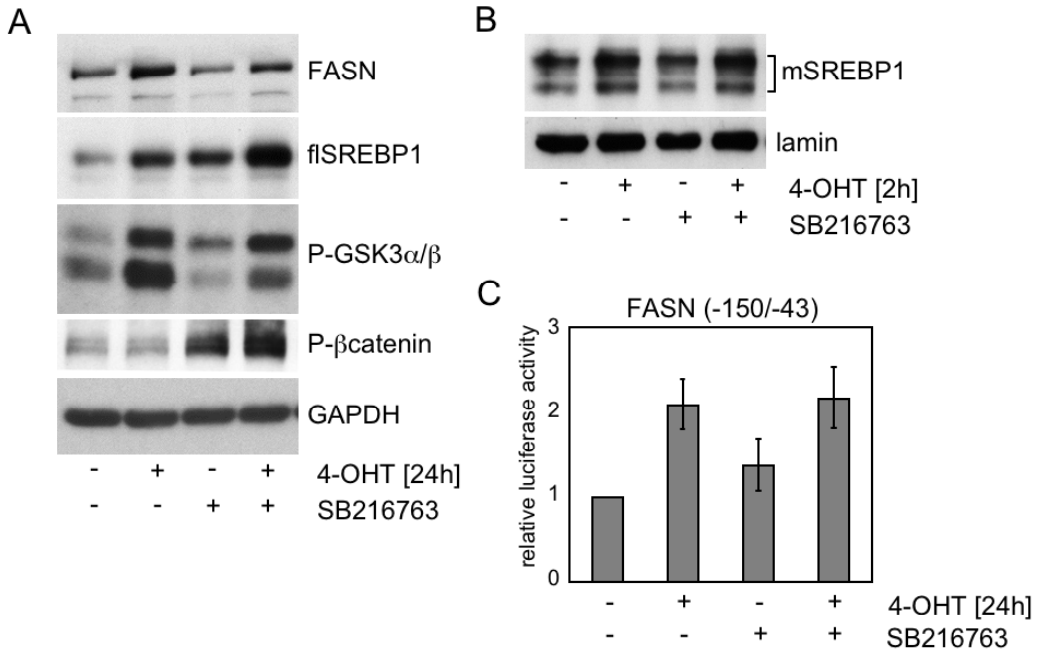

Figure S6: Activation of SREBP1 by Akt is independent of GSK3 activity

A) RPE myrAkt-ER cells were cultured in 1% LPDS and treated with 100 nM 4-OHT or solvent for 24 hours in the absence or presence of 5 μM SB216763. Whole cell lysates were analysed for expression of FASN and full length SREBP1. Activation of myrAkt-ER by phosphorylation of Akt substrates GSK3α/β was detected using a phosphospecific antibody. Stabilisation of ß-catenin in response to GSK3 inhibition was monitored using a phosphospecific ß-catenin antibody. GAPDH is shown as a loading control. **B)** RPE myrAkt-ER cells were starved in 1% LPDS for 16 hours, pre-treated with 5 μM SB216763 for 30 mins and stimulated with 100 nM 4-OHT for 2 hours. Nuclear extracts were prepared and analysed for the presence of mature SREBP1. Lamin is shown as a loading control. **C)** U2OS myrAkt-ER cells were transfected with 0.1 μg of a luciferase reporter construct containing a fragment of the FASN promoter [FASN (-150/-43)]. 24 hours post-transfection, cells were treated as in (A). Data represent three independent experiments performed in duplicate and relative luciferase activity was normalised to the activity of a co-transfected lacZ expression plasmid. Error bars represent SD.

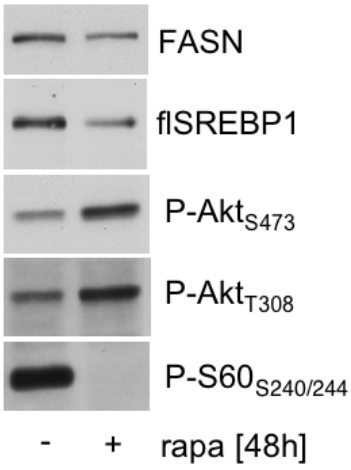

Figure S7: Long-term inhibition of mTORC1 in parental RPE cells leads to activation of Akt phosphorylation but blocks FASN and SREBP1 expression

A) RPE cells were treated with 50 nM rapamycin or solvent (DMSO) for 48 hours. Whole cell lysates were analysed for expression of FASN and full length SREBP1. Activation of myrAkt-ER was detected using S473 and T308 phosphospecific antibodies. Inhibition of mTOR was controlled using a phosphospecific S6 antibody.

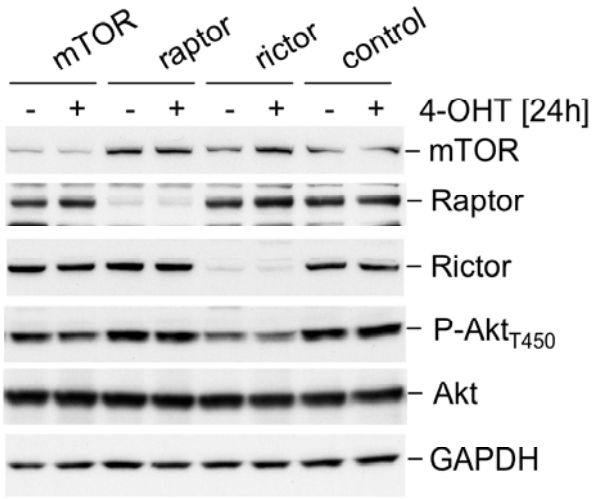

Figure S8: Silencing of mTOR or rictor reduces Akt threonine 450 phosphorylation

RPE myrAkt-ER cells were transfected with 100 nM siRNA oligonucleotides specific for mTOR, raptor, rictor or an unspecific control. 72 hours post-transfection, cells were placed in medium containing 1% LPDS and treated with 4-OHT for 24 hours. Whole cell lysates were analysed for Akt phosphorylation on threonine 450 using a phosphospecific antibody.

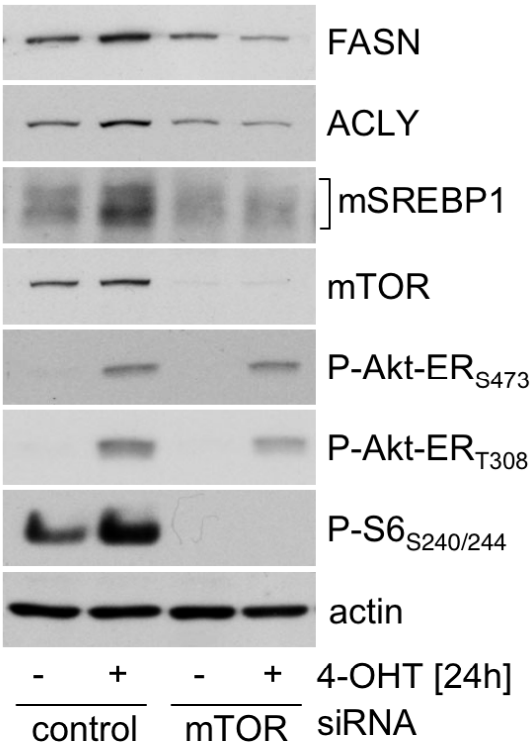

Figure S9: Silencing of mTOR blocks activation of SREBP1 by Akt in U2OS cells

U2OS myrAkt-ER cells were transfected with 70 nM siRNA oligonucleotides specific for mTOR or an unspecific control oligonucleotide as indicated. 24 hours post-transfection, medium was replaced with medium containing 0.5% LPDS and cells were treated with 100 nM 4-OHT (+) or solvent (-) for 48 hours. Whole cell lysates were analysed for expression of FASN, ACLY and mature SREBP1. Activation of myrAkt-ER and S6 were detected using phosphospecific antibodies. mTOR activity was detected using a phosphospecific S6 antibody.

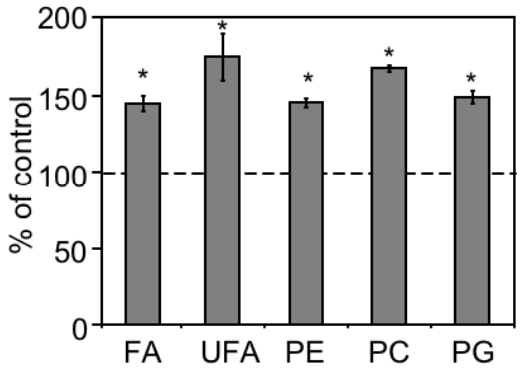

Figure S10: Insulin induces accumulation of fatty acids and phosphoglycerides in Kc167 cells

Kc167 cells were treated with 1 μM insulin for 48 hours. Lipids were extracted and metabolite concentrations were analysed by NMR spectroscopy. Data are shown as mean percentage changes ± SD of insulin-treated cells compared to control cells from three replicate samples (*$p \leq 0.01$). (FA = saturated fatty acids; UFA = unsaturated fatty acids; PE = phosphatidylethanolamine; PC = phosphatidylcholine; PG = phosphatidylglycerol).

Error bars represent SEM.

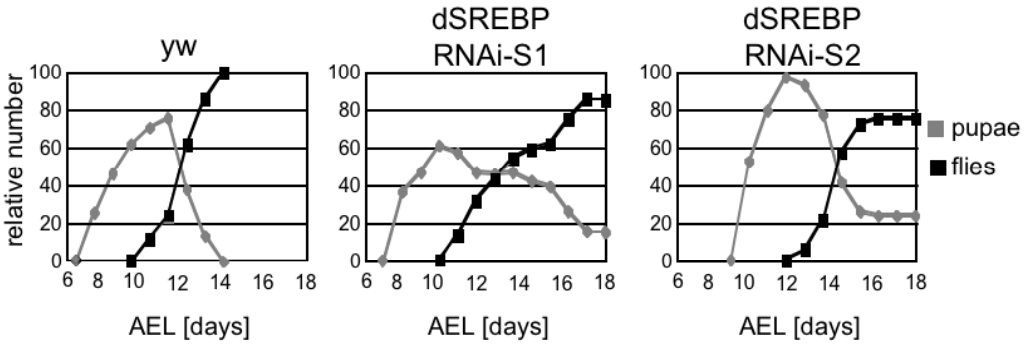

Figure S11: Silencing of dSREBP results in developmental delay and lethality

Control (yw), dSREBP$^{RNAi\text{-}S1}$ and dSREBP$^{RNAi\text{-}S2}$ flies were crossed with *da-GAL4* flies to analyse development. Appearance of pupae and flies was recorded for 18 days after egg laying (AEL). At day 18 total hatched and dead pupae were counted. Relative numbers of pupae/flies are normalised to total number of flies/pupae.

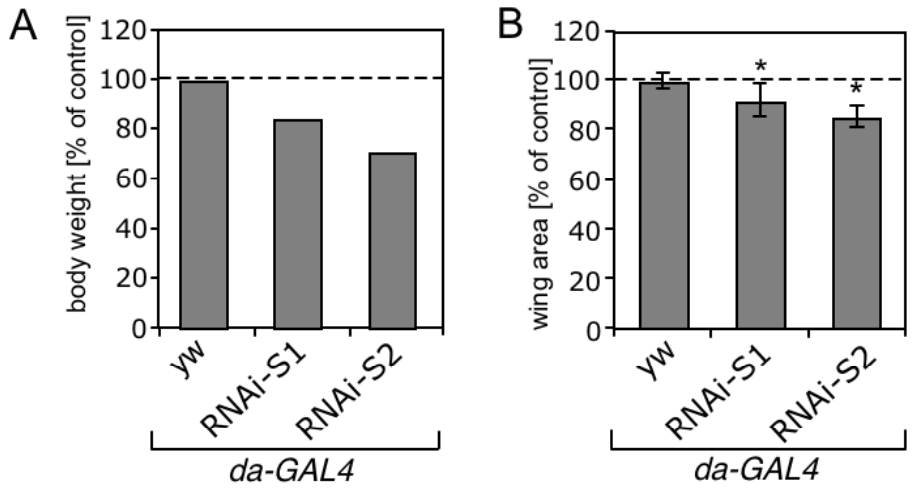

Figure S12: Silencing of dSREBP leads to reduced body weight and wing size

A) Body weight of adult males expressing *dSREBP* RNAi 3 days after eclosion. Data represent cumulative weight of 30 flies and are compared to controls. **B)** Relative wing area of flies expressing dSREBP RNAi. Data are shown as mean percentage changes $\pm$ SD. (*) $p \leq 1.0x10^{-3}$. Error bars represent SD.

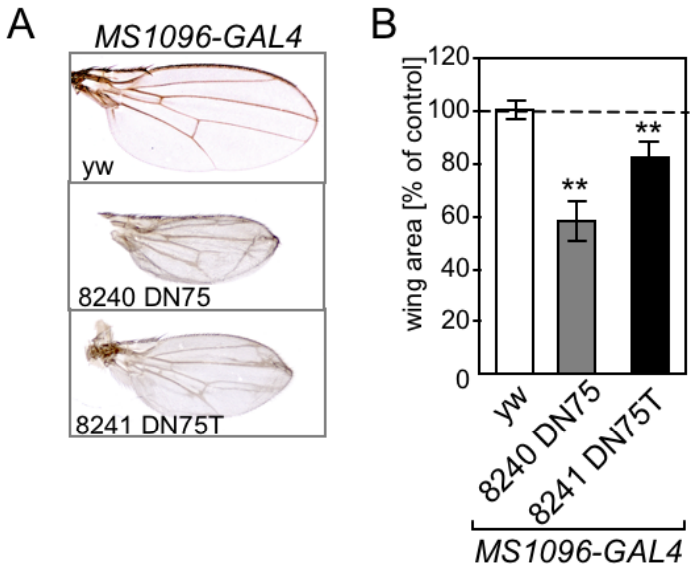

Figure S13: Expression of dominant negative dSREBP reduces wing size

A) Wings of flies expressing dominant negative alleles of dSREBP in the dorsal wing layer using the *MS1096-GAL4* driver. **B)** Wing area analysis of control, $dSREBP^{8240\text{-}DN75}$ and $dSREBP^{8241\text{-}DN75T}$ flies. Data are shown as mean percentage changes. (**) $p \leq 10^{-12}$. Error bars represent SD.

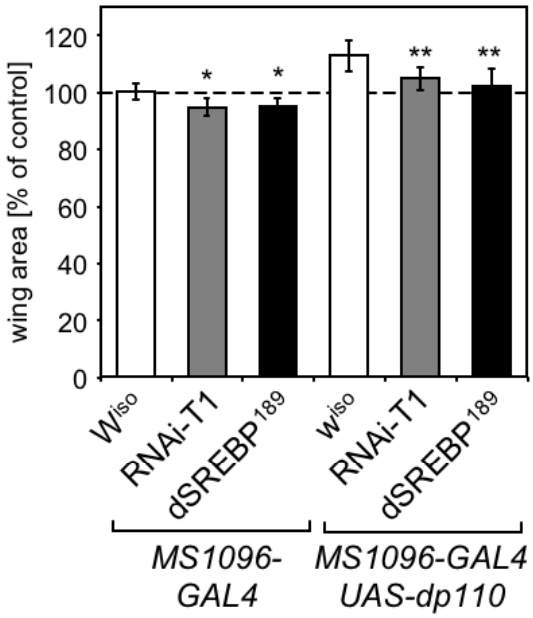

Figure S14: Silencing of dSREBP using an additional RNAi sequence or heterozygous deletion of dSREBP reduces wing size and blocks increase in wing size induced by over-expression of dp110

Control (w^{iso}), UAS-dSREBP-RNAi^{T1} or dSREBP^{189} flies were crossed to *MS1096-GAL4* or *MS1096-GAL4-UAS-dp110* flies. Wings of male flies were analysed for wing size. Data are shown as mean percentage changes compared to controls ($n \leq 20$ wings). (*) $p \leq 5x10^{-6}$ compared to MS1096-GAL4, (**) $p \leq 0.004$ compared to *MS1096-GAL4-UAS-dp110*. Error bars represent SD.