Supplementary Information

FoxO1 inhibits mTORC1 and activates Akt by inducing the expression of Sestrin3 and Rictor

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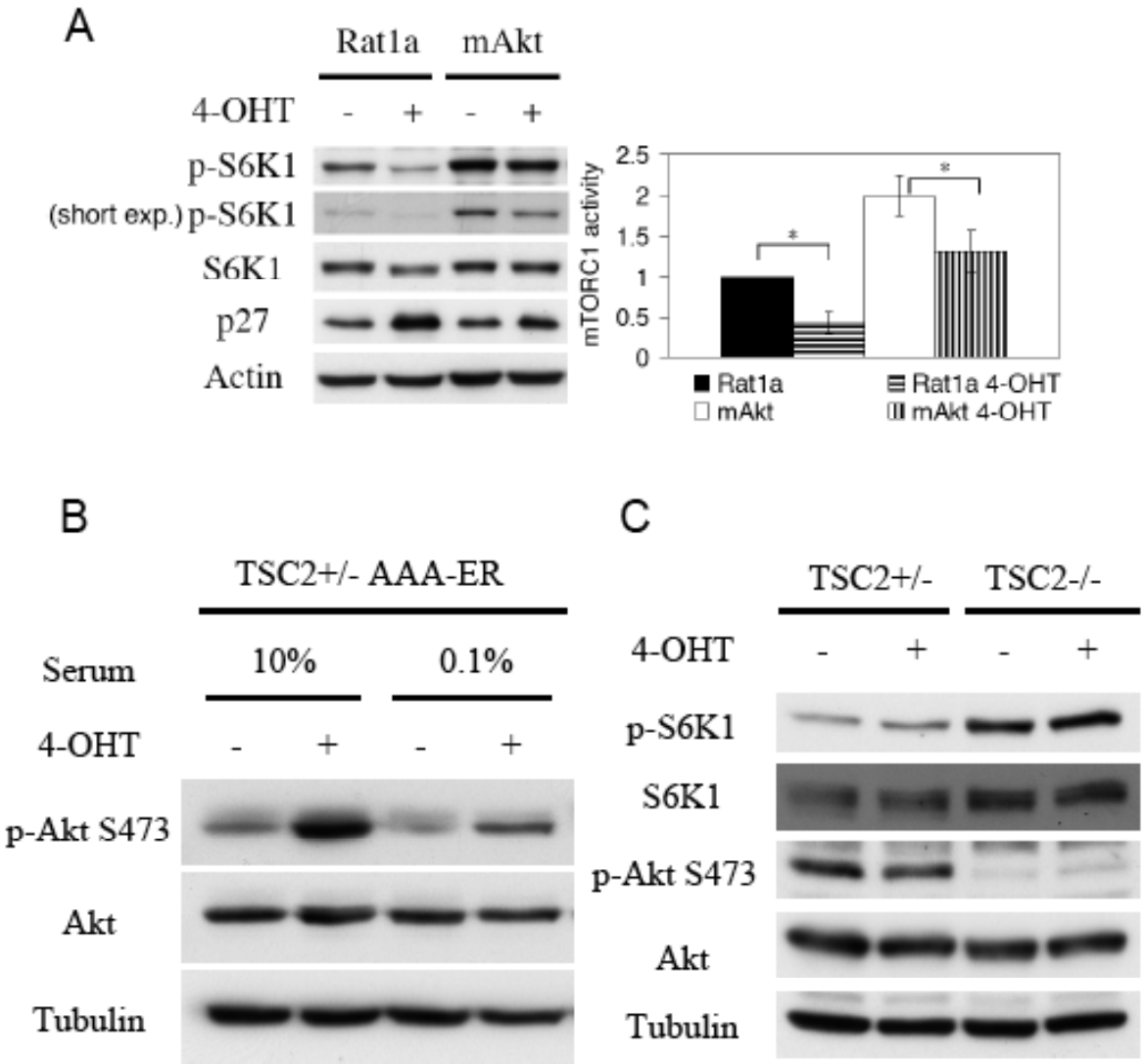

Figure S1: A. **Activation of FoxO1 down-regulates mTORC1 but elevates Akt activity – related to figure 1.** Activated FoxO1 represses mTORC1 activity. Rat1a, Rat1a-mAkt, cells stably expressing FoxO1(AAA)-ER were treated with 300 nM 4-hydroxy tamoxifen (4-OHT) to induce FoxO1(AAA) activity. Twenty-four hours later total protein was extracted and subjected to immunoblotting with the indicated antibodies. Bar graph represents quantification of relative mTORC1 activity (p-S6K) from four independent experiments. *,$P<0.05$. **B. Activated FoxO elevates Akt activity in absence of growth factors.** Sixteen hours prior to 4-OHT treatment, TSC2+/- FoxO1(AAA)-ER cells were cultured in growth medium with either 10% or 0.1% serum. Twenty-four hour after 4-OHT treatment, total protein was extracted and subjected to immunoblotting with the indicated antibodies**. C. 4-hydroxy tamoxifen (4-OHT) does not alter mTORC1 and Akt activity in TSC2+/- and TSC2-/- parental MEFs.** TSC2+/- and TSC2-/- MEFs were cultured in the absence or presence of 300 n 4-OHT for 24 hours. Total protein was extracted and subjected to immunoblotting with the indicated antibodies.

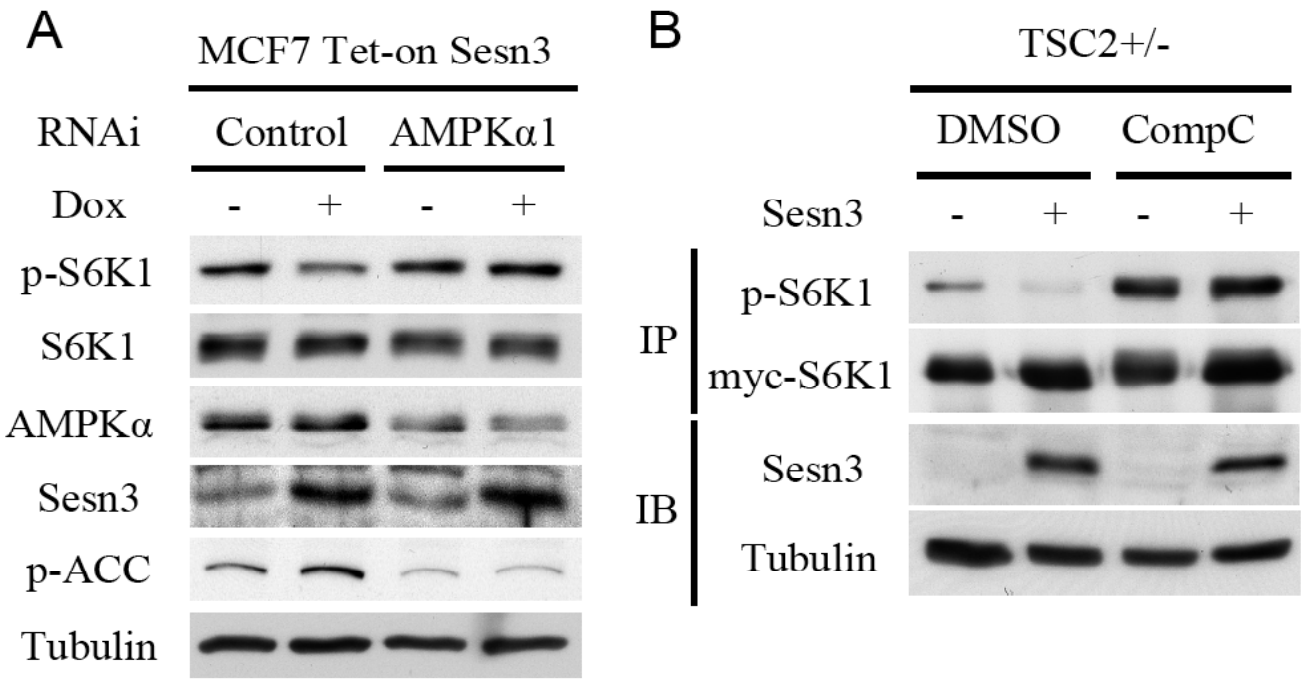

Figure S2: Sesn3 represses mTOR activity through AMPK – related to figure 3.
A. MCF7 Tet-on Sesn3 stable cell line was established by utilizing MCF7 Tet-On Advanced cell line (Clontech 632108) infected with retrovirus expressing pRetroX-Tight-Pur-Sesn3. Cells were transiently transfected with hAMPKα1 or control RNAi (Darmacon) 48 hours prior to Doxycycline treatment. Twenty-four hours after treated with Doxycyclin, total protein were harvested and subjected to immunoblotting analyses. **B.** TSC2+/- MEFs were transiently co-transfected with Sesn3 and myc-S6K1 plasmids. Sixteen hours after transfection, cells were treated with 20 μM compound C or vehicle (DMSO) for 8 hours. Total protein extracts were subjected to immunoprecipitation using 9B-11 myc-tag antibody followed by immunoblotting with anti-pS6K1, 9B-11, and anti-Sesn3 antibodies.

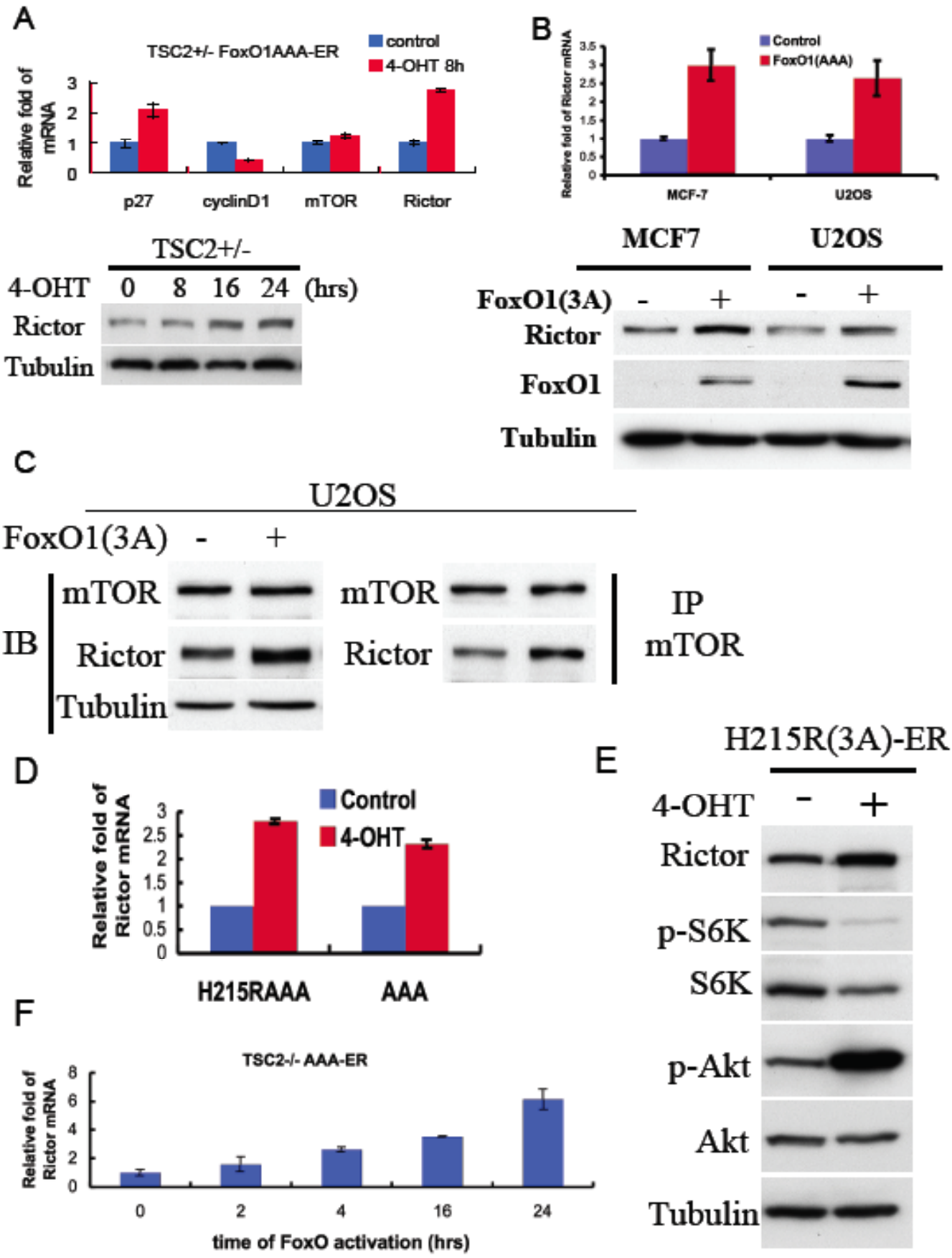
A
TSC2+/- FoxO1AAA-ER
control
4-OHT 8h
Relative fold of mRNA
p27
cyclinD1
mTOR
Rictor
B
Control
FoxO1(AAA)
Relative fold of Rictor mRNA
MCF-7
U2OS
TSC2+/-
4-OHT 0 8 16 24 (hrs)
Rictor
Tubulin
MCF7
U2OS
FoxO1(3A) - + - +
Rictor
FoxO1
Tubulin
C
U2OS
FoxO1(3A) - +
IB
mTOR
Rictor
Tubulin
mTOR
Rictor
IP
mTOR
D
Control
4-OHT
Relative fold of Rictor mRNA
H215RAAA
AAA
E
H215R(3A)-ER
4-OHT - +
Rictor
p-S6K
S6K
p-Akt
Akt
Tubulin
F
TSC2-/- AAA-ER
Relative fold of Rictor mRNA
time of FoxO activation (hrs)

Figure S3: FoxO1 upregulates Rictor mRNA and protein levels and increases mTOR-Rictor association – related to figure 4. A. TSC2+/- FoxO1(AAA)-ER cells were treated with or without 4-OHT and were harvested at the indicated time points for RNA and protein analyses. Total RNA was analyzed by qRT-PCR as described in Experimental Procedures. Total protein was subjected to immunoblotting with anti-Rictor antibody. **B.** MCF7 and U2OS cells were infected with either FoxO1(AAA) or control adenovirus. Forty-eight hour later, total RNA and protein were extracted and subjected to qRT-PCR and immunoblotting. **C.** U2OS cells samples were immunopricipitated with mTOR antibody and subjected to immunoblotting. **D.** Upregulation of Rictor mRNA by FoxO1 does not require its binding to DNA**.** TSC2-/- MEFs were infected with FoxO1(AAA)-ER or FoxO1H215R(AAA)-ER retrovirus to establish polyclonal stable cell lines. Twenty-four hour after 4-OHT treatment cells were harvested for RNA (D) and protein (E) analyses. **E.** FoxO1H215R mutant is sufficient to elevate Rictor, to inhibit S6K1 phosphoryaltion, and to activate Akt phosphryaltion in Tsc2-/- cells. **F.** The kinetics of Rictor induction by FoxO1. TSC2-/- FoxO1(AAA)-ER cells were untreated or treated with 4-OHT, and were harvested at the indicated time points for Rictor mRNA analyses using qRT-PCR.

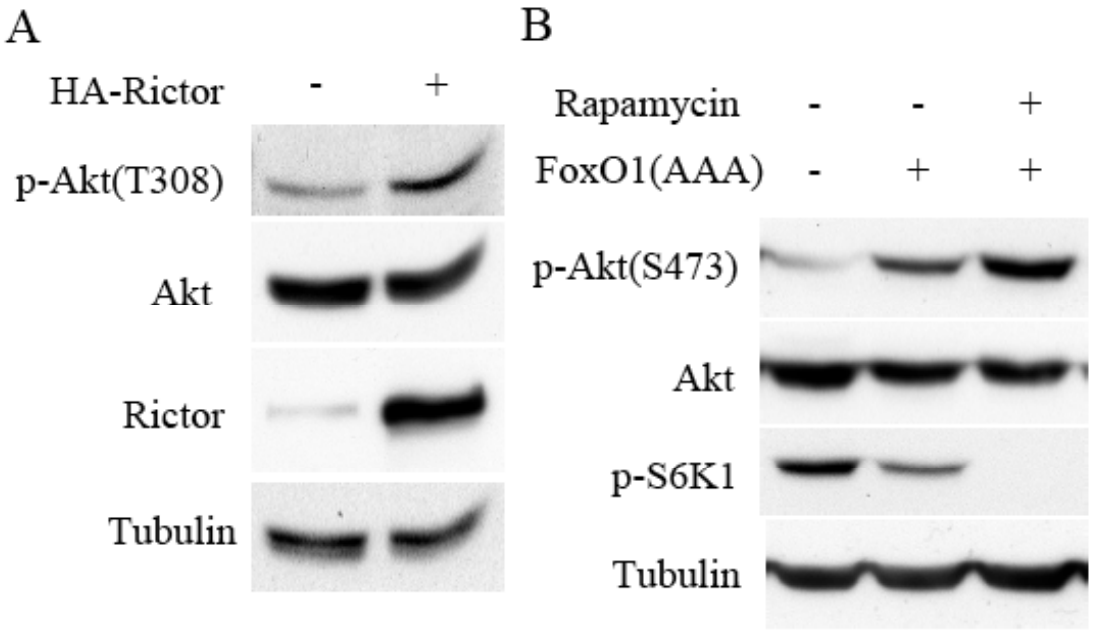

Figure S4. A. Overexpression of Rictor increases T308 phosphorylation of Akt – related to figure 5. HEK293 cells were transiently transfected with HA-Rictor plasmid. Thirty-six hour after transfection, total protein were extracted and subjected to immunoblotting with the specified antibodies. **B. Rapamycin treatment further increased Akt activity when FoxO is active.** TSC2-/- FoxO1(AAA)-ER cells were treated with either 300 nM 4-OHT or 100 nM Rapamycin or both. Total protein extracts were subjected to immunoblotting with indicated antibodies.

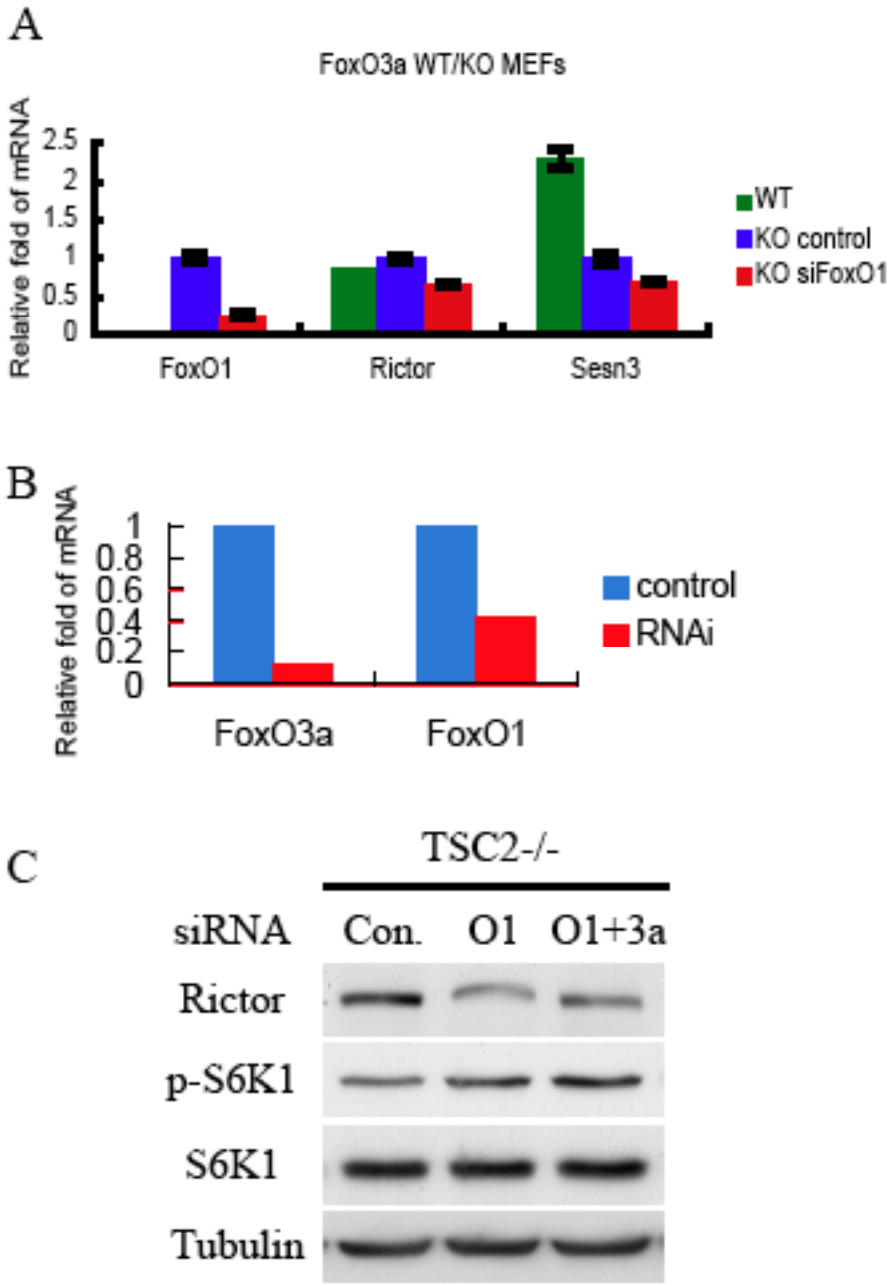

Figure S5: A. The effect of FoxO1 knockdown in FoxO3a-/- cells on the expression of Rictor and Sesn3 – related to figure 6. B. The efficiency of FoxO1 or FoxO3a knockdown in TSC2-/- MEFs. TSC2-/- cells were transiently transfected with FoxO1, FoxO3a, or control RNAi. Forty-eight hour after transfection, total RNA was extracted and subjected to q-PCR. The results show about 90% efficiency for FoxO3a and 60% efficiency for FoxO1. **C. The effect of FoxO1 and FoxO1 + FoxO3a knockdowns in Tsc2-/- cells on the expression of Rictor and the phosphorylation of S6K1.**

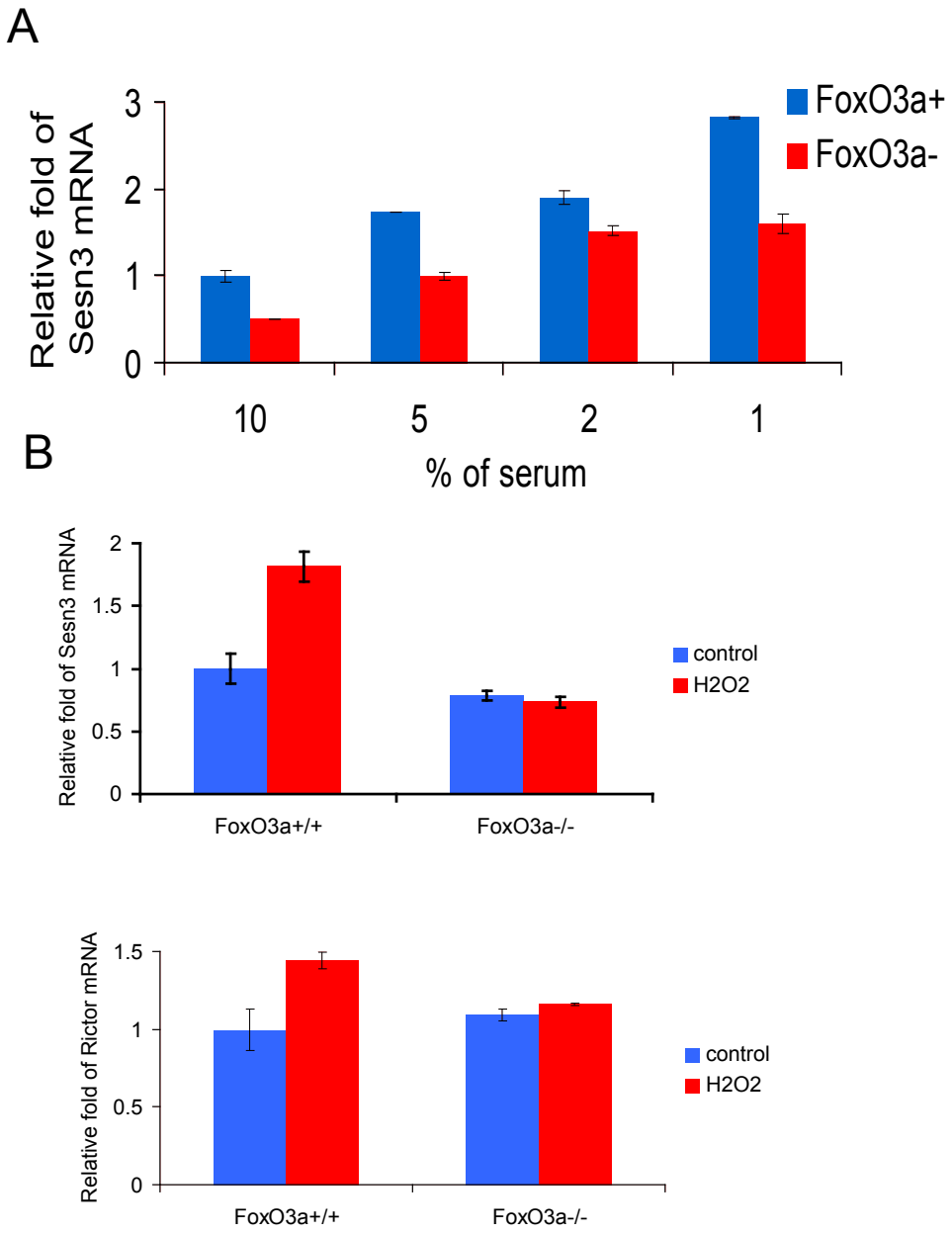

Figure S6. A. Levels of Sesn3 mRNA in different serum concentration-related to figure 7. The level of Sesn3 was quantified by qRT-PCR under the same conditions described in Fig. 8A. **B. Changes in Sesn3 and Rictor mRNA levels by oxidative stress in a FoxO-dependent manner – related to figure 7.** Immortalized isogenic wild-type or FoxO3a-/- MEFs were treated with 0.5mM H_2O_2 and were harvested after 15 minutes at 26°C, for RNA extraction and qRT-PCR.

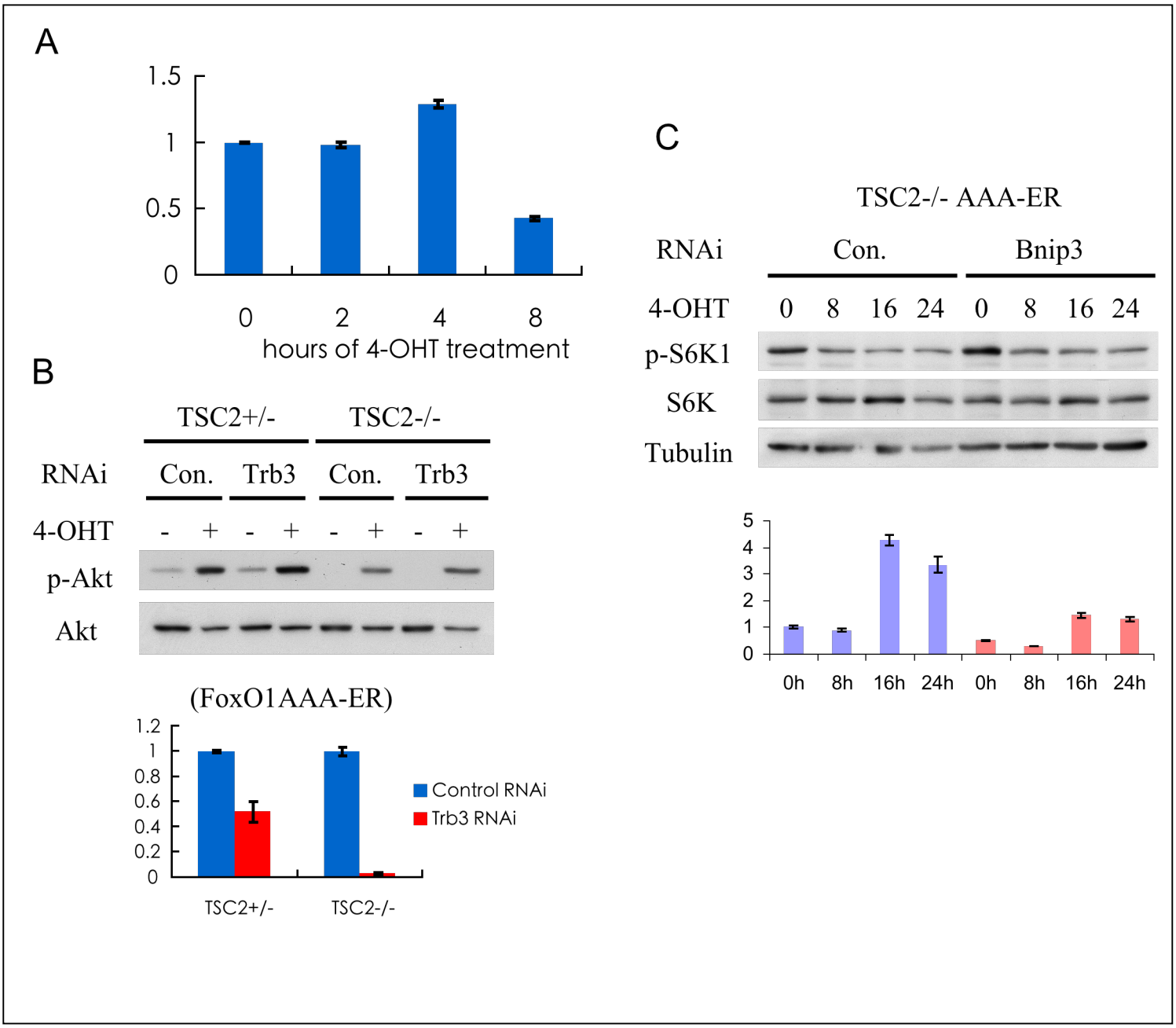

Figure S7: The knockdown of Trb3 does not affect FoxO-mediated Akt phosphoryaltion – related to figure 6 and figure 7. A. TSC2-/- FoxO1(AAA)-ER cells were treated with 4-OHT at the indicated time points. Total RNA was extracted and subjected to qRT-PCR. **B.** TSC2+/- and TSC2-/- FoxO1(AAA)-ER cells were transiently transfected with Trb3 or control RNAi. Total RNA was extracted for qRT-PCR. Forty-eight hour after transfection, cells were treated with 4-OHT for 16 hours prior to protein analyses. **C. The knockdown of Bnip3 does not affect FoxO mediated mTORC1 activity – related to figure 6 and figure 7.** TSC2-/- FoxO1(AAA)-ER cells were transiently transfected with Bnip3 or control RNAi. Forty-eight hour after transfection, cells were treated with 4-OHT and harvested at indicated time points for protein and RNA analyses. The onset of Bnip3 mRNA induction by FoxO is observed at 16 hours after 4-OHT treatment, while p-S6K is reduced as early as 8 hours after FoxO activation shown by immunoblotting.

Supplemental Experimental Procedures

Reagents and antibodies

Dulbecco's modified Eagle medium (DMEM, 25 mM Glucose) and MEM were purchased from Invitrogen; fetal bovine serum (FBS) from Germini; protein A/G agarose from Santa Cruz Biotechnology; antibodies against Sesn3 from Proteintech, p27, ERα (HC-20) and FoxO1 antibodies (H-128, for ChIP assay) from Santa Cruz Biotechnology, actin and tubulin antibodies from sigma, Rictor antibody (A300-459A, for IP) from Bethyl, HA tag antibody from Covance, 4E-BP1 antibody is a gift from Dr. Nahum Sonenberg, and antibodies to mTOR, Rictor, Raptor, Rheb, Akt, phospho-Akt(S473), phospho-Akt(T308), S6K1, phospho-S6K1(S371), S6, phospho-S6, phospho-4E-BP(S65), phospho-4E-BP(T37/46), TSC2, FoxO1, and myc-Tag (9B-11) from Cell Signaling.

Plasmids

pLA-cmv hSesn3-1 (long form) and hSesn3-2 (short form) were gifts from Dr. Peter Chumakov; pRK5-S6K1 from Dr. Nahum Sonenberg; pRL-mAVO3 (Rictor) from Dr. Michael Hall; FoxO1(AAA)-ER were cloned into pBabe-puro vector. To generate the Sesn3 promoter reporter plasmid, human Sesn3 intron 1 (+746~ +865) region was synthesized by Intergarted DNA Technologies (IDT) (sense 5'- CGA ATT CCT TAG CAC CCC CCT GCC CCC GTG TTG ATC CAG AAA AAT GAG GCG CAC CAT CAG TTT ATT GCT GCT TCC TAG AGT GAC TGA TAG AAA GTT TAC TCC AGT ATT GTT TAG CTT ACT TGG- 3' and antisense 5'- CTA GCC AAG TAA GCT AAA CAA TAC TGG AGT AAA CTT TCT ATC AGT CAC TCT AGG AAG CAG CAA TAA ACT GAT GGT GCG CCT CAT TTT TCT GGA TCA ACA CGG GGG CAG GGG GGT GCT AAG GAA TTC GGT AC- 3'). Double strand DNA was formed by annealing two single strand DNA and cloned into pGL3-TATA vector (KpnI- NheI).

Retrovirus, lentivirus and adenovirus production and infection

High titer retrovirus expressing FoxO1(AAA)-ER, FoxO1H215R(AAA)-ER, dominant-negative p53, and mAkt were produced by transfecting phoenix cells. Forty-eight hours after transfection, medium was collected and concentrated by amicon ultrafiltration membrane (Millipore). For virus infection, $1x10^5$ cells were seeded in 6 cm dish the day before and incubated with concentrated virus in presence of polybrene. Lentivirus expressing Rictor shRNA was prepared by using Block-iT Lentiviral RNAi expression system (Invitrogen) and concentrated to obtain higher titer (Rictor shRNA sense- CAC CGA GAC AAG GCC AAT CTT CAT GCG AAC ATG AAG ATT GGC CTT GTC, antisense- AAA AGA CAA GGC CAA TCT TCA TGT TCG CAT GAA GAT TGG CCT TGT CTC). Human AMPKα1 shRNA lentiviral vector is a gift from Dr. Michael Karin. For each experiment, cells were seeded the day before and infected by high titer virus 48 hours prior to FoxO activation. FoxO1(AAA) and control adenovirus were amplified by infecting 293A cells and concentrated by using Adenopack (Vivascience). Virus titer was determined by Adeno-X titer kit (Clontech). For adenovirus infection, high titer virus stock was diluted into culture medium at optimal MOI (5000 MOI for NIH3T3, 10 MOI for DOV13, 500 MOI for MCF7, and 50 MOI for U2OS).

Real- time quantitative PCR

Total RNA was extracted at indicated time point and condition by using TRIzol reagent (Invitrogen), and first strand cDNA was produced with SuperScrip III reverse transcriptase (Invitrogen). Quantitative PCR was performed with iQ SYBR green super mix (BIO-RAD) and quantified with iQ5 real-time PCR detecting system. Relative levels of mRNA were compared by using actin or gapdh primers to normalize for total RNA input. Primer sequences are the followings: mouse and rat p27 5'-CAG CTT GCC CGA GTT CTA CT -3' and 5'- AGA GTT TGC CTG AGA CCC AA -3';

mCyclinD1 5’- AGT GCG TGC AGA AGG AGA TT -3’ and 5’- CTC TTC GCA CTT CTG CTC CT -3’; rCyclinD1 5’- AGC AGA AGT GCG AAG AGG AG -3’ and 5’- GCT CCA GAG ACA AGA AAC GG -3’; mouse mTOR 5’- AAG GAA ATG CAG AAG CCT CA -3’ and 5’- CTC CGT GGA TTC GAT CAT CT -3’; rat mTOR 5’- ACA TGC TGC AGG ACT CCT CT -3’ and 5’- GCA GCA CTT CAA GCA GAG TG -3’; mRictor 5’- GTG CTA GAT CCA GGC CAG AC -3’ and 5’- GGT CGC CCT CAC TGA GAT GT -3’; rRictor 5’- GTG CTA GAT CCA GGC CAG AC -3’and 5’- GGT TGC CCT CAC TGA GAT GT -3’; mRaptor 5’- GCC TGG AGT CAC ACT GGA TT -3’ and 5’- CAG TTC AGC TCT CCC AGA GG -3’; rRaptor 5’- AAC CTG AGC TTG ACG GAA GA -3’ and 5’- AAG GTG CTG CTG GCA CTA CT -3’; mouse and rat Rheb 5’- GCT TTG GCA GAA TCT TGG AA -3’ and 5’- ACA TCA CCG AGC ACG AAG AC -3’; mSin1 5’- TGG CAG TAC ACG AGT GAA GG -3’ and 5’- CAA TAT GCA GGC AGT AGG CA -3’; mPRAS40 5’- TGC TCC TAG TCC ACC ACC TC -3’ and 5’- CGT CCT CAT CTT CCT CCT CA -3’; mFoxO1 5’- CAA TCT GTC CCT TCA CAG CA -3’ and 5’- CTC CCT CTG GAT TGA GCA TC -3’; mSesn1 5’- GGA CGA GGA ACT TGG AAT CA -3’ and 5’- ATG CAT CTG TGC GTC TTC AC -3’; hSesn1 5’- GCA TGT TCC AAC ATT TCG TG -3’ and 5’- TCC CAC ATC TGG ATA AAG GC -3’; mSesn2 5’- TAG CCT GCA GCC TCA CCT AT -3’ and 5’- TAT CTG ATG CCA AAG ACG CA -3’; hSesn2 5’- GAC CAT GGC TAC TCG CTG AT -3’ and 5’- GCT GCC TGG AAC TTC TCA TC -3’; mSesn3 5’- CAT GCG TTT CCT CAC TCA GA -3’ and 5’- GGC AAA GTC TTC GTA CCC AA -3’; hSesn3 5’- ATG CTT TGG CAA GCT TTG TT -3’ and 5’- GCA AGA TCA CAA ACG CAG AA -3’; mActin 5’-AGA GGG AAA TCG TGC GTG AC -3’ and 5’-CAA TAG TGA TGA CCT GGC CGT -3’; hActin 5’-CCA TCA TGA AGT GTG ACG TGG -3’ and 5’-GTC CGC CTA GAA GCA TTT GCG -3’; rat GAPDH 5’-ATG TTC GTC ATG GGT GTG AA -3’ and 5’-GGT GCT AAG CAG TTG GTG GT -3’.