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Supplementary Information for manuscript,

"Regulation of GSK3 cellular location by FRAT modulates mTORC1-dependent cell growth and sensitivity to rapamycin"

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Table 1

List of proteins whose phosphorylation is potentially suppressed by mTORC1 (data obtained from SILAC-based mass spectrometry analysis of the mTORC1-regulated phosphoproteome as described [12].

Gene	Log2(	Annotation	
Hmgn1;L	-4.0893	Non-histone chromosomal protein HMG-14	Chromosome remodel
Atrx	-2.6173	Transcriptional regulator ATRX	Chromosome remodel
Mett10d	-2.1007	Isoform 1 of Putative methyltransferase METT10D	Chromosome remodel
Chd4	-1.7479	Chromodomain-helicase-DNA-binding protein 4	Chromosome remodel
Srcap	-1.4029	Putative uncharacterized protein	Chromosome remodel
Ezh2	-1.1269	Isoform ENX-1B of Histone-lysine N-methyltransferase EZH2	Chromosome remodel
Tip60	-0.57	Isoform 1 of Histone acetyltransferase HTATIP	Chromosome remodel
Pds5b	-2.0744	Isoform 1 of Sister chromatid cohesion protein PDS5 homolog B	DNA repair
Cdt1	-1.3039	DNA replication factor Cdt1	DNA replication
Dgcr8	-2.0617	Protein DGCR8	miRNA
Lmnb1	-2.2046	Lamin-B1	Nucleopore
Nup153	-1.6324	Nucleoporin 153	Nucleopore
Tcof1	-2.2181	Treacle protein	rDNA transc
Senp3	-2.0671	Sentrin-specific protease 3	rDNA transc
Zcchc8	-2.3802	Zinc finger CCHC domain-containing protein 8	splicing
Srrm2	-1.4846	Isoform 3 of Serine/arginine repetitive matrix protein 2	splicing
Prpf4b	-1.3629	Serine/threonine-protein kinase PRP4 homolog	splicing
Rprd2	-1.2854	Isoform 1 of Regulation of nuclear pre-mRNA domain-containing pro	splicing
Acin1	-1.071	Isoform 1 of Apoptotic chromatin condensation inducer in the nucleu	splicing
2610101N	-1.0703	Isoform 3 of U2-associated protein SR140	splicing
Ahnak	-1.4738	AHNAK nucleoprotein isoform 1	splicing
Gtf2f1	-4.2298	General transcription factor IIF subunit 1	Transcription
Foxk1	-3.9502	Forkhead box protein K1	Transcription
Foxk2	-3.2075	Isoform 1 of Forkhead box protein K2	Transcription
Tcea1	-2.4464	Isoform 2 of Transcription elongation factor A protein 1	Transcription
Tcfeb	-1.7984	Transcription factor EB	Transcription
Fam117b	-1.7549	Protein FAM117B	Transcription
Grlf1	-1.6907	Glucocorticoid receptor DNA-binding factor 1	Transcription
Gatad2b	-1.2476	Isoform 1 of Transcriptional repressor p66-beta	Transcription
Jun	-1.1254	Transcription factor AP-1	Transcription
Junb	-1.0586	Transcription factor jun-B	Transcription
Pcif1	-2.1367	Phosphorylated CTD-interacting factor 1	Transcription

Figure S1

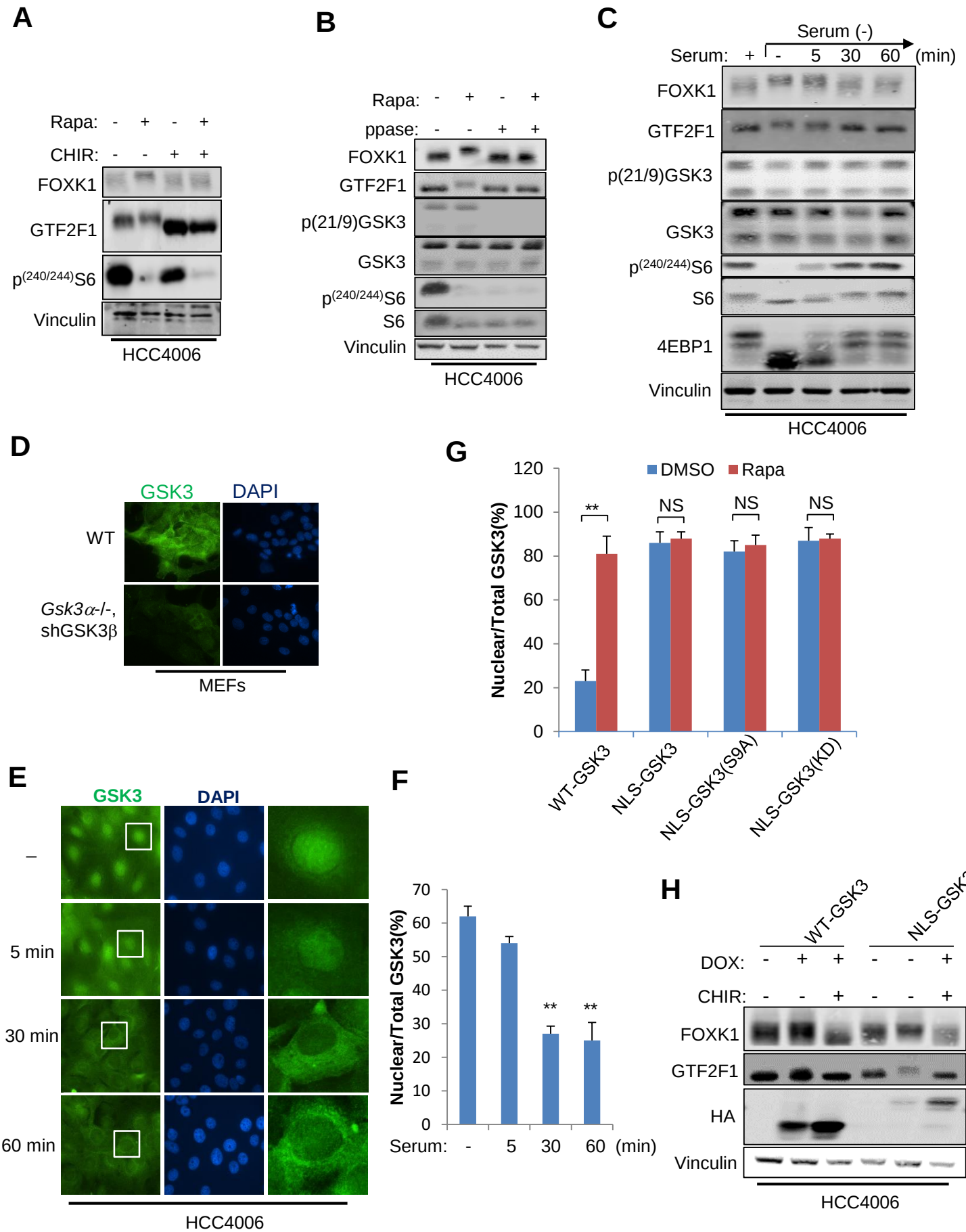
A
Rapa: - + - +
CHIR: - - + +
FOXK1
GTF2F1
p(240/244)S6
Vinculin
HCC4006
B
Rapa: - + - +
ppase: - - + +
FOXK1
GTF2F1
p(21/9)GSK3
GSK3
p(240/244)S6
S6
Vinculin
HCC4006
C
Serum (-)
Serum: + - 5 30 60 (min)
FOXK1
GTF2F1
p(21/9)GSK3
GSK3
p(240/244)S6
S6
4EBP1
Vinculin
HCC4006
D
GSK3
DAPI
WT
Gsk3α-/-, shGSK3β
MEFs
G
DMSO
Rapa
Nuclear/Total GSK3(%)
120
100
80
60
40
20
0
**
NS
NS
NS
WT-GSK3
NLS-GSK3
NLS-GSK3(S9A)
NLS-GSK3(KD)
E
GSK3
DAPI
–
5 min
30 min
60 min
HCC4006
F
Nuclear/Total GSK3(%)
70
60
50
40
30
20
10
0
**
**
Serum: - 5 30 60 (min)
H
WT-GSK3
NLS-GSK3
DOX: - + + - - +
CHIR: - - + - - +
FOXK1
GTF2F1
HA
Vinculin
HCC4006

Figure S1

(A) HCC4006 cells were exposed to 20nM Rapamycin and/or 10μM CHIR for 4hrs. Whole cell lysate was extracted and subjected to WB analysis with the indicated antibodies. (B) HCC4006 cells were exposed to DMSO or 20nM Rapamycin for 4hrs. Whole cell lysate was exposed to vehicle or l-phosphatase for 20 minutes prior to WB analysis with the indicated antibodies. (C) Whole cell lysates from growing, serum starved(16hrs) cells or cells with 10% serum restimulation for indicated times were subjected to WB analysis with the indicated antibodies. (D) WT or GSK3a/b-deficient MEFs established as described previously[9]. Cells were subjected to immunostaining with GSK3 antibodies. (E) HCC4006 Cells were serum-starved for 16hrs prior to restimulation for the indicated times and subsequently subjected to immunostaining with anti-GSK3 antibody and (F) GSK3 nuclear translocation was quantified and assessed as the ratios between nuclear and total fluorescence in at least 10 cells and values are expressed as mean ± SEM. One-way ANOVA with Dunnett's post-test, $^{**}p<0.001$, n=3. (G) GSK3 nuclear translocation was quantified from Figure 1D and assessed as the ratios between nuclear and total fluorescence in at least 10 cells and values are expressed as mean ± SEM. One-way ANOVA with Dunnett's post-test, $^{**}p<0.001$, n=3. (H) Established cells from Figure 1E were exposed to 1μM Dox with or without 1μM CHIR for 24hrs. Whole cell lysates were subjected to WB analysis with the indicated antibodies. Data are representative of at least three independent experiments.

Figure S2

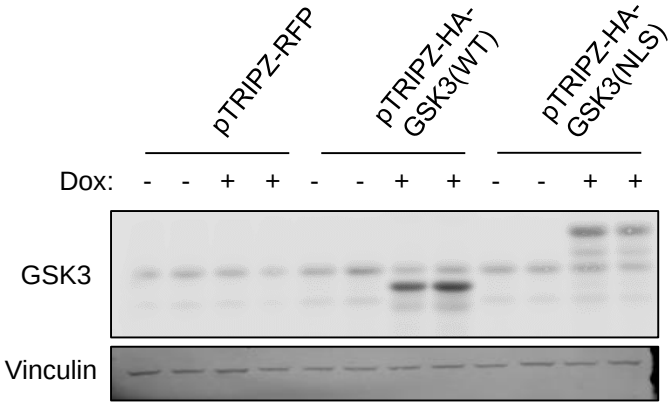

Figure S2

Cell lines were established with pTRIPZ-RFP, pTRIPZ-HA-GSK3-(WT), pTRIPZ-HA-NLS-GSK3b. Cells were injected into mice through subcutaneous injection as described in Figure 2. Dox (625 mg per kg) was included in the food where indicated and after 3 weeks, whole cell lysates from tumor tissues were subjected to WB analysis with the antibodies against GSK3 and vinculin.

Figure S3

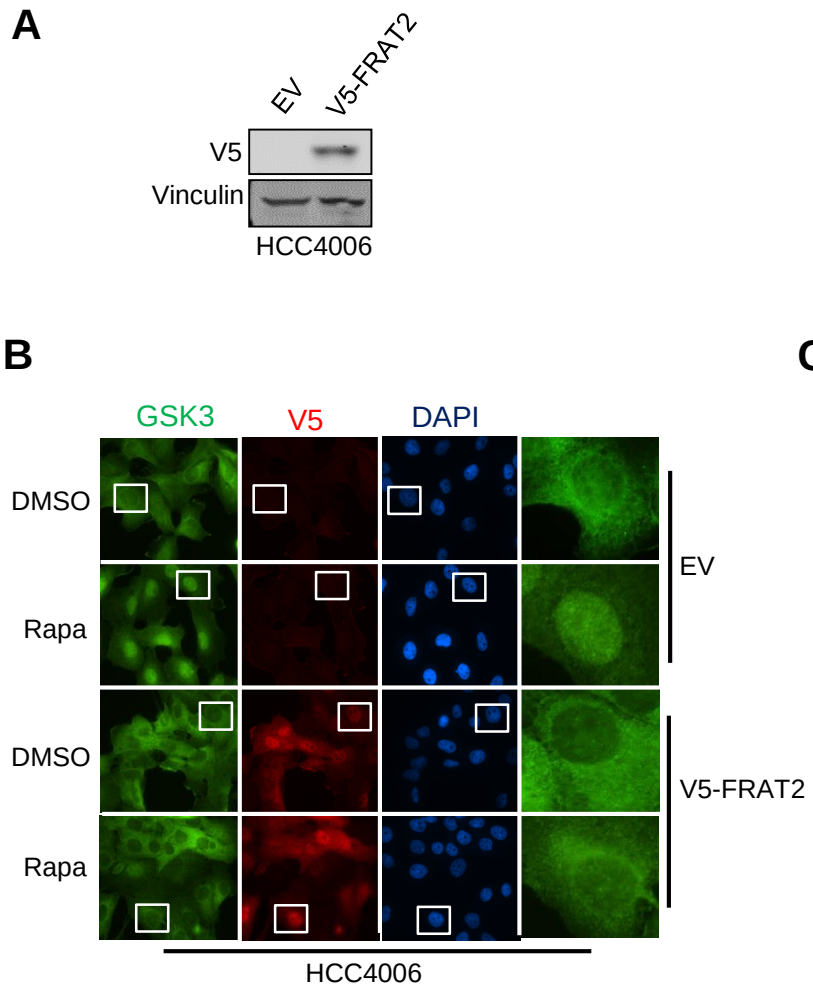

Figure S3

Cell lines were established with ectopically expressing FRAT2 in HCC4006 cells. (A) Whole cell lysates of growing cells were extracted and subjected to WB analysis with the indicated antibodies. Data are representative of at least three independent experiments. (B) Cells were exposed to 20nM rapamycin for 4hrs and subjected to Immunostaining with the indicated antibodies and (C) GSK3 nuclear translocation was quantified and assessed as the ratios between nuclear and total fluorescence in at least 10 cells and values are expressed as mean ± SEM. One-way ANOVA with Dunnett's post-test, $^{**}p<0.001$, n=3.

Figure S4

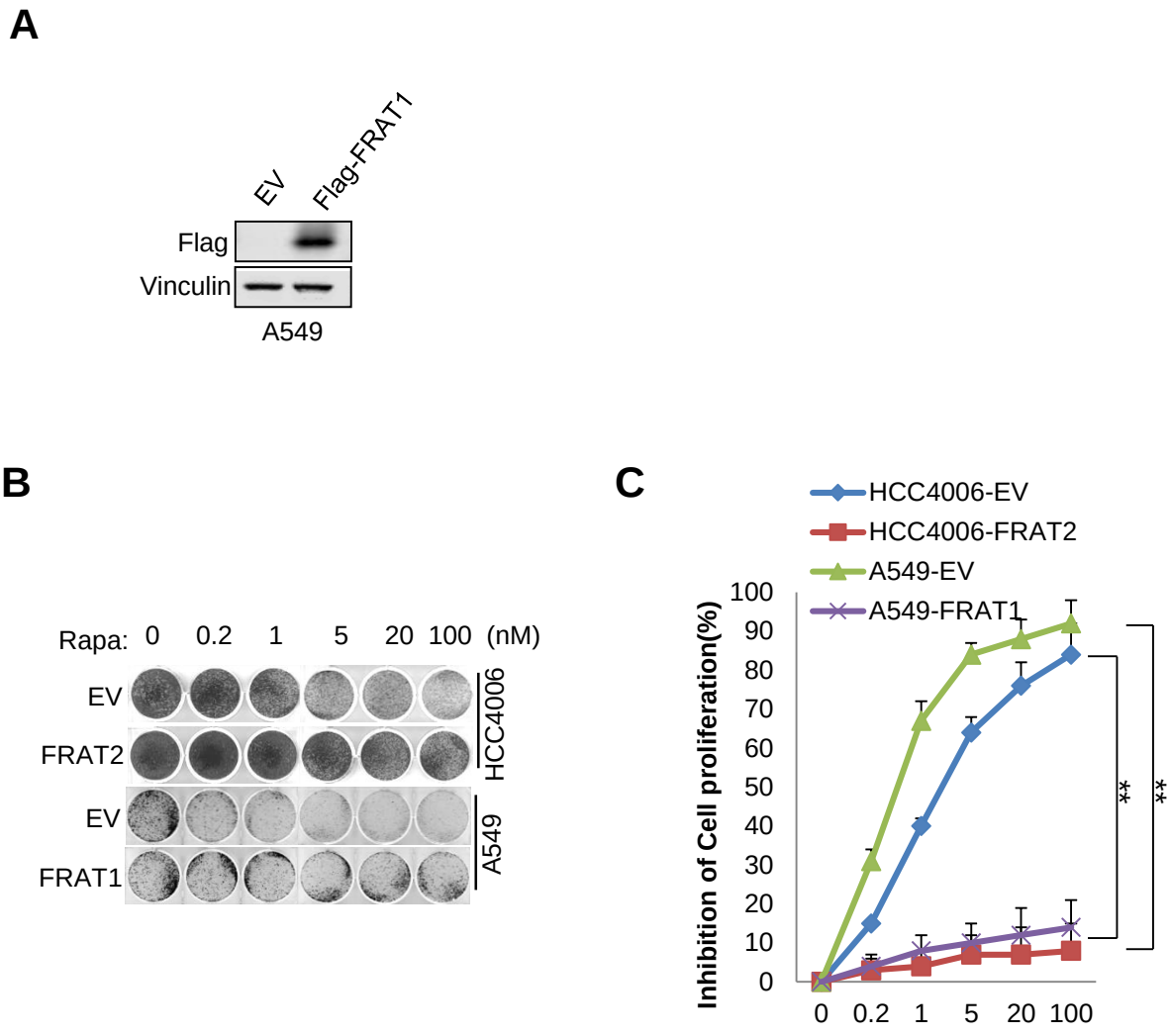

Figure S4

Cell lines were established with ectopic expression of FRAT1/2 in HCC4006 or A549 cells. (A) Whole cell lysates were subjected to WB analysis with the indicated antibodies. Data are representative of at least three independent experiments. (B) Clonogenic assay was performed using indicated cells with the indicated concentrations of rapamycin and (C) inhibition of cell proliferation was estimated as described in *Materials and Methods.* Values are expressed as mean ± SEM. Student T-Test, ***p*<0.001, n=3.

Figure S5

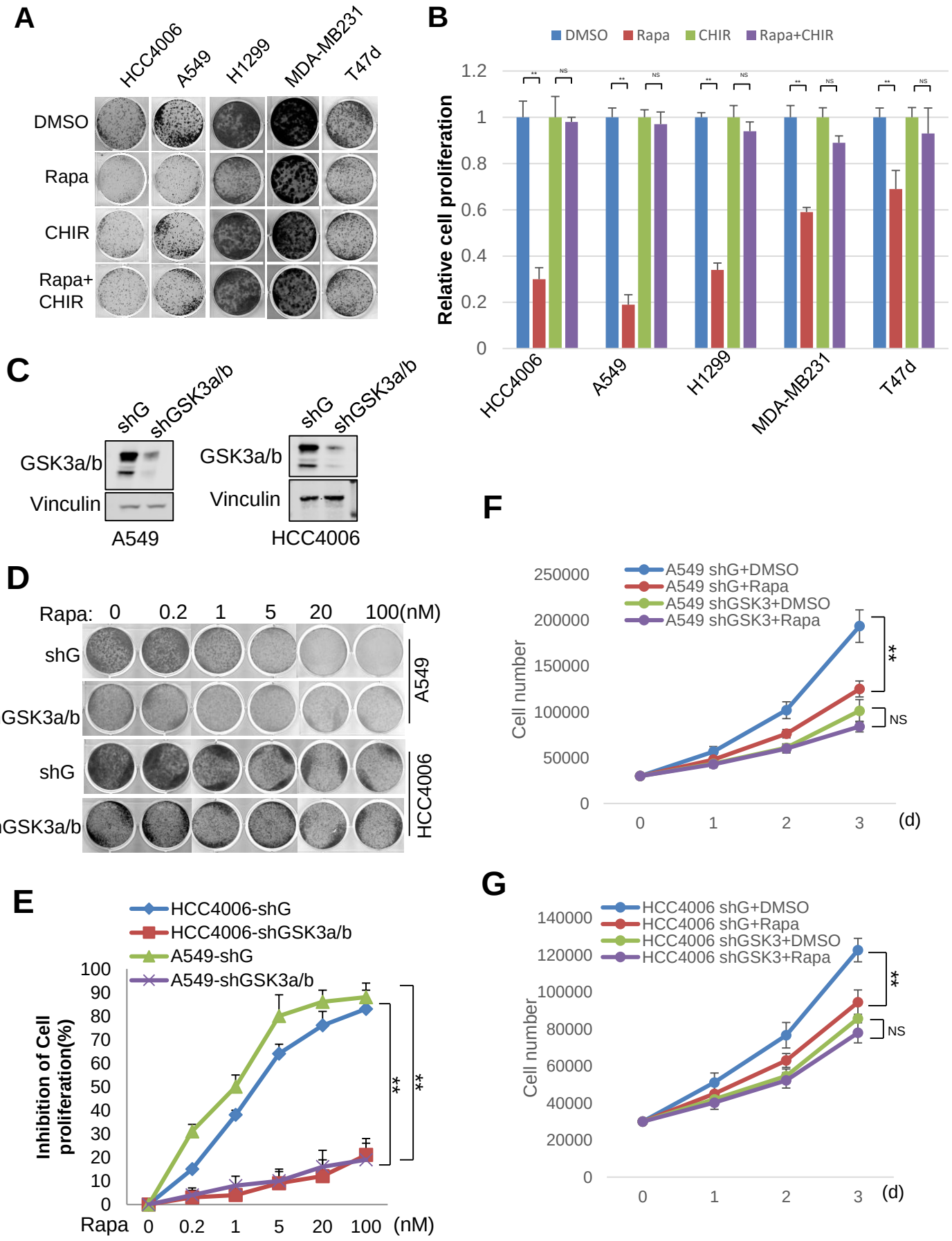
A
HCC4006
A549
H1299
MDA-MB231
T47d
DMSO
Rapa
CHIR
Rapa+ CHIR
B
DMSO
Rapa
CHIR
Rapa+CHIR
Relative cell proliferation
1.2
1
0.8
0.6
0.4
0.2
0
**
NS
HCC4006
A549
H1299
MDA-MB231
T47d
C
shG
shGSK3a/b
GSK3a/b
Vinculin
A549
HCC4006
D
Rapa: 0 0.2 1 5 20 100(nM)
shG
shGSK3a/b
A549
HCC4006
F
A549 shG+DMSO
A549 shG+Rapa
A549 shGSK3+DMSO
A549 shGSK3+Rapa
Cell number
250000
200000
150000
100000
50000
0
**
NS
0
1
2
3
(d)
E
HCC4006-shG
HCC4006-shGSK3a/b
A549-shG
A549-shGSK3a/b
Inhibition of Cell proliferation(%)
100
90
80
70
60
50
40
30
20
10
0
**
**
Rapa 0 0.2 1 5 20 100 (nM)
G
HCC4006 shG+DMSO
HCC4006 shG+Rapa
HCC4006 shGSK3+DMSO
HCC4006 shGSK3+Rapa
Cell number
140000
120000
100000
80000
60000
40000
20000
0
**
NS
0
1
2
3
(d)

Figure S5

(A) Clonogenic assay was performed with rapamycin (20nM) or CHIR (0.5μM) or both using the indicated cell lines. (B) Cell proliferation was estimated as described in *Materials and Methods*. (C-G) Cell lines were established with knock-down of GSK3a/b in A549 and HCC4006 cells. (C) Whole cell lysates from growing cells were subjected to WB analysis with antibodies against GSK3 and Vinculin. Data are representative of at least three independent experiments. (D) Clonogenic assay was performed with the indicated concentrations of rapamycin and (E) inhibition of cell proliferation were estimated as described in *Materials and Methods*. (F-G) cell growth curves were determined with or without 20nM rapamycin in each cell lines. Values are expressed as mean ± SEM. One-way ANOVA with Dunnett's post-test, $^{**}p<0.001$, n=3.

Figure S6

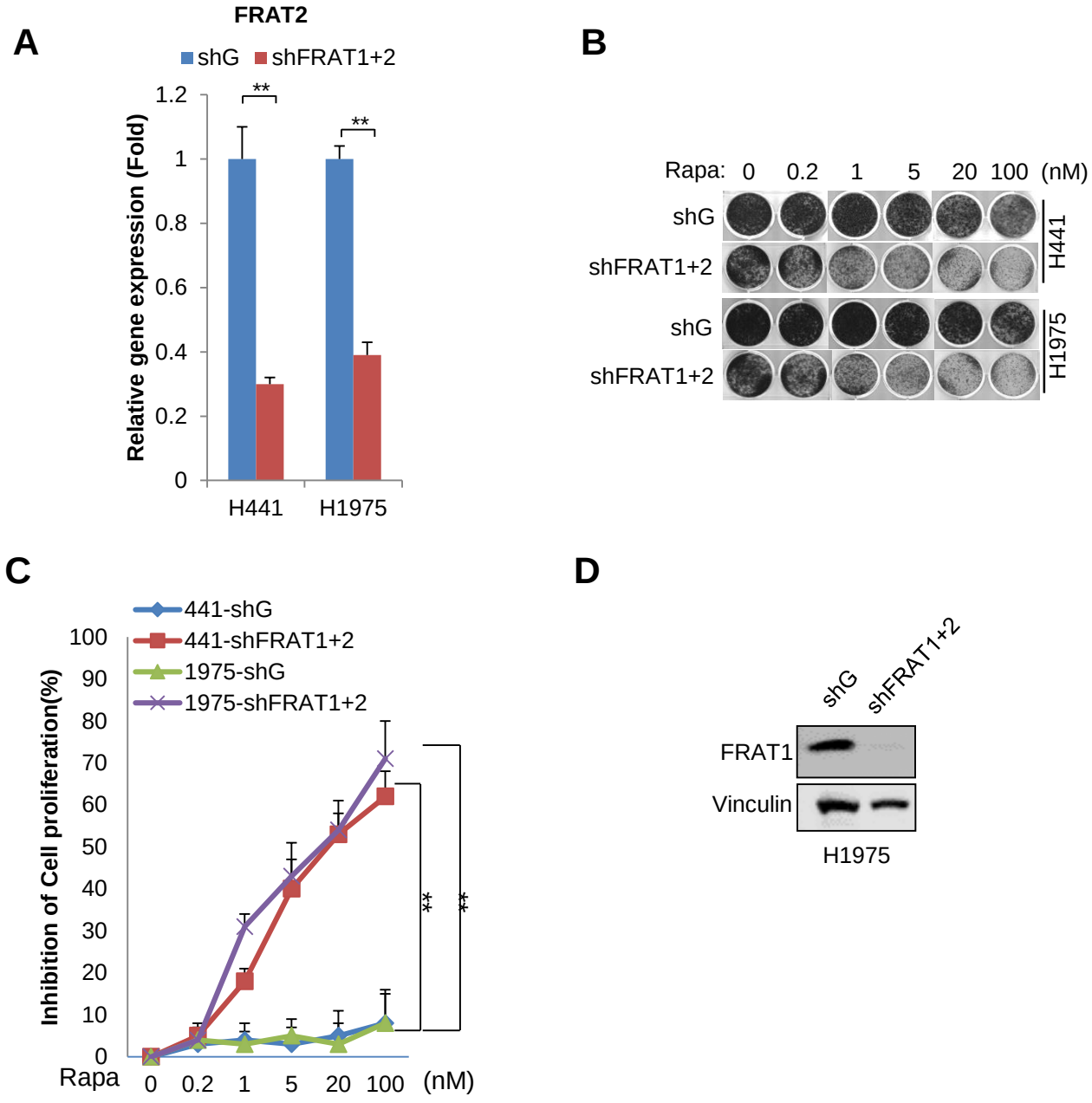

Figure S6

Cell lines were established with suppression of FRAT1/2 expression in H441 or H1975 cells. (A) Total RNA was extracted from growing cells and subjected to RT-qPCR analysis with primers against FRAT2. (B) Clonogenic assay was performed using the indicted cell lines with the indicated concentrations of rapamycin and (C) inhibition of cell proliferation were estimated as described in *Materials and Methods*. Values are expressed as mean ± SEM. One-way ANOVA with Dunnett's post-test, $^{**}p<0.001$, n=3. (D) Whole cell lysates were subjected to WB analysis with anti-FRAT1 and anti-Vinculin antibodies. Data are representative of at least three independent experiments.

Figure S7

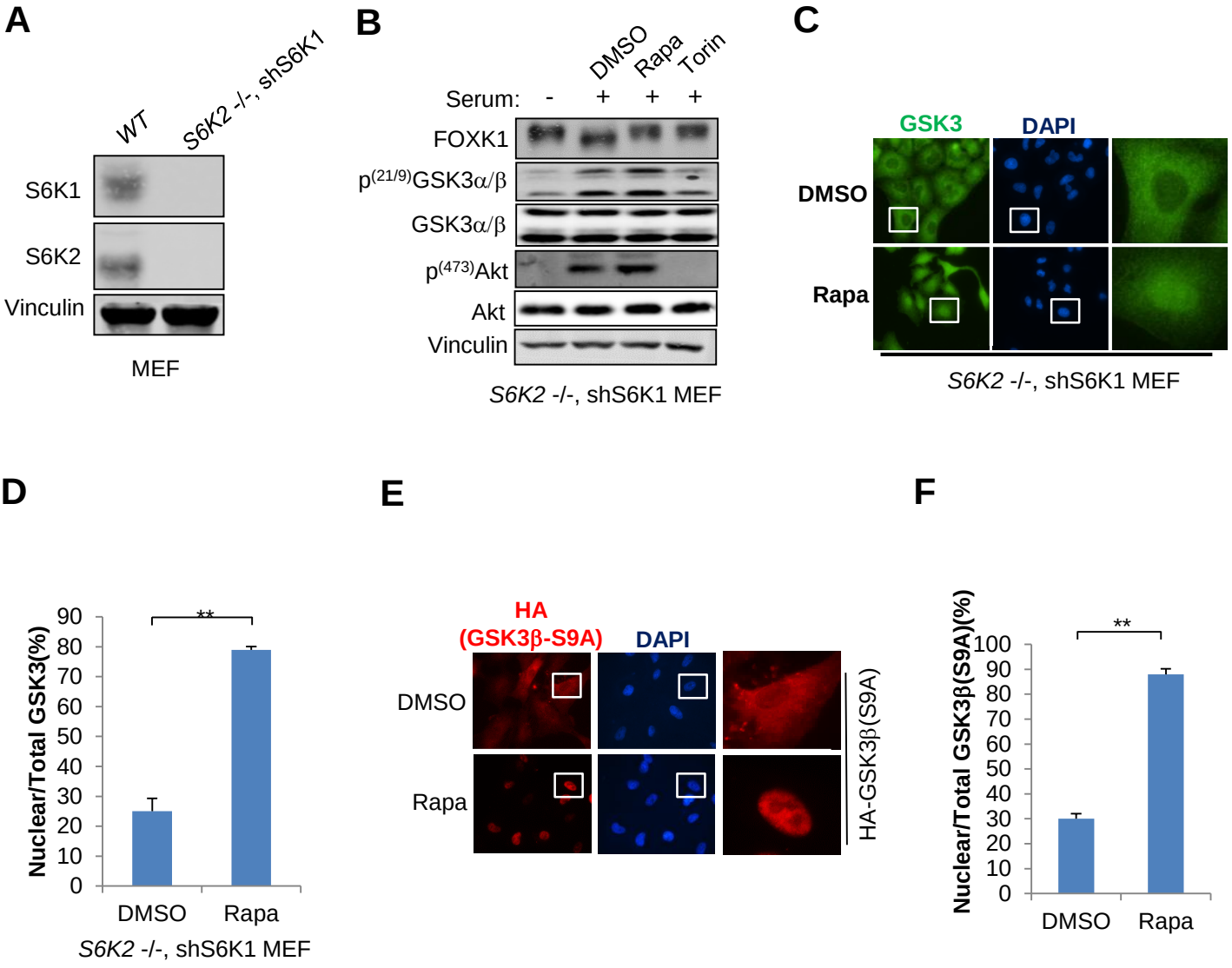

Figure S7

(A-D) S6K1/2 double deficient cell lines were established by suppressing S6K2 with RNAi in *S6K1* -/- MEFs. (A, B) Whole cell lysates from growing cells or cells which were exposed to Rapamycin (20nM) or Torin (200nM) for 4hrs were subsequently subjected to WB analysis with the indicated antibodies. Data are representative of at least three independent experiments. (C) immunostaining was performed with anti-GSK3 antibody and (D) GSK3 nuclear translocation was quantified and assessed as the ratios between nuclear and total fluorescence in at least 10 cells and values are expressed as mean ± SEM. (E) HCC4006 cells transiently expressing GSK3b(S9A) were subjected to immunostaining with anti-HA antibody and (F) GSK3(S9A) nuclear translocation was quantified and assessed as the ratios between nuclear and total fluorescence in at least 10 cells and values are expressed as mean ± SEM. One-way ANOVA with Dunnett's post-test, $^{**}p<0.001$, n=3.

Figure S8

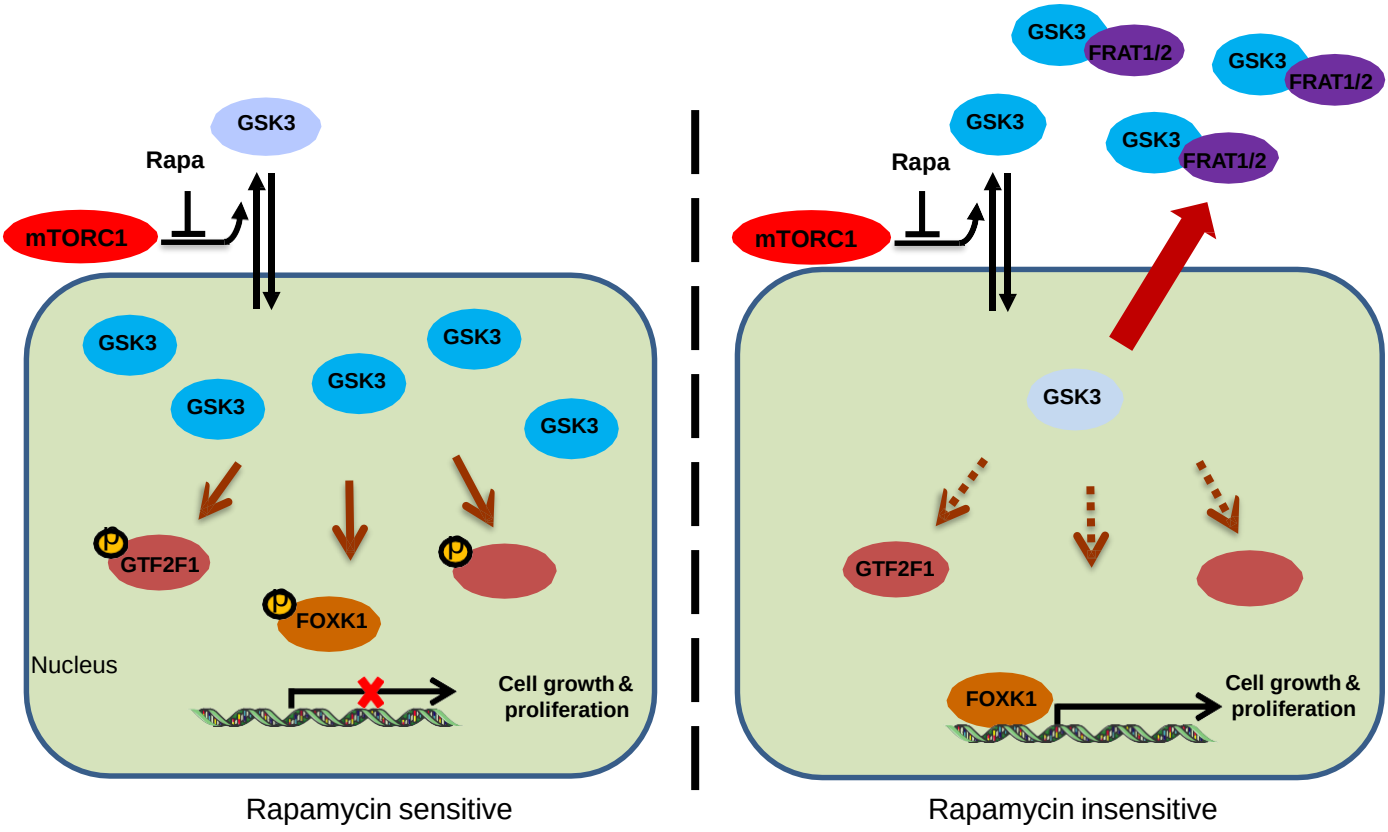

Figure S8. GSK3 and FRAT contribute to mTORC1-dependent regulation of nuclear protein phosphorylation events.
Model: mTORC1 regulates nuclear-cytoplasmic localization of GSK3 and thus promotes differential phosphorylation and regulation of target proteins involved in cell growth/cell cycle regulation. Upon mTORC1 suppression, GSK3 accumulates in the nucleus where it promotes phosphorylation of nuclear proteins including FOXK1 and GTF2F1. Increased expression of FRAT1/2 facilitate nuclear exclusion of GSK3 resulting in decreased GSK3-dependent phosphorylation of FOXK1 and GTF2F1, and potentially many additional targets identified in our previous phosphoproteomics analysis (see Table 1).

Experimental Procedures

Clonogenic assay

1,000 or 2,000 Cells were seeded in 12 or 6 well/plates and incubated for 1~2 weeks. After colonies were clearly observed, they were fixed with 4% Formaldehyde, stained with crystal violet (0.5% w/v). After rinsing four times with PBS buffer, the images of the wells were scanned. For quantification, methanol was added to each well plate and O.D was measured at 570nm as described [22].

Immunofluorescence staining

Cells were plated on cover glass and the next day, cells were fixed with 4% paraformaldehyde for 10 min at room temperature. The cells were rinsed with PBS four times and incubated with a blocking solution containing 5% BSA in PBS for 15 min. The cells were then incubated with indicated antibodies in blocking solution for 3.5 hrs followed by 4 times washing with PBS. Secondary antibodies conjugated to a fluorochrome (Alexa Fluor, Thermo Fisher Scientific) in blocking buffer were then added to cover glasses and incubated for another 1.5 hrs at room temperature. Cells were rinsed with PBS four times and incubated with Hoechst 33258 solution (DNA staining) for 15 min. After washing with PBS four times, cells were mounted with mounting buffer and images observed by fluorescence microscopy.

Animal studies

For our mouse xenograft studies, we followed Institutional Animal Care and Use Committee (IACUC)-approved protocols and guidelines. Indicated amount of cells ($1X10^6$ cells) were injected subcutaneously into 5-6-week-old female nude mice (Envigo or Taconic). After subcutaneous tumors formed, mice were randomly divided into 2~3 groups for intraperitoneal injection 3

days/week with vehicle or rapamycin (1 or 5 mg/kg body weight) or fed food containing Doxycyclin and grown for additional 3~4 weeks. Rapamycin was dissolved with PEG400: water (50%: 50%).

Establishment of stable cell lines

To generate lentiviruses, shRNA plasmids or overexpression plasmids were transfected into 293T cells together with the packaging (Δ8.9) and envelope (VSVG) plasmids, and medium was changed the next day. After 24 hrs, viral supernatants were harvested, and new medium was added. For infection, cells were infected with viral supernatants in the presence of a serum-containing medium supplemented with 4 μg/ml polybrene. After 16 hrs, viral-containing medium was removed and cells were grown in serum-containing medium for 24 hrs. Cells were treated with puromycin (2 μg/ml) or blasticidin (10μM) for selection. The knock-down or overexpression of target protein was confirmed by immunoblot analysis.

Statistics

Data were expressed as average ± SEM of at least three independent experiments performed in triplicate. One-way ANOVA or two-tailed Student's t test was used to determine differences between each group, followed by the Dunnett's or Tukey's post-test or pairwise comparisons as appropriate.