Supplementary Figures

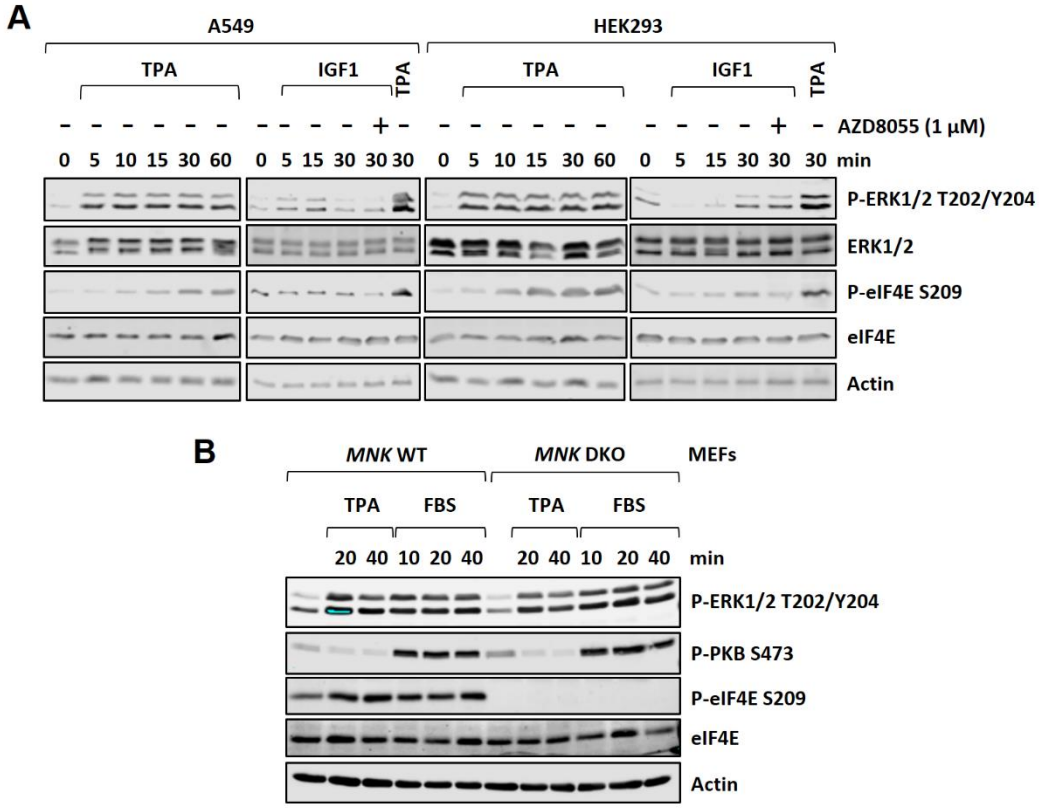

Figure S1

Figure S1. Responses of PKB, ERK and eIF4E phosphorylation to TPA, IGF-1 and FBS. Related to Fig. 1.

(A) A549 and HEK293 cells were starved of serum for 16 h, where indicated, preincubated with AZD8055 for 30 min in KRB, before treatment with either 1 μM TPA or 10 nM IGF-1 for the indicated periods of time.

(B) MEFs were starved of serum for 16 h, before treatment with either 1 μM TPA or 10% FBS for the indicated periods of time. Cells were then lysed and protein lysates were immunoblotted with antisera against indicated P-/total proteins.

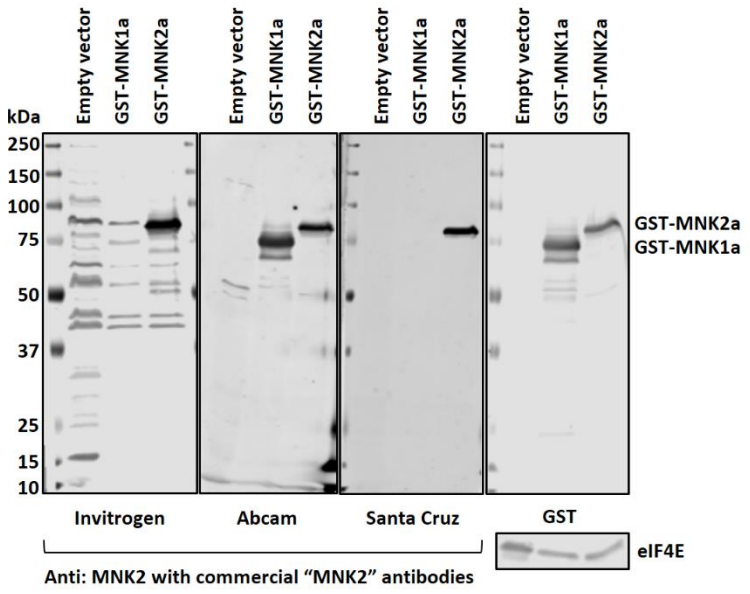

Figure S2

Figure S2. Validation of commercial total MNK2 antibodies. Related to Fig. 1.

HEK293 cells were transfected with empty vector, or vectors encoding GST-MNK1a[WT] or GST-MNK2a[WT]. After 48 h, cells were lysed and samples were analysed by SDS-PAGE/WB using indicated antisera for MNK2, GST or eIF4E. Note that Invitrogen's MNK2 antibody (PA5-13953) detected many non-specific bands, Abcam's MNK2 antibody (Ab84345) recognized both MNK1 and MNK2, Santa Cruz' MNK2 antibody (sc-7445) recognized only exogenously expressed MNK2, not endogenous MNK2. We have tested these antibodies in other cell lines with similar results (Merrett J & Proud CG, data not shown).

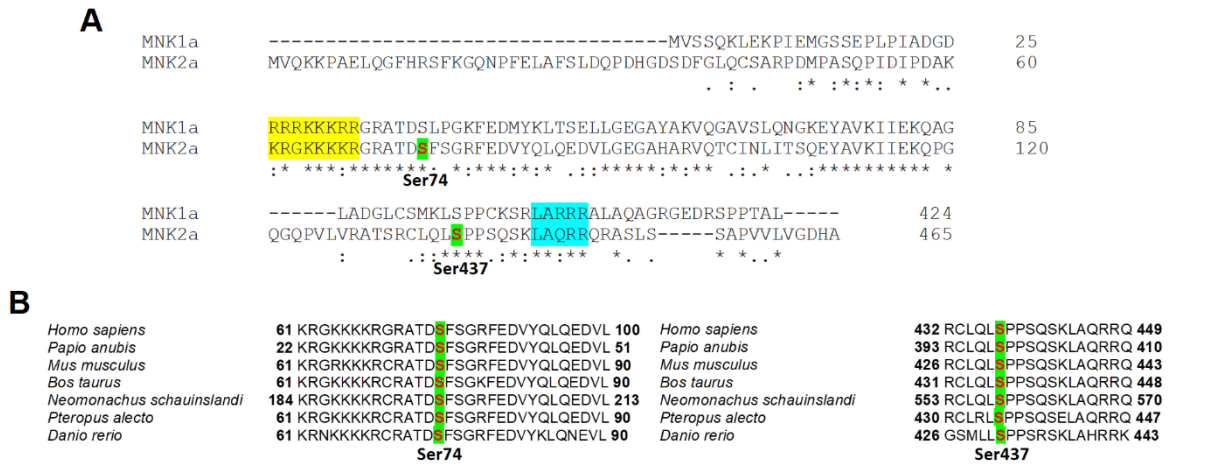

Figure S3

Figure S3. Sequence alignment of MNK1a and MNK2a regarding the newly discovered phosphorylation sites. Related to Fig. 2.

(A) Sequence alignment of human MNK1a and MNK2a. Rapamycin-sensitive phosphorylation sites in MNK2a (*1*) and the corresponding residues in MNK1a are highlighted in green. The eIF4G-binding domain is highlighted in yellow and the MAPK-binding motif in blue. "*": fully conserved residues; ":": residues with strongly similar properties; ".": residues with weakly similar properties.

(B) Alignment of sequences surrounding Ser74 and Ser437 of human MNK2a across different species.

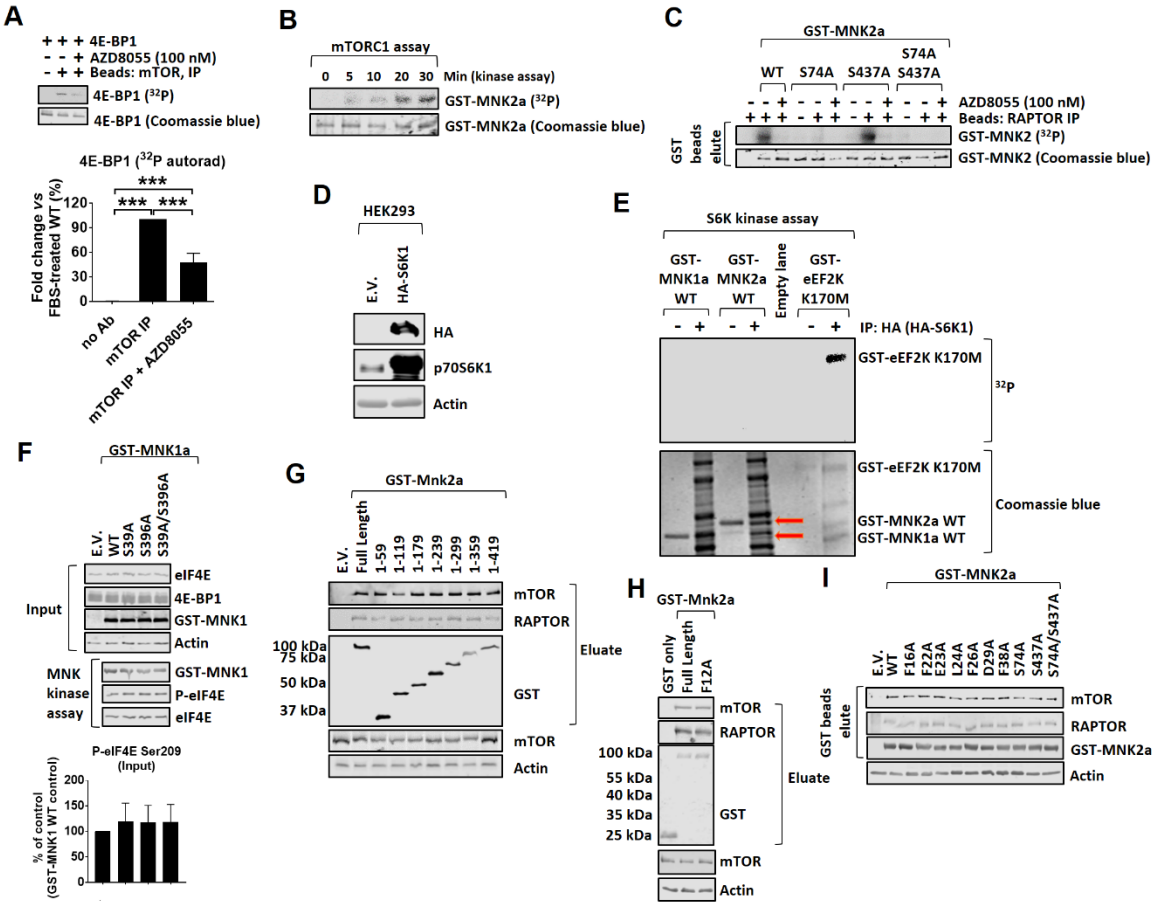

Figure S4

Figure S4. mTORC1 but not S6K1 phosphorylates MNK2 at Ser74. Related to Fig. 2.

(A) mTOR kinase assays were performed with mTOR immunoprecipitated (IPed) as in Fig. 1B, and with recombinant GST-4E-BP1 as substrates.

(B) mTORC1 was purified by IP from HEK293 cell lysates using anti-RAPTOR. mTORC1 kinase assays were performed with recombinant GST-MNK2a as substrates and samples were removed for analysis at the indicated times.

(C) mTORC1 kinase assays were performed as in C, but with WT or the indicated mutants of GST-MNK2a.

(D) HEK293 cells were transfected with empty vector (E.V.) or a vector for HA-S6K1. Proteins from cell lysates were immunoblotted using antibodies for HA, S6K1 and actin.

(E) HA-S6K1 from **c** were IPed using anti-HA antibody, IPed HA-S6K1 were then subjected to S6K1 kinase assays using GST-MNK1a, GST-MNK2a or GST-eEF2K K170M as substrates.

(F) HEK293 cells were transfected with indicated GST-MNK1a constructs. Cells were of starved of serum for 16 h. GST-MNK1a were then pulled down with glutathione beads and assayed for kinase activity against recombinant eIF4E as substrate.

(G-I) HEK293 cells were transfected with vectors for WT or mutant GST-MNK2a. GST-MNKs were pulled down with glutathione beads. Binding of mTOR and RAPTOR to GST-MNK2a was analysed by SDS-PAGE/WB.

For panels A and F, data are quantified as means ± S.D., *n* = 3. ***: P < 0.001 (one-way ANOVA).

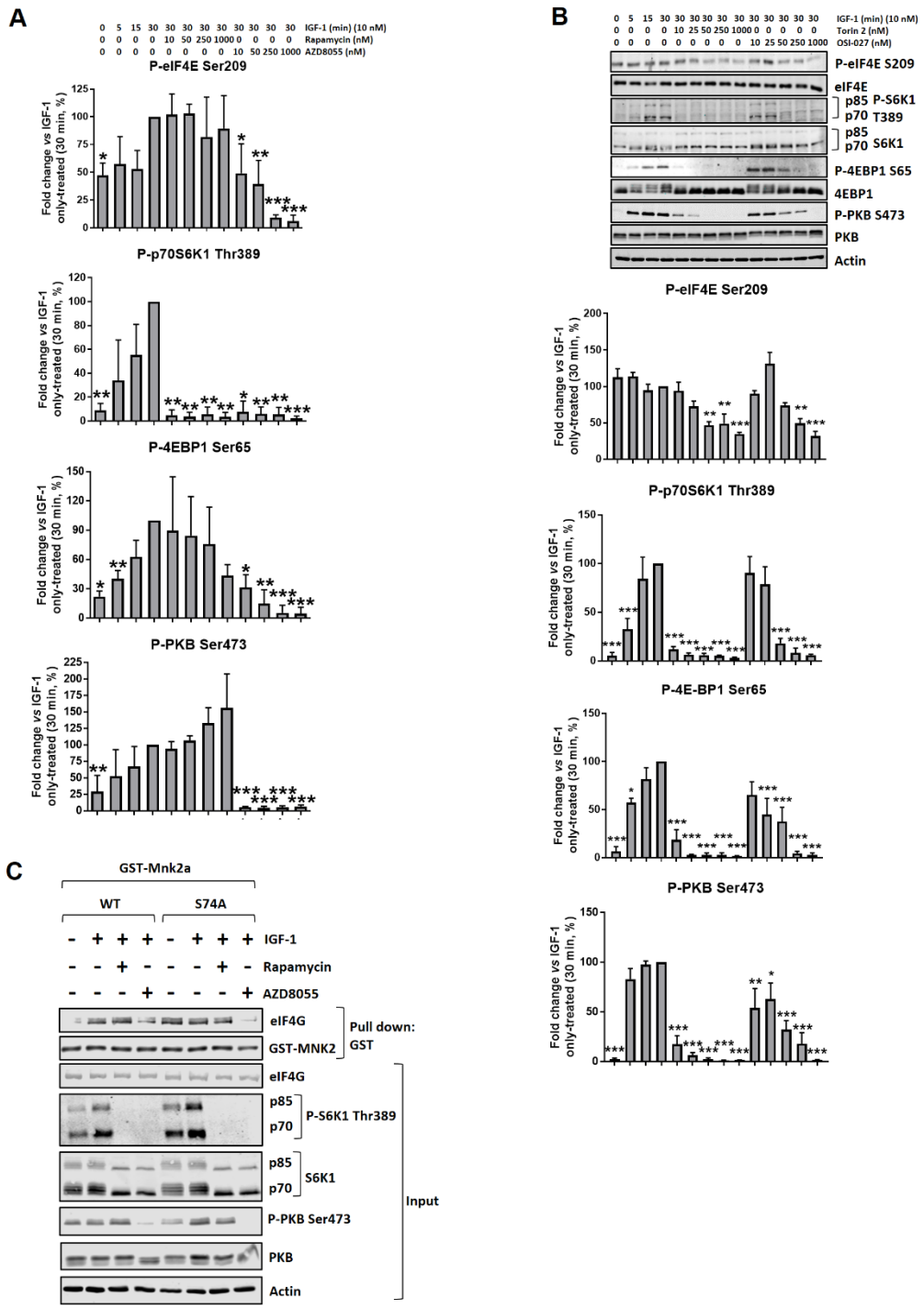

Figure S5

Figure S5. AZD8055 inhibits eIF4E phosphorylation by preventing the association between MNK2 and eIF4G. Related to Fig. 3.

(A) Quantitation of Fig. 3A.

(B) HEK293 cells were starved of serum for 16 h, kept in KRB in the presence of the indicated concentrations of Torin 2 or OSI-027 for 30 min, before stimulation with 10 nM IGF-1 for the indicated times. Levels of P-eIF4E Ser209, P-p70S6K1 Thr389, P-4E-BP1 Ser65 and P-PKB Ser473 are quantified.

(C) HEK293 cells were transfected with vectors encoding GST-MNK2a[WT] or GST-MNK2a[S74A]. 32 h later, cells were starved of serum for 16 h, and then pre-treated with 200 nM rapamycin or 1 μM AZD8055 for 30 min, before stimulation with 10 nM IGF-1 for 30 min. GST-MNK2as were then purified with glutathione beads. Both eluates and input lysates were subjected to immunoblotting analysis for the indicated proteins.

For panels A and B, data are quantified as means ± S.D., $n = 3$. *: $0.01 \leq P < 0.05$; **: $0.001 \leq P < 0.01$; ***: $P < 0.001$ (one-way ANOVA).

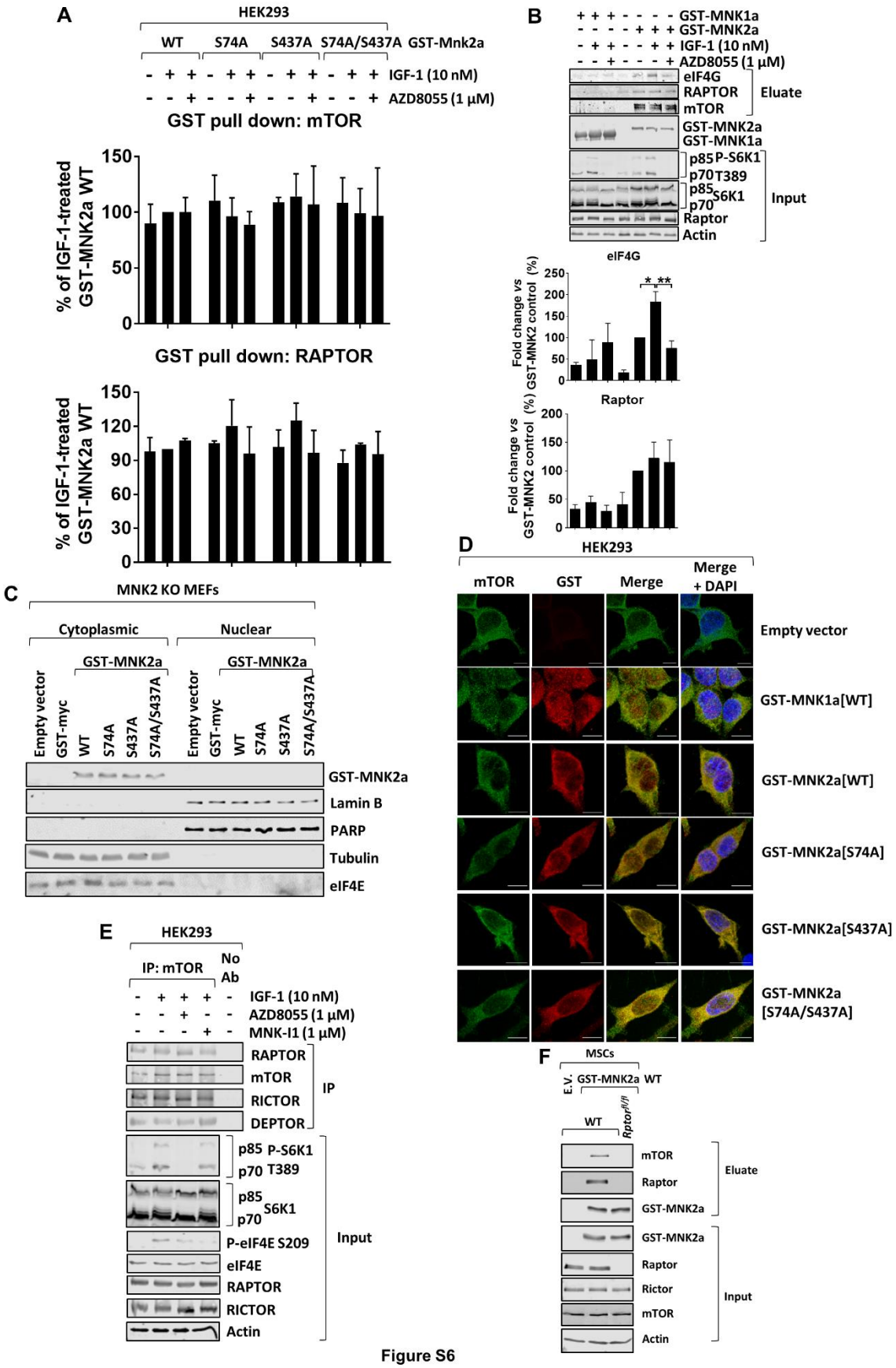

Figure S6

Figure S6. MNK2 but not MNK1 associates with mTORC1. Related to Fig. 3.

(A) Quantification of mTOR and RAPTOR from the GST pull down in Fig. 3B.

(B) HEK293 cells were transfected with vectors for WT GST-MNK1a or WT GST-MNK2a. Cells were starved of serum for 16 h and then kept in KRB for 30 min in the absence or presence of AZD8055, followed by stimulation with IGF1 for another 30 min. GST-MNKs were pulled down with glutathione beads. Immunoblotting analysis were performed against indicated P-/total proteins.
(C) MNK2$^{-/-}$ MEFs were transfected with vectors for GST-MNK2a[WT], GST-MNK2a[S74A], GST-MNK2a[S437A] or GST-MNK2a[S74A/S437A]. After 48 h, cells were lysed and cytoplasmic/nuclear fractions were isolated; proteins were then analysed by SDS-PAGE and immunoblotted for indicated phosphorylated- (P-) or total proteins.
(D) HEK293 cells expressing the empty vector, GST-MNK1[WT] or indicated WT or mutant GST-MNK2 were immunostained to visualize their intracellular locations in comparison with mTOR. Scale bar = 10 μm.
(E) HEK293 cells were starved of serum for 16 h and then kept in KRB for 30 min in the absence or presence of AZD8055, followed by stimulation with IGF1 for another 30 min. mTORCs were IPed from cell lysates and both immunoprecipitates and input lysates were subjected to immunoblotting analysis for the indicated proteins. No Ab = IP without antibody as a negative control.
(F) WT and *Rptor*$^{-/-}$ mouse mesenchymal stem cells (MSCs) were transfected with GST-MNK2a (mouse). 48 h later, GST-MNK2a were then pulled down with glutathione beads. Binding of mTOR and RAPTOR to GST-MNK2a was analysed by SDS-PAGE/WB.

Data Information: For panels A and B, data are quantified as means ± S.D., $n = 3$. *: $0.01 \leq P < 0.05$; **: $0.001 \leq P < 0.01$ (one-way ANOVA). For simplicity, not all statistical significances were shown.

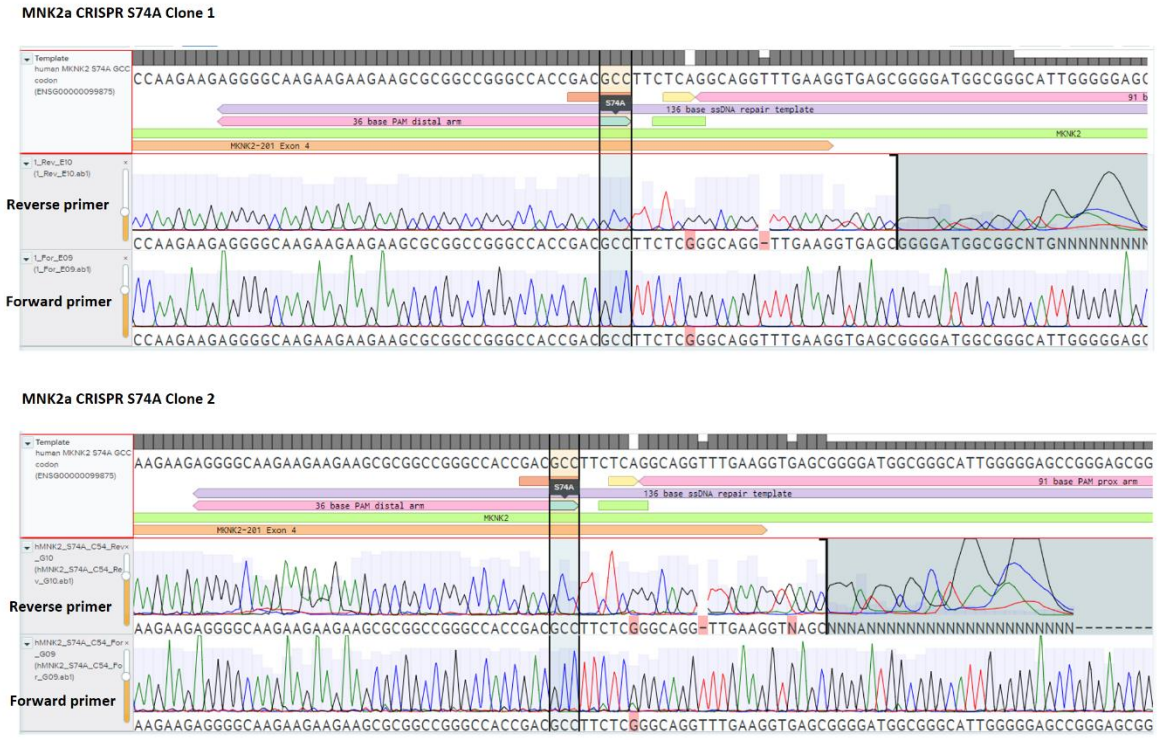

Figure S7

Figure S7. Sanger sequencing results of MNK2 S74A cells. Related to Fig. 5.

Forward sequencing primer: 5’-CGGGCCACCGACAGCTTCTCGTTTT-3’; reverse sequencing primer: 5’-GAGAAGCTGTCGGTGGCCCGCGGTG-3’.

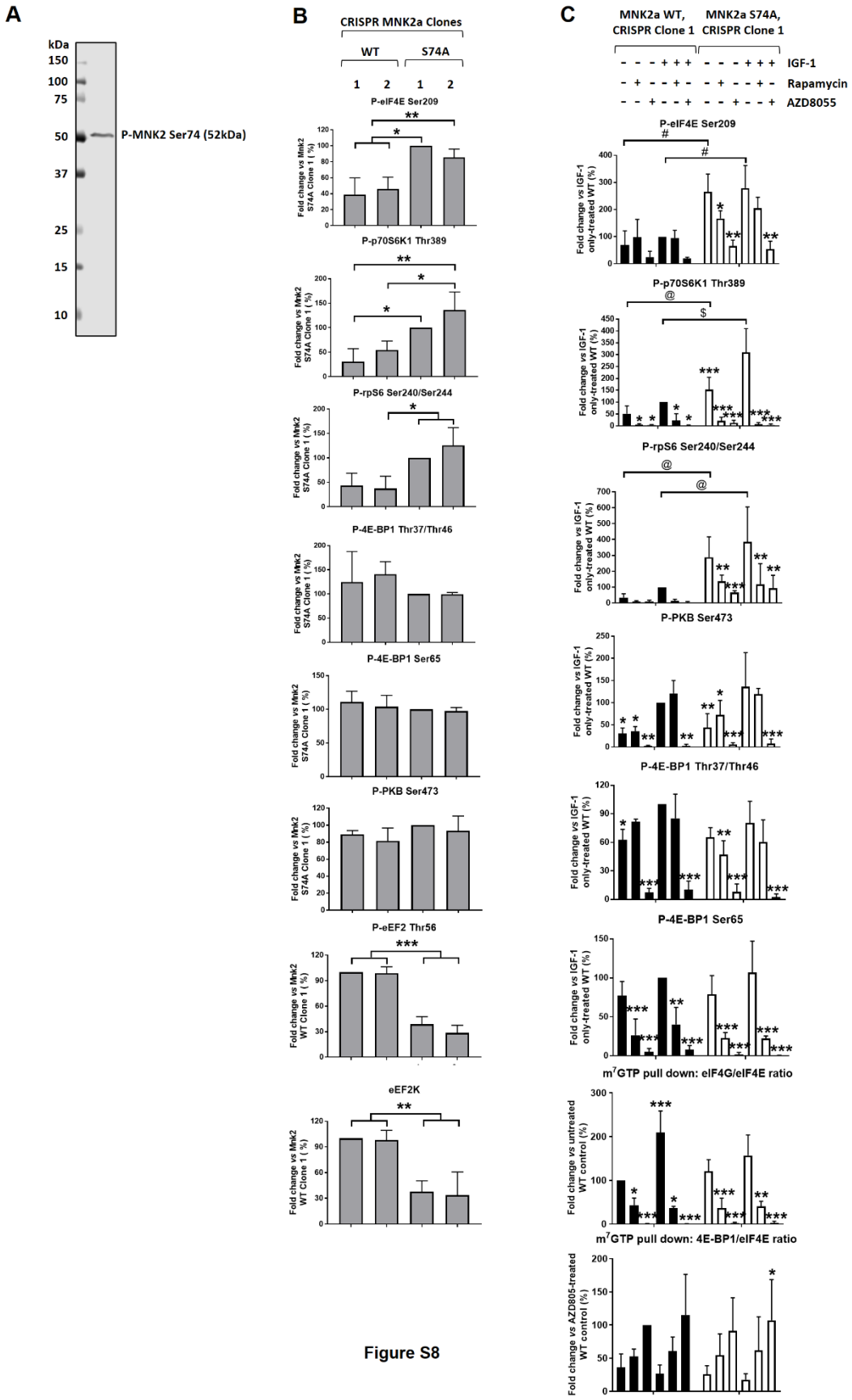

Figure S8

Fig. S8. Quantitation of Fig. 5D and E. Related to Fig. 5D.

(A) P-MNK2 Ser74 only recognized one band (52 kDa, HEK293 cell lysate) on an SDS-PAGE gel.

(B) Quantification of Fig. 5D.

(C) Quantification of Fig. 5E.

Data are presented as means ± S.D., $n = 3$. *: $0.01 \leq P < 0.05$; **: $0.001 \leq P < 0.01$; ***: $P < 0.001$ (one-way ANOVA), or; @: $0.01 \leq P < 0.05$; #: $0.001 \leq P < 0.01$; $: $P < 0.001$ (two-way ANOVA).

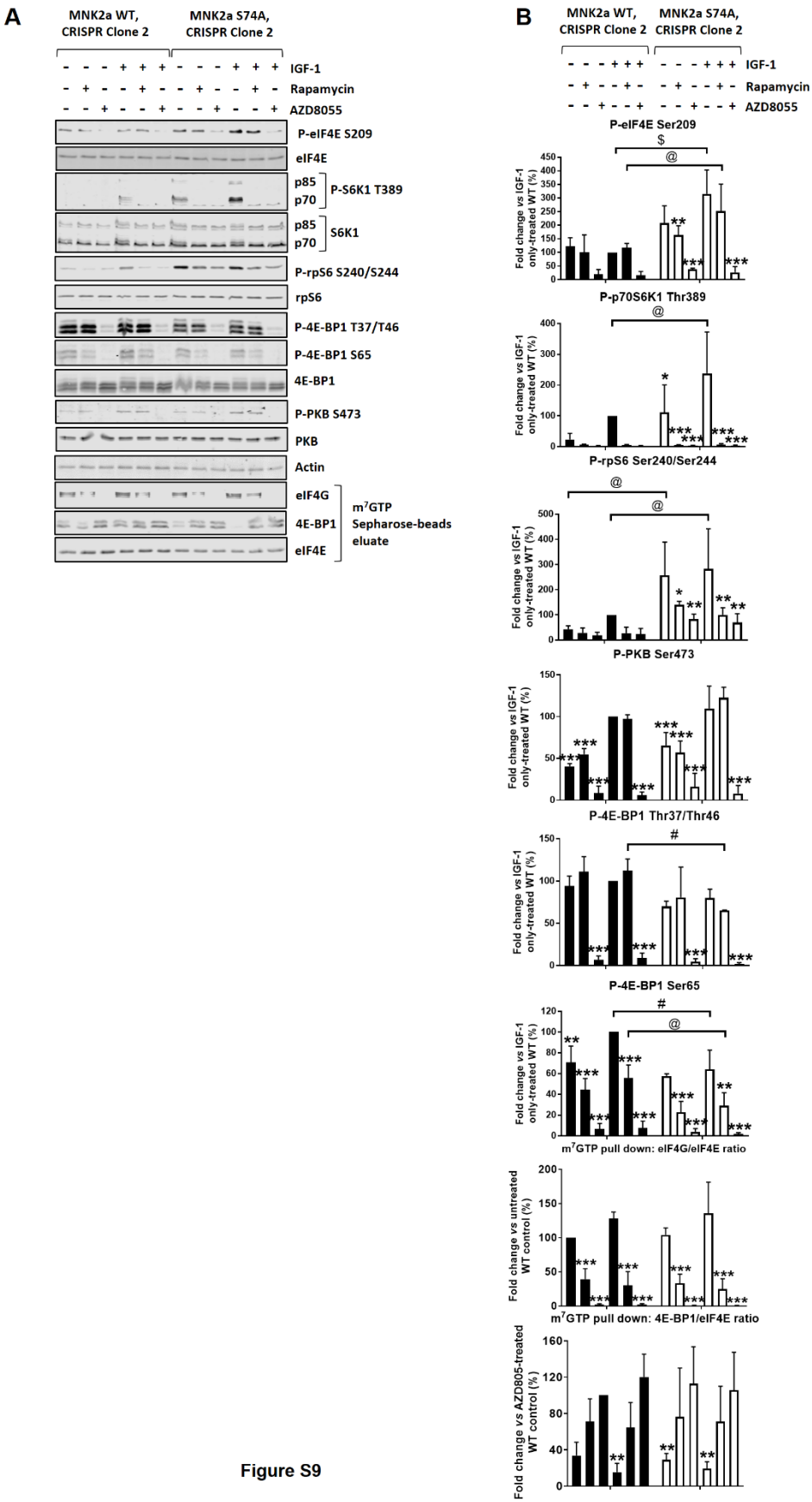

Figure S9

Figure S9. Genetic knock-in of MNK2a Ser74 to alanine in HEK293 cells enhance eIF4E and S6K1 phosphorylations, clone 2. Related to Fig. 5.

(A) Cells starved of serum for 16 h, pre-treated with rapamycin (200 nM) or 1 μM AZD8055 for 30 min, before stimulation with 100 nM IGF-1 for 30 min. Protein lysates were immunoblotted using antisera against indicated P- or total proteins.
(B) Quantitation of A.

For panel B, data are presented as means ± S.D., $n = 3$. *: $0.01 \leq P < 0.05$; **: $0.001 \leq P < 0.01$; ***: $P < 0.001$ (one-way ANOVA), or; @: $0.01 \leq P < 0.05$; #: $0.001 \leq P < 0.01$; $: $P < 0.001$ (two-way ANOVA).

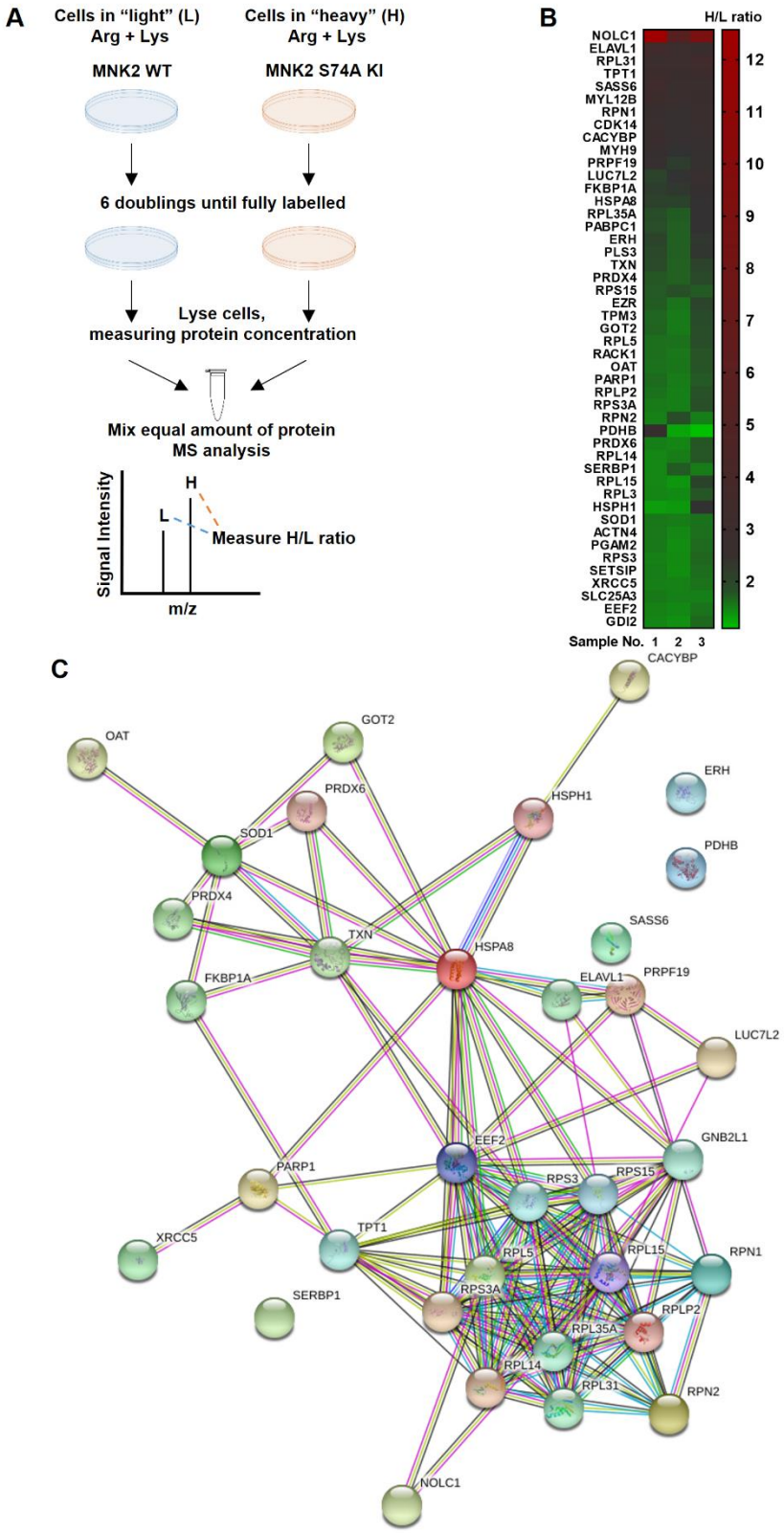

Figure S10

Figure S10. Proteins related to mRNA translation control are highly expressed in MNK2 S74A KI cells. Related to Fig. 6.

(A) Flow diagram of the SILAC/MS method. MNK2 S74A knock-in (KI) HEK293 cells previously maintained in normal medium were placed in medium containing stable isotope-labelled arginine and lysine (and non-labelled versions of all other amino acids) and maintained for 6 doublings until fully labelled. Cells were then lysed, mixed with an equal amount of WT cell lysates, proteins were subjected to tryptic digestion followed by MS analysis. For any given tryptic peptide, two species are observed, one containing normal amino acids (light or "L"), and one containing heavy (H) arginine and/or lysine. The latter peptides are derived from MNK2 S74A KI cells. The H/L ratio reflects the relative abundance of the parent protein between WT and MNK2 S74A KI cells.

(B) Heat map representation of proteins with expression levels equal or higher than 1.5-fold in MNK2 S74A knock-in cells compared to the WT cells.

(C) STRING functional association analysis of candidate proteins described in B.

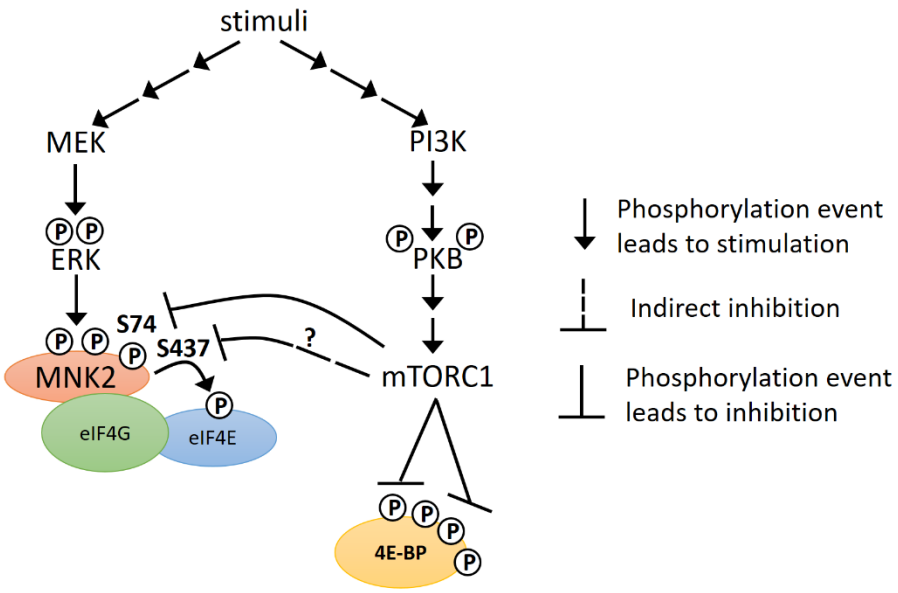

Figure S11

Figure S11. Schematic summary of crosstalk between MNKs and mTORC1. Related to Fig. 1-6.

See Discussion for further information.

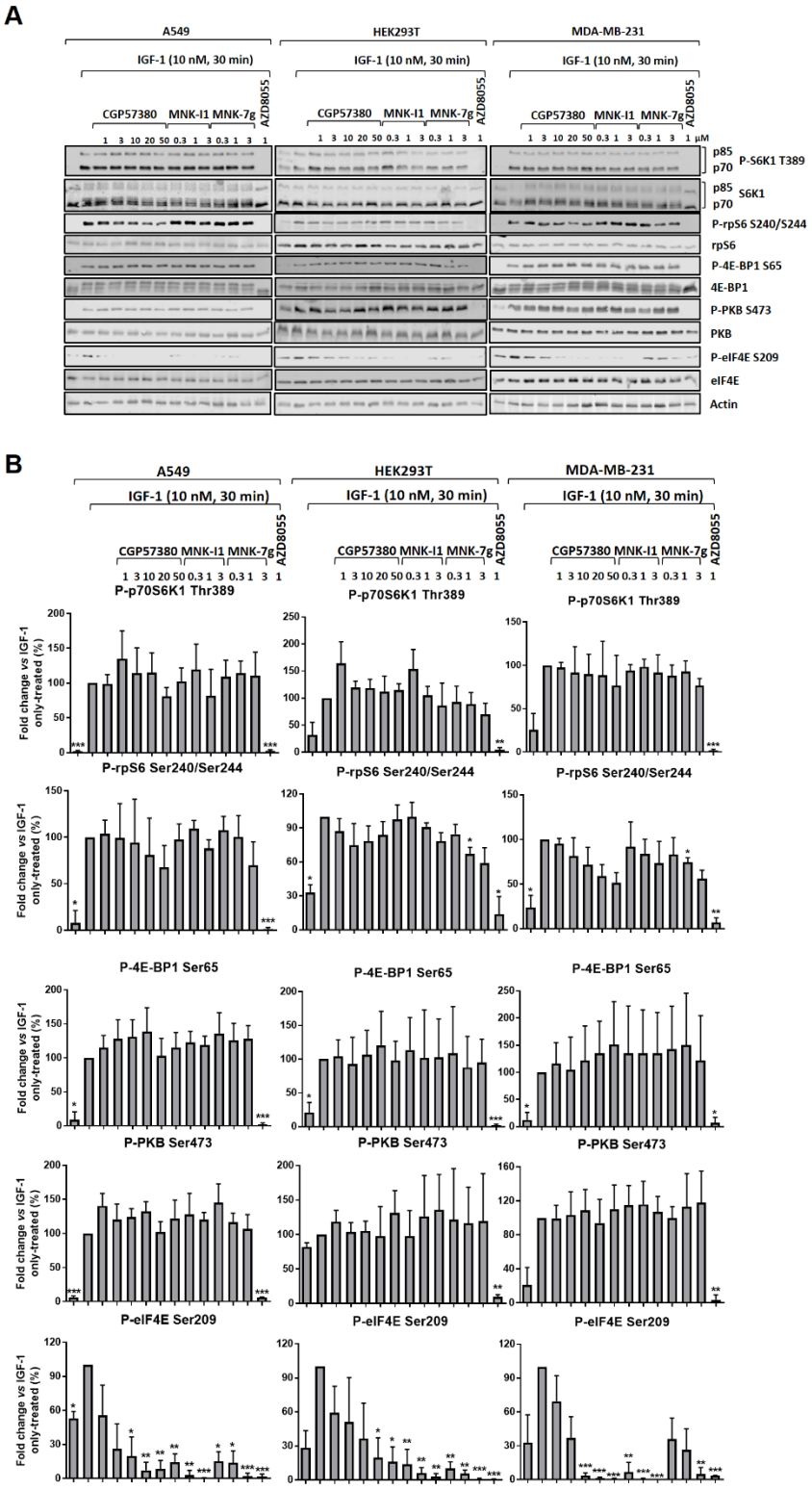

Figure S12

Figure S12. MNK inhibitors do not affect signaling downstream of mTORC1 and 2. Related to Fig. 5 and 6.

(A) A549, HEK293T and MDA-MB-231 cells were starved of serum for 16 h, pre-treated with indicated concentrations of CGP57380, MNK-I1, MNK-I2 or AZD8055 for 30 min, before stimulation

with IGF-1 (10 nM) for another 30 min. Protein lysates were immunoblotted using antisera against indicated P- or total proteins.
(B) Quantification of A.

For panel B, results are presented as means ± S.D., $n = 3$. *: $0.01 \leq P < 0.05$; **: $0.001 \leq P < 0.01$; ***: $P < 0.001$ (one-way ANOVA).

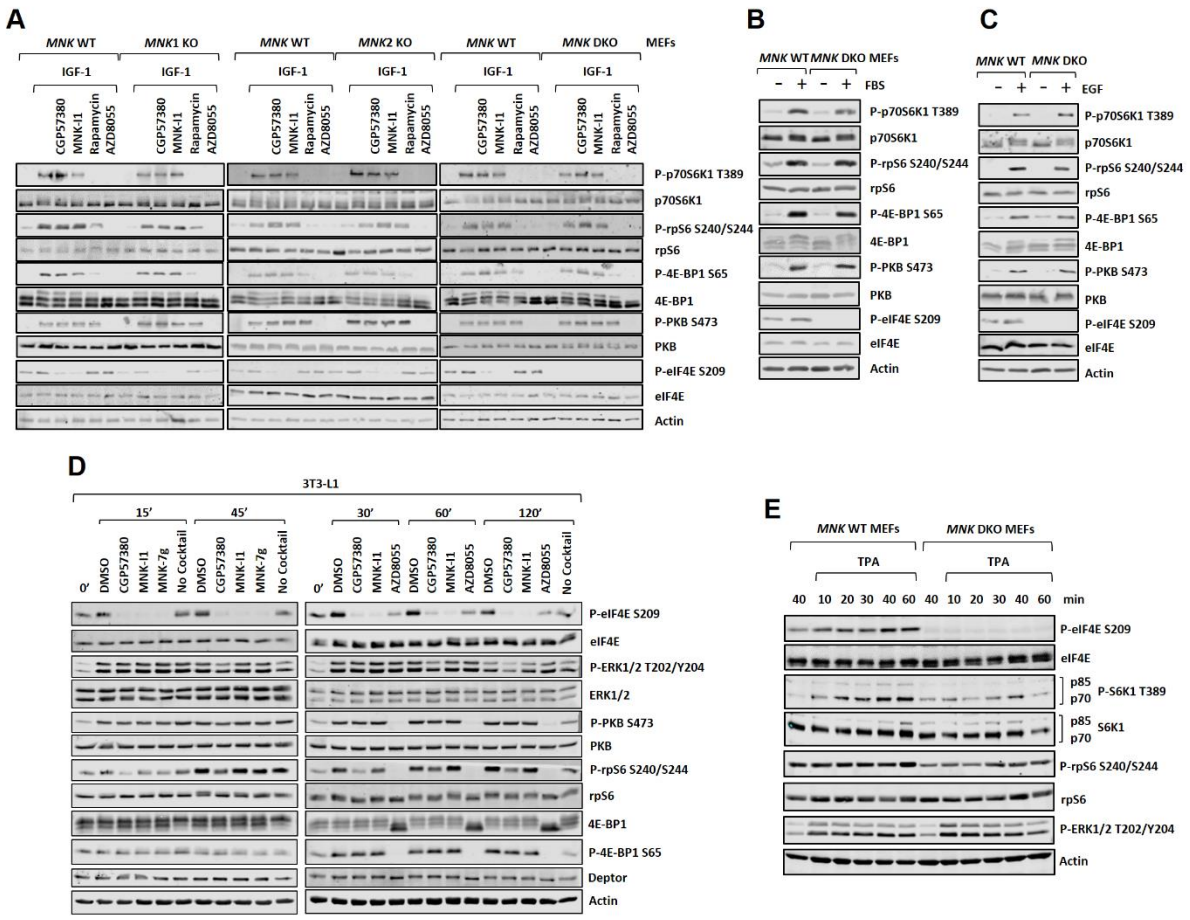

Figure S13

Figure S13. Acute MNK inhibition attenuates TPA-induced mTORC1 stimulation, yet does not affect signalling downstream of mTORC1 and 2 in response to other stimuli tested. Related to Fig. 5 and 6.

(A) WT, MNK1-KO, MNK2-KO and DKO MEFs were serum starved for 16 h, pre-treated with 20 μM CGP57380, 1 μM MNK-I1, 200 nM rapamycin or 1 μM AZD8055 for 30 min, before stimulation with IGF-1 (10 nM) for another 30 min.

(B) WT and DKO MEFs were serum starved for 16 h before stimulation with EGF (10 nM) for 30 min.

(C) WT and DKO MEFs were serum starved for 16 h before stimulation with 10% FBS for 30 min.

(D) 2-day post-confluent 3T3-L1 fibroblasts were pre-treated with 3 μM MNK-I1, 3 μM MNK-7g, 50 μM CGP57380, 1 μM AZD8055 or DMSO for 60 min before being stimulated with adipogenic cocktail for the indicated times.

(E) WT and MNK DKO MEFs were serum starved for 16 h before treatment with vehicle control or TPA for the indicated times. Proteins from cell lysates were analysed by immunoblotting as shown.

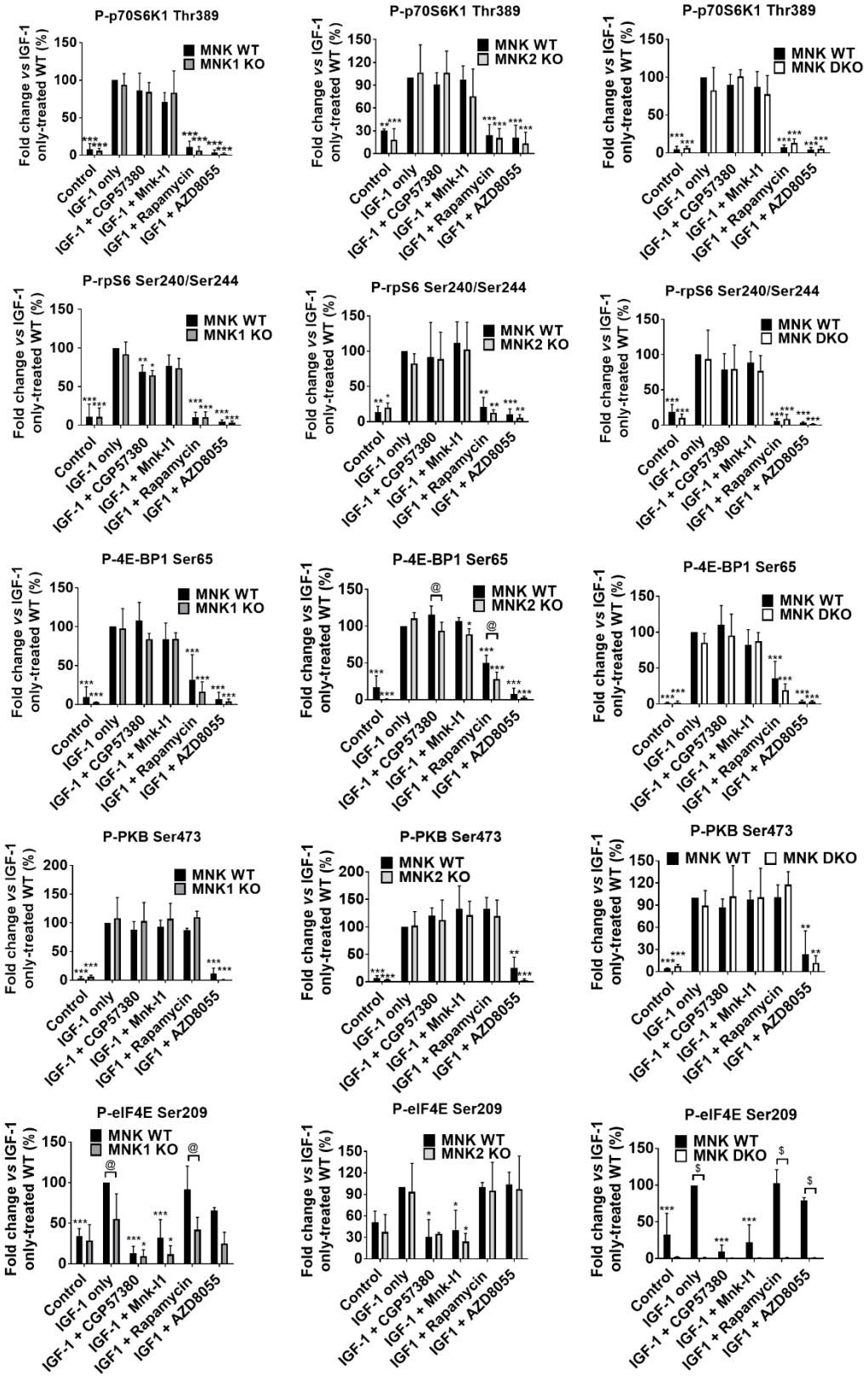

Figure S14

Figure S14. Quantitation of Fig. S13A.

Results are presented as means ± S.D., $n = 3$. *: $0.01 \leq P < 0.05$; **: $0.001 \leq P < 0.01$; ***: $P < 0.001$ (one-way ANOVA), or; @: $0.01 \leq P < 0.05$; $: $P < 0.001$ (two-way ANOVA).

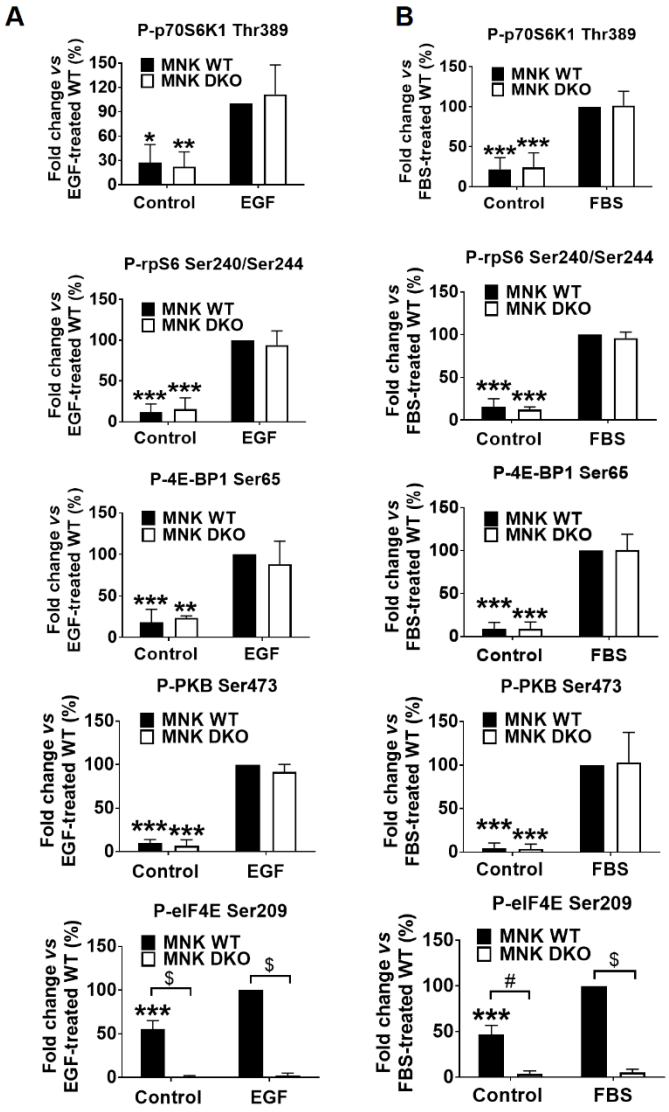

Figure S15

Figure S15. Quantitation of Fig. S13B and C.

Data are presented as means ± S.D., *n* = 3. *: 0.01 ≤ P < 0.05; **: 0.001 ≤ P < 0.01; ***: P < 0.001 (one-way ANOVA), or; #: 0.001 ≤ P < 0.01; $: P < 0.001 (two-way ANOVA).

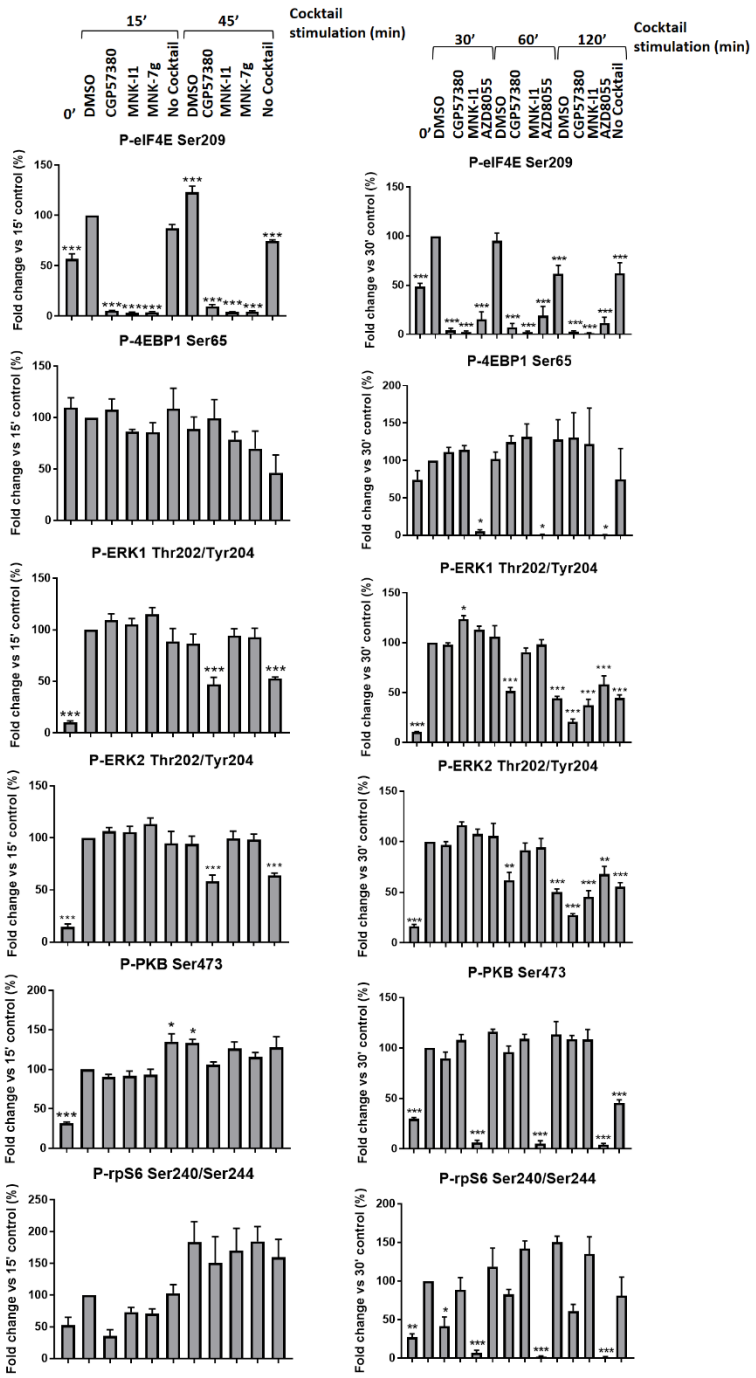

Figure S16

Figure S16. Quantitation of Fig. S13D.

Data are presented as means ± S.D., $n = 3$. *: $0.01 \leq P < 0.05$; **: $0.001 \leq P < 0.01$; ***: $P < 0.001$ (one-way ANOVA).

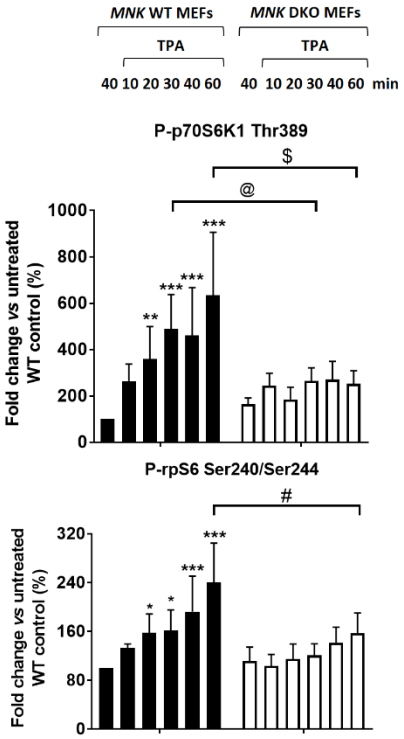

Figure S17

Figure S17. Quantitation of Fig. S13E.

Data are presented as means ± S.D., *n* = 5. *: 0.01 ≤ P < 0.05; **: 0.001 ≤ P < 0.01; ***: P < 0.001 (one-way ANOVA), or; @: 0.01 ≤ P < 0.05; #: 0.001 ≤ P < 0.01; $: P < 0.001 (two-way ANOVA).

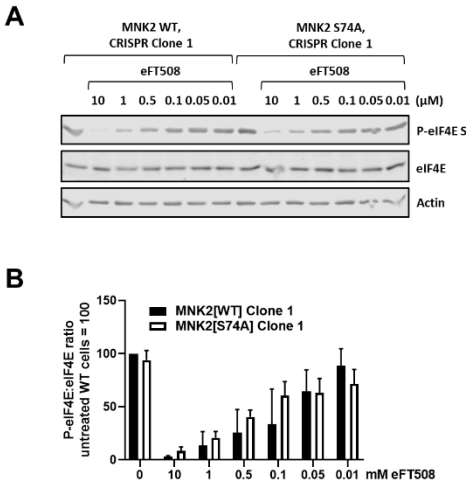

Figure S18

Figure S18. IC50 of eFT508 did not alter in MNK2[S74A] HEK293 cells.

(A) HEK293 cells were serum starved overnight, cultured in KRB for 30 min before the addition of indicated concentrations of eFT508. After 30 min, cells were treated with 1 μM TPA and were then incubated for another period of 30 min before lysis. Proteins from cell lysates were analysed by immunoblotting as shown.

(B) Quantification of A. Data are means ± S.D.; n = 3.

Table S2. Age/Gleason score statistics of prostate cancer patient samples on tissue-microarray chip. Average age: 70.8±7.9 (means ± S.E.M.) years old; range: 55-90 years old.

		Number of Patients (%)
		n=84
Age (years)	50-59	7 (8.3%)
	60-69	31 (36.9%)
	70-79	35 (41.7%)
	80-89	10 (11.9%)
	90-99	1 (1.2%)
Tumor Stage	Gleason 5	1 (1.2%)
	Gleason 6	24 (28.6%)
	Gleason 7	34 (40.5%)
	Gleason 8	13 (15.4%)
	Gleason 9	12 (14.3%)

Table S3. Effect of eFT508 on human prostate tumor cell proliferation. *Ex vivo* human prostate tumor tissues were cultured in medium containing vehicle control or indicated concentrations of eFT508. Data are expressed as % of proliferating cells as assessed by Ki-67 staining.

Patient ID	***Vehicle control***	***eFT508 (5 µM)***	***eFT508 (10 µM)***
33635L	40.19	32.71	17.52
33641RA	26.28	2.69	7.83
33592R	30.44	52.21	39.89
33633R	50.5	54.77	55.47
33646L	34.26	Poor cellularity	30.01

Table S4. List of mutagenesis primers used in this study. F: forward primer; R: reverse primer.

Protein / mutation	***F/ R***	***Primer sequence***
Mnk1 / S39A	F	5'- CCGGGCCACTGACGCCTTGCCAGGAAAG -3'
	R	5'- CTTTCCTGGCAAGGCGTCAGTGGCCCGG -3'
Mnk1 / S396A	F	5'- CTCCATGAAGCTTGCCCCTCCCTGCAAG -3'
	R	5'- CTTGCAGGGAGGGGCAAGCTTCATGGAG -3'
Mnk2 / V2-STOP	F	5'- GGGCCCCTGGGATCCTAGCAGAAGAAACCAG -3'
	R	5'- CTGGTTTCTTCTGCTAGGATCCCAGGGGCCC -3'
Mnk2 / F16A	F	5'- GTTTCCACCGTTCGGCCAAGGGGCAGAACC -3'
	R	5'- GGTTCTGCCCCTTGGCCGAACGGTGGAAAC -3'
Mnk2 / F22A	F	5'- GGGCAGAACCCCGCCGAGCTGGCCTTC -3'
	R	5'- GAAGGCCAGCTCGGCGGGGTTCTGCCC -3'
Mnk2 / E23A	F	5'- CAGAACCCCTTCGCGCTGGCCTTCTC -3'
	R	5'- GAGAAGGCCAGCGCGAAGGGGTTCTG -3'
Mnk2 / L24A	F	5'- GAACCCCTTCGAGGCGGCCTTCTCCCTAG -3'
	R	5'- CTAGGGAGAAGGCCGCCTCGAAGGGGTTC -3'
Mnk2 / F26A	F	5'- CTTCGAGCTGGCCGCCTCCCTAGACCAG -3'
	R	5'- CTGGTCTAGGGAGGCGGCCAGCTCGAAG -3'
Mnk2 / D29A	F	5'- CCTTCTCCCTAGCCCAGCCCGACCAC -3'
	R	5'- GTGGTCGGGCTGGGCTAGGGAGAAGG -3'
Mnk2 / F38A	F	5'- CGGAGACTCTGACGCTGGCCTGCAGTGC -3'
	R	5'- GCACTGCAGGCCAGCGTCAGAGTCTCCG -3'
Mnk2 / K60-STOP	F	5'- GACATCCCGGACGCCTAGAAGAGGGGCAAGAAG -3'
	R	5'- CTTCTTGCCCCTCTTCTAGGCGTCCGGGATGTC -3'
Mnk2 / S74A	F	5'- CGGGCCACCGACGCCTTCTCGGGCAG -3'
	R	5'- CTGCCCGAGAAGGCGTCGGTGGCCCG -3'
Mnk2 / G120-STOP	F	5'- CATTGAGAAGCAGCCATGACACATTCGGAGCAGGG -3'
	R	5'- CCCTGCTCCGAATGTGTCATGGCTGCTTCTCAATG -3'

Mnk2 / L180-STOP	F	5’- GGCACTTCAACGAGTAGGAGGCCAGCGTGGTG -3’
	R	5’- CACCACGCTGGCCTCCTACTCGTTGAAGTGCC -3’
Mnk2 / S240-STOP	F	5’- CTCAACGGGGACTGCTAACCTATCTCCACCCC -3’
	R	5’- GGGGTGGAGATAGGTTAGCAGTCCCCGTTGAG -3’
Mnk2 / G300-STOP	F	5’- CGTGGGCCGCTGTTGAAGCGACTGCGGCTG -3’
	R	5’- CAGCCGCAGTCGCTTCAACAGCGGCCCACG -3’
Mnk2 / A360-STOP	F	5’- CAGAGGCTGAGTGCCTAACAAGTCCTGCAGCAC -3’
	R	5’- GTGCTGCAGGACTTGTTAGGCACTCAGCCTCTG -3’
Mnk2 / G420-STOP	F	5’- GAGGAGGCCGCGTGACAGGGCCAGCC -3’
	R	5’- GGCTGGCCCTGTCACGCGGCCTCCTC -3’
Mnk2 / S437A	F	5’- CTGCCTGCAGCTGGCTCCACCCTCCC -3’
	R	5’- GGGAGGGTGGAGCCAGCTGCAGGCAG -3’

Table S5. List of primary antibodies used in this study. Abbreviations: Abcam: Abcam, Cambridge, Cambridgeshire, UK; Abclonal: Abclonal Biotechnology, Wuhan, Hubei, China; CST: Cell Signaling Technology, Danvers, MA, USA; DAKO: Agilent Technologies Pathology (Dako), Mulgrave, VIC, Australia. Dundee: Dundee cell products, Dundee, UK. EG: Eurogentec, Seraing, Belgium. IHC: immunohistochemistry; IP: immunoprecipitation; LSBio: LifeSpan BioSciences, Seattle, WA. USA; Millipore: Merck Millipore, Bayswater, Australia; P-: phosphorylated; SA: Sigma-Aldrich, Castle Hill, NSW, Australia; SCB: Santa Cruz Biotechnology, Dallas, USA; TFS: Thermofisher Scienfic, Adelaide, SA, Australia; WB: western blotting.

1^{ry} antibody	*From*	*Cat. No.*	*Application*	*Dilution*
4EBP1	CST	9452S	WB	1:1000
Cleaved caspase 3	Abcam	Ab4051	IHC	1:1000
eEF2	NEB	2332S	WB	1:1000
eEF2K	EG	Customized	WB	1:1000
eIF4E	CST	9742S	WB	1:1000
eIF4G	CST	2617S	WB	1:1000
DEPTOR	LSBio	LS-C143540	WB	1:1000
ERK1/2	CST	9102S	WB	1:1000
GST	SA	GE27-4577-01	WB	1:2000
HA	SA	H3663-100UL	WB/IP	1:1000/-
Ki67	DAKO	M7240	IHC	1:200
Lamin B	SCB	sc-6216	WB	1:1000
MNK2	Abcam	Ab84345	WB	1:1000
MNK2	TFS	PA5-13953	WB	1:1000
MNK2	SCB	sc-7445	WB	1:1000
mTOR	CST	2972S	WB	1:1000
mTOR	Dundee	Customized	IP	-
P-4EBP1 Ser65	CST	9451S	WB	1:1000
P-4EBP1 Thr37/Thr46	CST	8459S	WB	1:1000
PARP	CST	9542S	WB	1:1000
P-eEF2 Thr56	EG	Customized	WB	1:1000
P-eIF4E Ser209	Millipore	7823	WB	1:1000

P-eIF4E Ser209 (for human)	TFS	44-528G	IHC	1:100
P-eIF4E Ser209 (for mouse)	Abcam	ab76256	IHC	1:100
P-Erk1/2 Thr202/Tyr204	CST	9101S	WB	1:1000
PKB	CST	9272S	WB	1:1000
P-MNK2 Ser74	Abclonal	Customized	WB/IHC	1:1000/1:50
P-PKB Ser473	CST	9271S	WB	1:1000
P-PKB Ser473	CST	3787S	IHC	1:100
P-rpS6 Ser235/Ser236	CST	2211S	IHC	1:100
P-rpS6 Ser240/Ser244	CST	2215S	WB	1:5000
P-S6K1 Thr389	CST	9205S	WB	1:500
RAPTOR	CST	2280S	WB	1:1000
RAPTOR	Dundee	Customized	IP	-
RICTOR	CST	9476S	WB	1:1000
RICTOR	Dundee	Customized	IP	-
rpS6	SCB	SC74459	WB	1:2000
S6K1	SC	SC230	WB	1:1000
Tubulin	SA	T5168	WB	1:2000
β-Actin	SA	A5441	WB	1:2000

Reference

1. R. L. Stead, C. G. Proud, Rapamycin enhances eIF4E phosphorylation by activating MAP kinase-interacting kinase 2a (Mnk2a). *FEBS Lett* **587**, 2623-2628 (2013).