Supplemental Materials and Methods:

Cell culture, constructs, viral infections and lentiviral shRNA silencing.

Primary MEFs were isolated from WT and S6K1/2 DKO littermate mice (*1*). MEFs were serially passaged every 3 to 5 days. After 20 passages, MEFs were considered immortalized and used for experiments. WT ($p53^{-/-}$) and 4E-BP1/4E-BP2 DKO ($p53^{-/-}$) MEFs were previously described (*2*). 4E-BP1/2 DKO cells expressing exogenous 4E-BP1 (DKO 4E-BP1) were described (*3*). Cells were maintained in DMEM (Invitrogen), except for Jurkat and U937, (RPMI, Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin (all from Invitrogen) at 37^{o}C and 5% CO_2. For serum starvation, cells were washed twice with DMEM and maintained in the same media supplemented with low serum (0.5% FBS) for the indicated time periods. For amino acid depletion experiment, cells were washed twice in Hank's Balanced Salt Solution (HBSS, Invitrogen) and maintained in HBSS supplemented with D-glucose (4.5g/L) and 10% dyalized FBS (Invitrogen) for 14 hours. As a control, cells were maintained in the same media supplemented with 10% FBS. pBABE/4E-BP1 WT, 4Ala and Δ4EBS constructs and infection conditions were described previously (*3*). Lentiviral vectors were from Sigma (St. Louis, MO). shRNA vector accession numbers are: human 4E-BP1 (Sigma: TRCN0000040203), human 4E-BP2 (Sigma: TRCN0000117814), human raptor (AddGene: plasmid 1857), mouse raptor (Sigma: TRCN0000077471), human mTOR (Addgene: plasmid 1856) and human rictor (Addgene: plasmid 1854) the Non-Target shRNA Control (Sigma: SHC002 or Addgene: plasmid 1864). shRNA vectors were co-transfected into HEK293T cells with the lentivirus packaging plasmids PLP1, PLP2, and PLP-VSVG (Invitrogen) using Lipofectamine 2000 (Invitrogen). Supernatants were collected 48 and 72 hours post-transfection, passed through a 0.45 μm nitrocellulose filter and applied on target cells with polybrene (5 μg/ml). Cells were re-infected the next day and selected with puromycin for 48 hours (1μg/ml, Sigma). The adenovirus encoding $S6K1^{CA}$ (AV-$S6K1^{CA}$) was a gift from Mario Pende and was described previously (*4*). For expression, S6K DKO MEFs were infected with AV-$S6K1^{CA}$ in 6 well plates at a multiplicity of infection (MOI) of 100. Additional cells were infected with an adenovirus encoding Green Fluorescent

Protien (AV-GFP) as a control. 48 hours after infection, cells were transferred to 10 cm dishes and maintained for experimental purposes.

Cell size determination and cell cycle analysis.

Cells were seeded in 100 mm dishes, grown overnight, and treated as indicated. 48 hours later, cells were harvested by trypsinization, washed twice with PBS containing 2% FBS and once in PBS. For cell cycle analysis, cells were resuspended in 200 ul of NPE NIM-DAPI (Beckman Coulter, Mississauga, ON) and analyzed using a Cell Lab Quanta SC (Beckman Coulter) flow cytometer. For cell size determination, cells were incubated with Hoechst 33342 (1.5μg/ml in PBS) for 15 minutes at 37^{o}C. Reactions were stopped on ice for 2 minutes and the size of G1 cells was determined by measuring electronic volume (EV) using a Cell Lab Quanta SC (Beckman Coulter) flow cytometer. Kernel density estimations for the univariate variable (electronic volume) were generated and plotted for each cell type and treatment using a bandwidth of 60.

Apoptosis assay.

WT and 4E-BP DKO MEFs in which raptor was depleted by shRNA were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin/streptomycin and 1μg/ml puromycin. Cells infected with scrambled shRNA served as a control. In addition, WT and 4E-BP DKO MEFs were serum starved (0.5% FBS) for 24h or treated with a vehicle (DMSO), PP242 (2.5μM) or Torin1 (250nM) for 48h. Media was collected, plates were washed twice with PBS and the cells were detached using Accutase solution (Sigma). Media, PBS washes and cells were pooled, spun at 1,200 rpm for 5 minutes at 4^{o}C. Cell pellets were resuspended and stained with annexin V–FITC/PI kit (Invitrogen) according to the manufacturer's instructions. Stained cells were analyzed by flow cytometry (LSRII, BD Biosciences) or Cell Lab Quanta SC (Beckman Coulter) and the data was processed using BD FACSDiva software (BD Biosciences).

Cell proliferation assay.

For BrdU incorporation assay (Cell Proliferation ELISA BrdU kit from Roche), cells were seeded in 96 well plates (1,000 cells/well, except for the amino acid depletion experiment 5,000 cells/well) and maintained as

indicated in the figure legends. Absorbance at 370nm (reference wavelength 492nm) was measured using a Varioskan microplate reader (Thermo Electron Corporation, Waltham, MA). For Trypan Blue exclusion, cells were seeded in 24 well plates (30,000 cells/well) overnight, and maintained under conditions indicated in the figure legends. Cell proliferation was determined by direct counting, where dead cells were excluded by Trypan Blue staining. Both methods yielded identical results (data not shown).

Western blot analysis.

Cell lysates were prepared, and Western blotting was carried out as described (*5*). Antibodies against 4E-BP1, 4E-BP2, phospho-4E-BP1 (Thr37/46, Ser65, Thr70), S6 kinase 1 and 2, phospho-S6 kinase 1 (Thr389), Akt, phospho-Akt (Ser473), rpS6, phospho-rpS6 (Ser240/244), raptor, mTOR, rictor, phospho-eIF4B (S422), p42/44 MAPK and cyclin D3 were from Cell Signaling Technology (Danvers, MA). Anti-PDCD4 antibodies were from Rockland Immunochemicals, and a generous gift of N. Colburn. Other antibodies used for the study were against VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), eIF4E (BD Biosciences, Mississauga, ON), ODC (BIOMOL International, Plymouth Meeting, PA), and β actin (AC-15, Sigma, St. Louis, MO). Polyclonal antibodies against eIF4G, anti-eIF4B and anti-4E-BP3 polyclonal antibody were described previously (*6*-*8*). Horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were from Amersham Biosciences (Baie d'Urfé, QC, Canada).

Polysome analysis, RNA isolation and sqRT-PCR.

Polysome profile analysis was carried out as previously described (*5*). Briefly, cells were cultured in 15 cm dishes and treated with 250 nM Torin1 or vehicle (DMSO) for 24 hours. Cells were washed with cold PBS containing 100μg/ml cycloheximide, collected, and lysed in a hypotonic lysis buffer (5 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 1.5 mM KCl, 100 μg/ml cycloheximide, 2 mM DTT, 0.5% Triton X-100, and 0.5% sodium deoxycholate). Lysates were loaded onto 10-50% sucrose density gradients (20 mM HEPES-KOH (pH 7.6), 100 mM KCl, 5 mM $MgCl_2$) and centrifuged at 35, 000 rpm for 2 hours at 4 ^{o}C. Gradients were fractionated and the optical density (OD) at 254 nm was continuously recorded using an ISCO fractionator (Teledyne ISCO; Lincoln, NE). RNA from each fraction was isolated using Trizol (Invitrogen) and treated

with DNaseTurbo (Ambion) according to the manufacturer's instructions. sqRT-PCR reactions were carried out using One-step RT-PCR kit (Invitrogen) according to the manufacturer's instructions. The list of primers and the number of cycles used for each of the transcripts are provided in supplementary table 1. To ensure that the reactions were in the linear (quantifiable) range, sqRT-PCRs were performed under the same conditions using a serial dilution of the RNA isolated from WT MEFs (Supp. Fig. 5G).

Soft agar assay

Experiments were carried out in 6 well plates coated with a base layer of 0.5% agarose (Agar Noble, Difco). E1A/ras transformed WT and 4E-BP DKO cells were seeded at a density of 5,000 cells/well in 0.35% agarose containing either vehicle (DMSO), or Torin1 (250nM). Cells were overlaid with DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin (all from Invitrogen) containing either DMSO, PP242 (2.5μM) or Torin1 (250nM) and incubated for 14 days. Media was changed every 2-3 days during the course of the experiment. Colonies were counted using a light microscope (4 fields per well per condition).

In vitro cap-binding affinity assay

Cells were seeded in 150mm plates and treated with a vehicle (DMSO), PP242 (2.5 μM) and Torin1 (250nM) for 24 hours. In addition, 4E-BP DKO MEFs were infected with pBABE, pBABE/WT4E-BP1, pBABE/4Ala and pBABE/Δ4EBS, selected with puromycin for 48 hours and incubated with a vehicle (DMSO) or Torin1 (250nM) for 24 hours. Cells were then washed with cold PBS, collected, and lysed in buffer containing 40mM HEPES-KOH (pH 7.5), 120mM NaCl, 1mM EDTA, 0.1 mM GDP and 10mM pyrophosphate, 10mM beta-glycerophosphate, 50mM NaF, and 0.3% CHAPS. Lysates were incubated with m^7GDP-agarose, washed 4 times with the lysis buffer and eIF4F complex formation was assessed as previously described (*9*).

Table 1: List of primers used for sqRT-PCR

mRNA	Primer Name	Primer Sequence (5'-3')	Exon	Amplicon size	# cycles
CyclinD3	CyclinD3MF	CGAGCCTCCTACTTCCAGTG	1	150	29
	CyclinD3MR	CCGAGCCTCCTACTTCCAGTG	2		
ODC	ODCMF	CGTCGGGCTTTGTCAGTC	1	230	29
	ODCMR	CCTTCTTGGAGTGGTGGAAA	2		
VEGFa1	VEGFMF	AGAGCAACATCACCATGCAG	3	176	29
	VEGFMR	TTTCTTGCGCTTTCGTTTTT	4		
β actin	β actinMF	GGCTGTATTCCCCTCCATCG	2	154	20
	β actinMR	CCAGTTGGTAACAATGCCATGT	3		

GAPDH primers were obtained form Applied Biosystems.

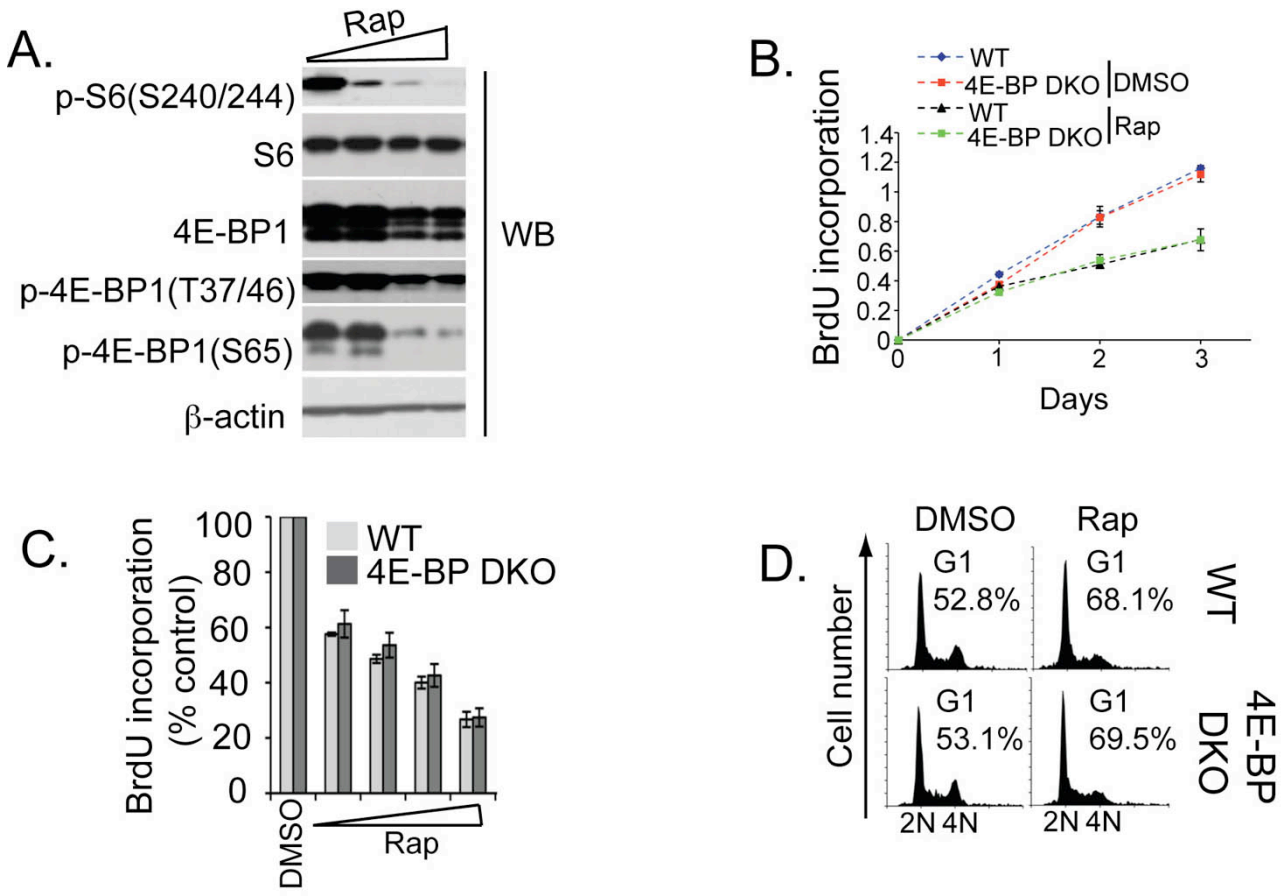

Figure S1. A WT MEFs were treated with rapamycin (5-100nM; Rap) or DMSO for 24 hours. Levels of the indicated proteins were determined by western blotting. β actin served as a loading control. **B** WT and 4E-BP DKO MEFs were treated with a vehicle (DMSO) or 50nM rapamycin (Rap) for the indicated time period and proliferation was measured by BrdU incorporation. Results are presented as mean value ± SD (n=3). **C** Proliferation of WT and 4E-BP DKO MEFs treated with rapamycin (5-100nM; Rap) or DMSO for 48 hours was monitored by BrdU incorporation. Results represent percentage of the values obtained in DMSO treated cells (set to 100%) ± SD values (n=3). **D** WT and 4E-BP DKO MEFs were treated with a vehicle (DMSO) or 50nM rapamycin for 24h and the cell cycle distribution was determined by measuring DNA content (N) using flow cytometry.

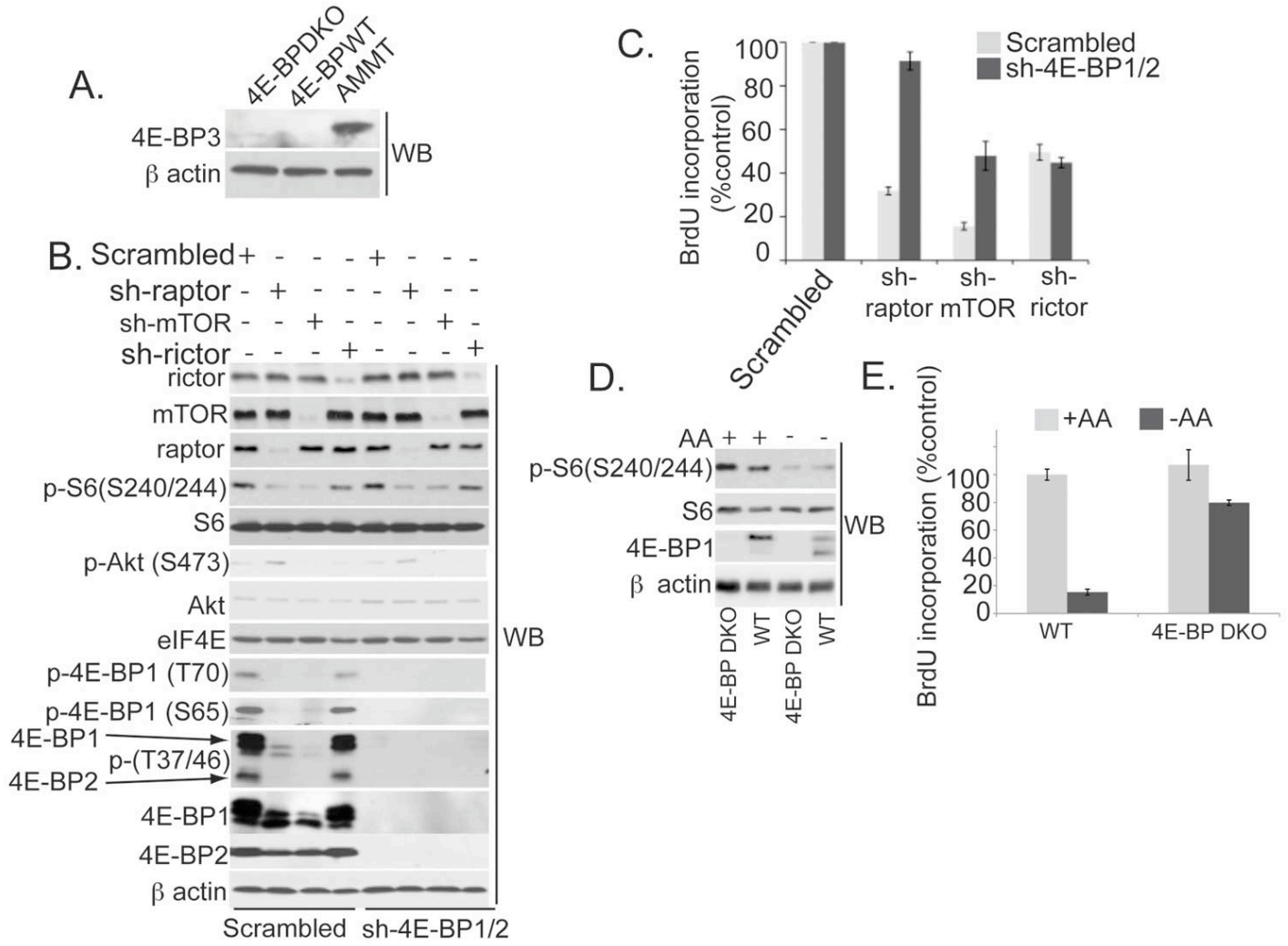

Figure S2. A Expression of 4E-BP3 protein in adult mouse mammary tissue (AMMT), 4E-BP WT, and DKO MEFs was determined by western blotting. β actin served as a loading control. **B** Stable HEK293 cell lines expressing 4E-BP1/2 shRNA (sh-4E-BP1/2) and a matched scrambled control (Mission, Sigma), were infected with lentiviruses encoding raptor, mTOR, or rictor shRNA or a matched scrambled control shRNA (Adgene). 72 hours post-infection, cells were screened by western blotting (**B**) and cell proliferation was determined by BrdU incorporation (**C**)**. D** WT and 4E-BP DKO MEFs were maintained in the presence (+AA) or absence (-AA) of amino acids for 14 hours. Levels and the phosphorylation status of indicated proteins were determined by western blotting. β actin served as a loading control. **E** Proliferation of WT and 4E-BP DKO MEFs maintained as in panel D was determined by BrdU incorporation. In panels D and E results are expressed as a percentage of the values obtained in DMSO treated cells, which were set to 100%. Bars represent SD values (n=3).

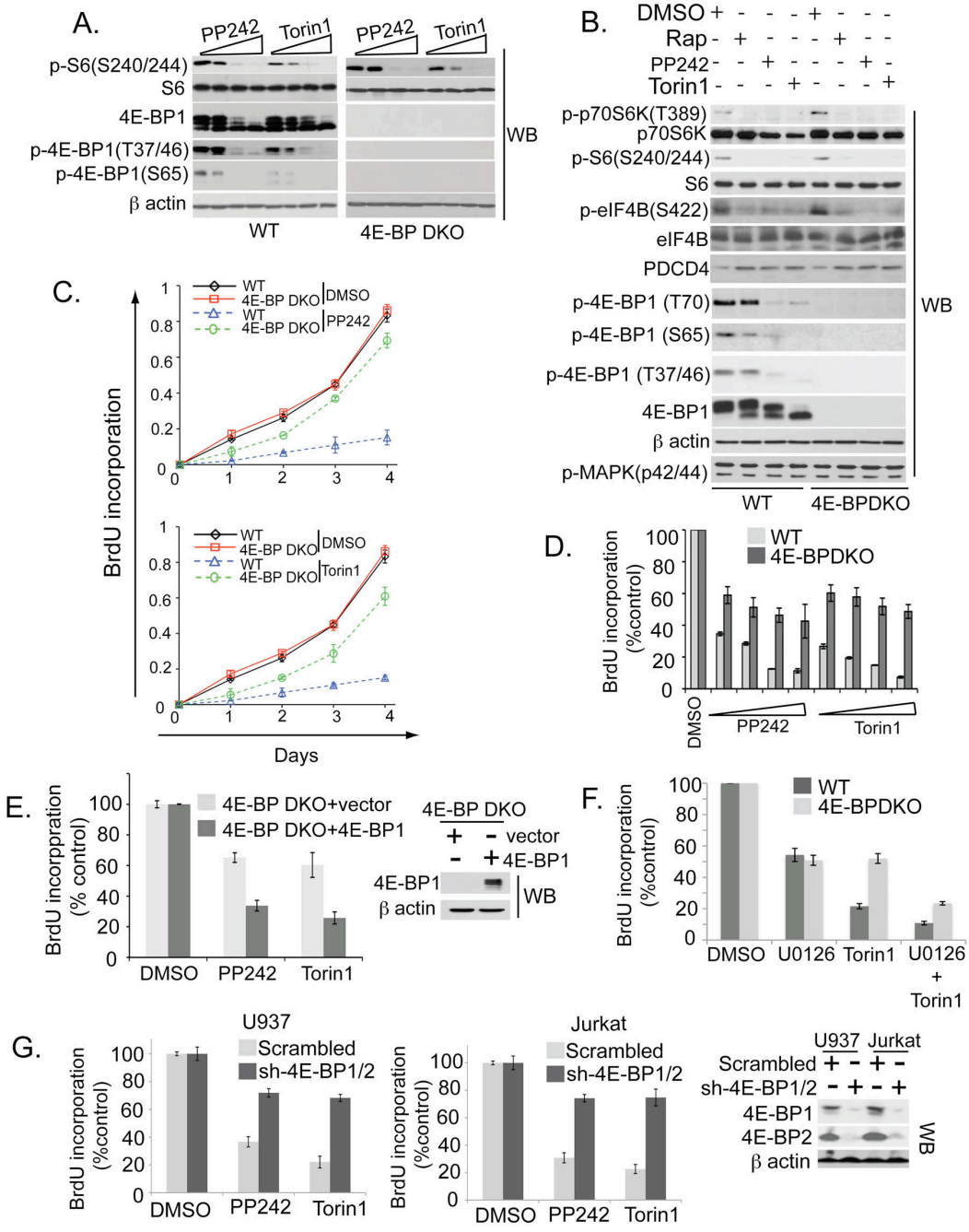

Figure S3. A WT and 4E-BP DKO MEFs were treated with PP242 (0.5-5μM), or Torin1 (25-500nM) for 24 hours. Levels of the indicated proteins were determined by western blotting. β actin served as a loading control. **B** WT and 4E-BP DKO MEFs were treated with 50nM rapamycin (Rap), 250nM Torin1, or 2.5μM PP242 and the levels of the indicated proteins were determined by western blotting. **C** Proliferation was measured by BrdU incorporation in WT and 4E-BP DKO MEFs treated with 2.5 μM PP242, 250 nM Torin1, or vehicle (DMSO) for the indicated time periods. Results are presented as mean values ± SD (n=3). **D** Proliferation of WT and 4E-BP DKO MEFs treated as in panel A for 48 hours was measured using BrdU incorporation. **E** WT and 4E-BP DKO MEFs were treated with the MEK1/2 inhibitor U0126 (40μM), 250nM Torin1, a combination of Torin1 and U0126 at the same concentrations, or vehicle (DMSO) for 48 hours. Proliferation was monitored by BrdU incorporation. **F** Proliferation of empty vector- or 4E-BP1–infected DKO MEFs (4E-BP1) treated with 2.5 μM PP242, 250 nM Torin1, or vehicle (DMSO) for 48h was measured by BrdU incorporation. The expression of exogenous 4E-BP1 was determined by western blotting. **G** Proliferation of U937 and Jurkat cells infected with sh-4E-BP1/2 or a scrambled shRNA was measured by BrdU incorporation. Cells were treated as in panel F. Depletion of 4E-BP1 and 2 was verified by western blotting. β actin-loading control in panels A, B, and G. In panels D, E, F and G results represent percentage of the values obtained in DMSO treated cells (set to 100%) ± SD (n=3).

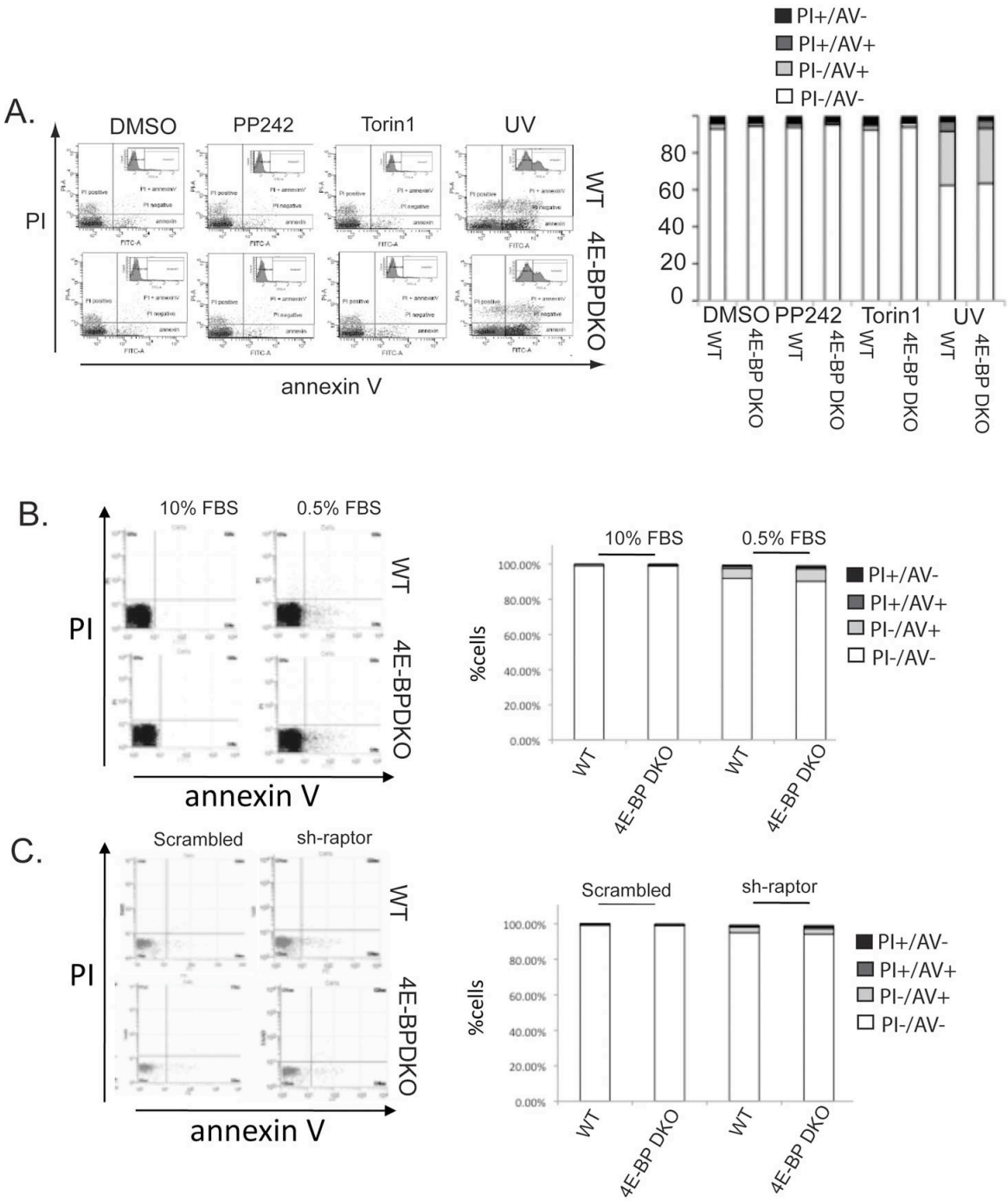

Figure S4. A The percentage of apoptotic cells in WT and 4E-BP DKO MEFs treated for 48 hours with 2.5μM PP242, 250nM Torin1, or vehicle (DMSO), was determined by flow cytometry using annexin V (AV)/propidium iodide (PI) staining. Exposure to UV irradiation (100J/m^2 for 90 minutes) induced a similar degree of apoptosis in both MEFs. **B** The percentage of apoptotic cells in WT and 4E-BP DKO MEFs maintained in 10% or 0.5% FBS, or **C** infected with a scrambled control or raptor-specific (sh-raptor) shRNA, was determined by flow cytometry using annexin V/propidium iodide (PI) staining.

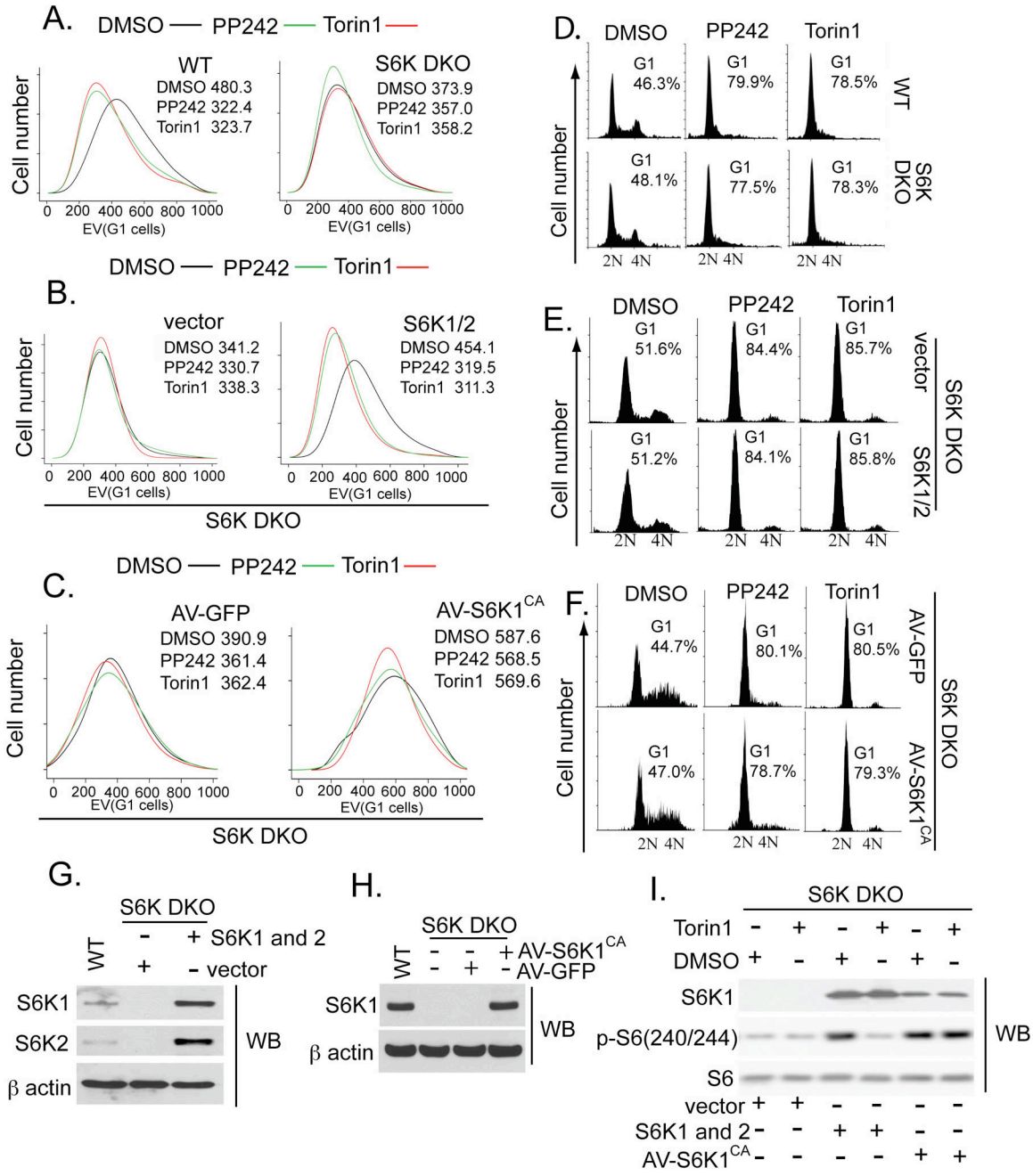

Figure S5. A WT and S6K DKO MEFs, **B** empty vector (vector) or S6K1- and S6K2-infected S6K DKO MEFs (S6K1/2), or **C** S6K DKO MEFs infected with adenovirus encoding Green Fluorescent Protein (AV-GFP) or constitutively active S6K1 (AV-S6K1^{CA}) were treated with a vehicle (DMSO), 2.5 μM PP242, or 250 nM Torin1 for 48 hours. The size of cells in G1 phase was determined by measuring electronic volume (EV) using flow cytometry. Numbers represent mean EV. WT and S6K DKO MEFs (**D**), S6K DKO MEFs infected with an empty vector or S6K1 and 2 (**E**), and S6K DKO MEFs infected with AV-GFP or AV-S6K1^{CA} (**F**) were treated as in panel C. Cell cycle distribution was determined by monitoring DNA content (N) using flow cytometry. Expression of exogenous S6Ks 1 and 2 (**G**), or S6K1^{CA} (**H**) in S6K DKO MEFs was assessed by western blotting. β actin served as a loading control. **I** Effects of Torin1 on the activity of exogenous S6K 1 and 2 and S6K1^{CA} was measured by monitoring S6 phosphorylation on Ser 240/244 by western blotting.

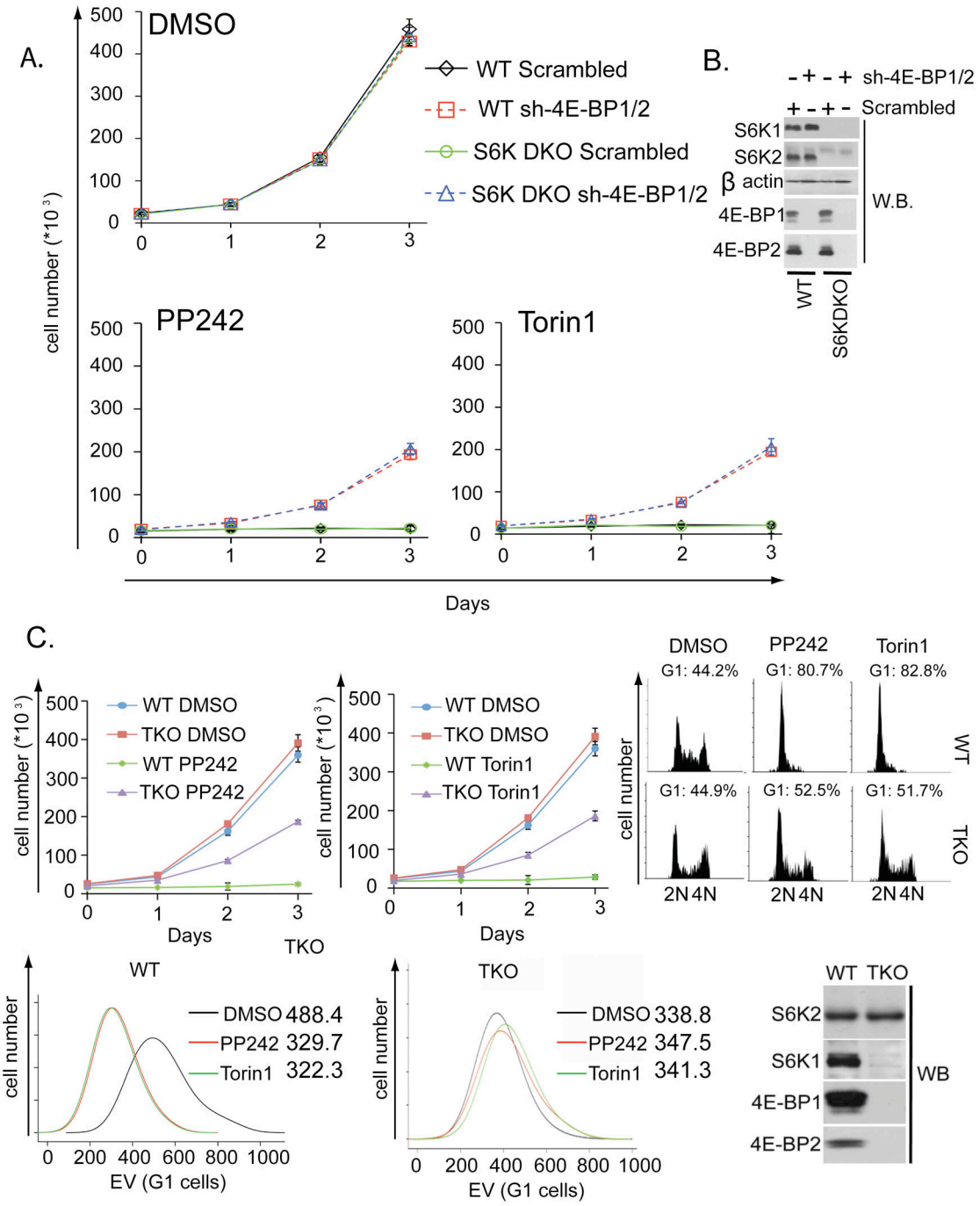

Figure S6. A WT and S6K DKO MEFs were infected with lentiviruses encoding scrambled control or 4E-BP1/2 shRNA (sh-4E-BP1/2). MEFs were treated with DMSO, PP242 (2.5μM) and Torin 1 (250nM) for the indicated time periods. Proliferation was determined by trypan blue exclusion. Results are presented as mean value ± SD (n=3) **B** Efficiency of 4E-BP1 and 2 depletion was determined by western blotting**. C** E1A/Ras-transformed WT and S6K1/4E-BP1/2 TKO MEFs were treated with DMSO, PP242 (2.5μM), and Torin 1 (250nM) for 48h (cell size and cell cycle), or for the indicated time periods (proliferation). Proliferation was determined by trypan blue exclusion. Results are presented as mean value ± SD (n=3). Cell cycle distribution was determined by measuring DNA content (N) using flow cytometry. Size of G1 cells was measured using electronic volume (EV). Numbers represent mean EV values. S6K and 4E-BP status in the WT and S6K1/4E-BP1/2 TKO MEFs was verified by western blotting.

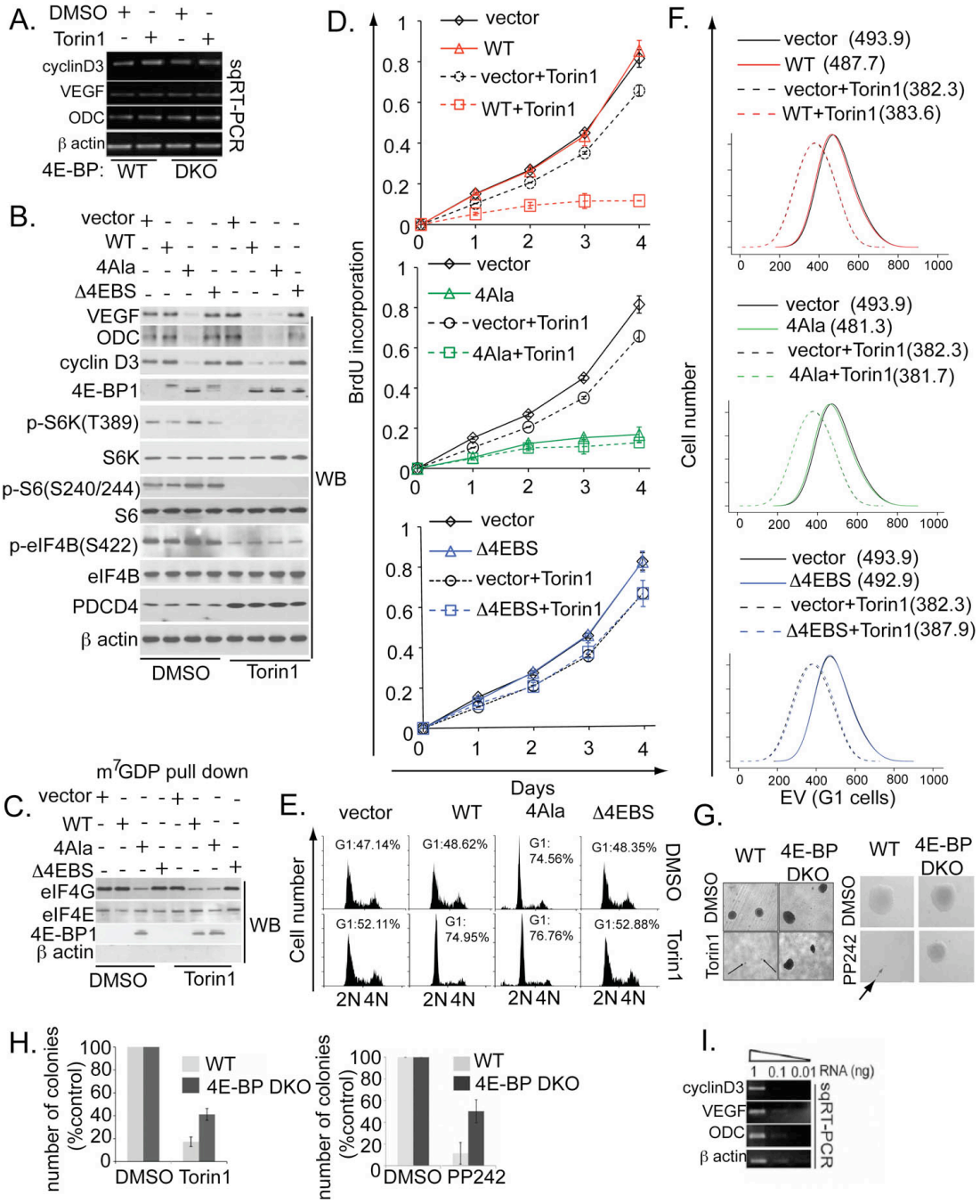

Figure S7. A Cyclin D3, VEGF, ODC, and β actin mRNAs in the cytoplasmic extracts of WT and 4E-BP DKO MEFs were quantied by sqRT-PCR. **B** 4E-BP DKO MEFs infected as indicated and treated with a vehicle (DMSO) or 250 nM Torin1 for 24 hours. Levels of the indicated proteins in these cells were determined by western blotting. **C** Association of eIF4E with eIF4G and 4E-BP1 in these cells was monitored by m^7GDP pull-down followed by western blotting. **D** Proliferation of MEFs from panel B was monitored by BrdU incorporation at the indicated time points. Results are presented as mean value ± SD (n=3). **E** Cell cycle distribution and **F** size of MEFs from panel B were determined by measuring DNA content (N) and electronic volume, respectively. **G** Effects of Torin1 (250 nM) or PP242 (2.5 μM) on anchorage-independent growth of E1A/Ras-transformed WT and 4E-BP DKO MEFs were monitored using a soft-agar assay. Arrows indicate the small size of colonies in WT MEFs treated with Torin1 or PP242. **H** Colonies in soft agar were counted after 14 days. Results represent a percentage of the number of colonies observed in DMSO treated cells (set to 100%). Mean numbers of colonies ± SD (n=4 for PP242 and n=6 for Torin1): WT MEFs+DMSO 45.2±2.5, WT MEFs+Torin1 9.6±3.5, 4E-BP DKO MEFs+DMSO 52.0±2.2 (right panel) and 4E-BP DKO MEFs+Torin1 30.5±2.4 and WT MEFs + DMSO 47.1±1.5, WT MEFs + PP242 5.3±4.0, 4E-BP DKO MEFs + DMSO 56.4±6.2 and 4E-BP DKO MEFs + PP242 29.3±2.7 (left panel). **I** sqRT-PCR reactions carried out in the experiments presented in Fig. 4C were in a linear (quantifiable) range. sqRT-PCRs were carried out using serial dilutions of RNA isolated from WT MEFs.

References:

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