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Phosphorylation by MNK1/2 Kinases in Prostate Cancer Development and Progression

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14. ABSTRACT The mRNA cap-binding protein eukaryotic initiation factor 4E (eIF4E) plays a key role in cancer progression. We have recently shown (Furic <i>et al.</i> , 2010) that phosphorylation of eIF4E at Ser209 by the MNK family of kinases promotes prostate cancer progression in a mouse model bearing a tissue-specific conditional PTEN deletion in prostatic epithelia. eIF4E activity is regulated at two levels: phosphorylation of Ser209 and binding to a family of small eIF4E-binding proteins aptly termed 4E-BPs. Binding of 4E-BPs to eIF4E precludes eIF4G binding thus preventing assembly of the eIF4F complex and recruitment of the ribosome to the messenger RNA. Our most recent data suggests that PTEN interacts genetically with 4E-BPs to induce cellular senescence in prostate cancer. In this report, we also begin to address the effects of simultaneous inhibition of MNK and mTORC1 activity in anchorage-independent prostate cancer cell lines. Our findings will hopefully yield important information for the design of effective therapeutic agents against human prostate cancer.					
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Annual Progress Report

Table of Contents

Page number

<i>1. Introduction</i>	<i>4</i>
<i>2. Body</i>	<i>5</i>
<i>3. Key research accomplishments</i>	<i>7</i>
<i>4. Reportable outcomes</i>	<i>8</i>
<i>5. Conclusion</i>	<i>9</i>
<i>6. References</i>	<i>10</i>
<i>7. Appendix</i>	<i>11</i>
<i>8. Supporting Data (Figure 1 to 3)</i>	<i>24</i>

1. Introduction

The mRNA cap-binding protein eukaryotic initiation factor 4E (eIF4E) is a key player in cancer development (Mamane *et al.*, 2004). Overexpression of eIF4E in NIH 3T3 and Rat-2 fibroblasts causes their oncogenic transformation (Lazaris-Karatzas *et al.*, 1990). Specifically, overexpression of eIF4E induces foci formation on a cell monolayer and allows for anchorage-independent growth. Moreover, injection of cell lines overexpressing eIF4E into nude mice leads invariably to tumor formation. eIF4E is phosphorylated on a single residue (Ser²⁰⁹) (Furic *et al.*, 2010). Phosphorylation of this amino acid is believed to regulate eIF4E binding to the mRNA cap and the rate of protein synthesis. While it is still not clear whether phosphorylation of eIF4E enhances or decreases the rate of protein synthesis, a recent study from our lab by Furic *et al.* (2010) has shown that phosphorylation of eIF4E promotes prostate cancer development. This study used a mouse model of prostate cancer in which the tumor suppressor PTEN (a PIP₃ phosphatase) is deleted in the prostate epithelium (*Pten*^{Flox/Flox}; Probasin-Cre4).

2. Body

Pten-null mice develop prostate invasive carcinoma by 5 to 8 months of age with complete penetrance (Trotman *et al.*, 2003). We observed that that *Pten*^{Flox/Flox}/*eIF4E*^{Ser209A/Ser209A} mice had a lower incidence of high grade PIN (prostatic intraepithelial neoplasia) than *Pten*^{Flox/Flox}/*eIF4E*^{WT/WT} mice (Furic *et al.*, 2010), suggesting that eIF4E phosphorylation plays an important role in promoting prostate cancer tumorigenesis in the context of *Pten*-deficiency. In addition to being regulated by phosphorylation, the activity of eIF4E is also regulated indirectly by a family of small eIF4E-binding proteins termed 4E-BPs, which comprise 4E-BP1, 4E-BP2 and 4E-BP3. In their dephosphorylated state, 4E-BPs bind to eIF4E thereby displacing eIF4G and inhibiting eIF4F assembly which mediates recruitment of the ribosome to the mRNA. 4E-BPs play an important role in restraining tumor development. Mice lacking 4E-BP1, 4E-BP2 and p53 exhibit lower tumor-free survival than p53-null mice (Petrolakis *et al.*, 2009). Notably, the deletion of 4E-BP1 and 4E-BP2 alone caused cellular senescence *via* p53 (unrestrained cell growth depends upon simultaneous deletion of 4E-BP1, 4E-BP2 and p53 [Petrolakis *et al.*, 2009]). Cellular senescence is an irreversible form of cell cycle arrest that can be triggered by inactivation of tumor suppressors or activation of proto-oncogenes. It occurs in aged prostate epithelia and can be readily monitored by SA- β -galactosidase staining (Fig. 2A). Deletion of PTEN (like 4E-BP1/4E-BP2) leads to p53-dependent cellular senescence (Chen *et al.*, 2005). PTEN regulates 4E-BP phosphorylation levels via mTORC1 (Fig. 1). We sought to investigate whether acute PTEN loss caused cellular senescence *via* the 4E-BPs. To address this possibility, 4E-BP1/4E-BP2 double knockout mice were crossed with *Pten*-conditional knockout mice to generate 4E-BP1/4E-BP2 WT/*Pten*^{Flox/Flox}, 4E-BP1/4E-BP2 heterozygous/*Pten*^{Flox/Flox} and 4E-BP1/4E-BP2 DKO/*PTEN*^{Flox/Flox} mice. The SA- β -galactosidase staining for 4E-BPs WT, heterozygous and null prostate epithelia was indistinguishable, *i.e.* no additive staining was observed upon deletion of 4E-BP1 and 4E-BP2 (Fig. 2B). These preliminary data suggest that loss of PTEN leads to senescence *via* the 4E-BPs. We have shown previously (Furic *et al.*, 2010) that eIF4E phosphorylation is required for anchorage-independent growth in *Pten*^{Flox/Flox} mouse embryo fibroblasts (MEFs) transduced with SV40-large T antigen. Since 4E-BP phosphorylation is regulated by PTEN, next we assessed whether 4E-BP phosphorylation plays a role in anchorage-independent growth. Phosphorylation of 4E-BPs is catalyzed by the serine/threonine protein kinase complex mammalian target of rapamycin complex 1 (mTORC1). Inhibition of mTORC1 with the allosteric inhibitor, rapamycin or the kinase inhibitor PP242 almost abolished anchorage – independent growth (Fig. 3A). Inhibition of MNK activity with compound 3 (CPD3) resulted in a substantial decrease in the number of colonies formed in soft agar. Simultaneous incubation of mTORC1 with MNK inhibitors did not have an additive effect on anchorage-independent growth, presumably because the mTORC1 inhibition alone almost completely abrogates anchorage independent growth. Therefore, it is difficult to conclude whether MNKs and mTORC1 regulate anchorage independence through the same molecular mechanism. As expected, rapamycin and PP242 (but not CPD3) markedly reduced the phosphorylation of ribosomal

protein S6, a marker for mTORC1 activation. Next we will confirm the inhibitory effect of CPD3 on MNK activity, using a phospho-specific antibody against eIF4E.

3. Key research accomplishments

- Demonstration that eIF4E phosphorylation is paramount for prostate cancer development in mouse models bearing a conditional PTEN deletion in prostatic epithelia (Furic *et al.*, 2010).
- Demonstration that cellular senescence in prostate cancer observed upon acute loss of PTEN loss likely results from increased phosphorylation of 4E-BPs (appendix, unpublished).
- Demonstration that inhibition MNK or mTORC1 signaling have similar outcomes with regards to inhibition of anchorage-independent growth (appendix, unpublished).

4. Reportable outcomes

Furic L, Rong L, Larsson O, Koumakpayi IH, Yoshida K, Brueschke A, Petroulakis E, Robichaud N, Pollak M, Gaboury LA, Pandolfi PP, Saad F, Sonenberg N. *EIF4E phosphorylation promotes tumorigenesis and is associated with prostate cancer progression*. Proc Natl Acad Sci U S A. 2010 Aug 10;107(32):14134-9.

5. Conclusion

In future work, we will repeat the preliminary data (shown in Fig. 3) to confirm our findings. We will also evaluate the effect of simultaneous MNK and mTORC1 inhibition on cell proliferation of human prostate cancer PC-3 cells. To this end, we will use a well-established BrdU labeling assay to quantify cells actively cycling through S-phase. We will validate our BrdU cell assay findings by trypan-blue exclusion viable cell counting. Should we observe an additive or synergistic effect of MNK and mTORC1 inhibition on cell proliferation, we will test the effect of simultaneous inhibition of these two pathways on tumor growth *in vivo*. Specifically we will inject PC-3 cells in nude mice subcutaneously, allow tumors to develop to a palpable size and subsequently inject rapamycin, PP242 and/or CPD3 into the tail vein. Tumors will be excised and size measured. Samples of tumors will be analysed for mTORC1 and MNK activation with phosphor-specific antisera against phosphorylated ribosomal protein S6 and eIF4E.

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7. Appendix

eIF4E phosphorylation promotes tumorigenesis and is associated with prostate cancer progression

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Translational regulation plays a critical role in the control of cell growth and proliferation. A key player in translational control is eIF4E, the mRNA 5' cap-binding protein. Aberrant expression of eIF4E promotes tumorigenesis and has been implicated in cancer development and progression. The activity of eIF4E is dysregulated in cancer. Regulation of eIF4E is partly achieved through phosphorylation. However, the physiological significance of eIF4E phosphorylation in mammals is not clear. Here, we show that knock-in mice expressing a nonphosphorylatable form of eIF4E are resistant to tumorigenesis in a prostate cancer model. By using a genome-wide analysis of translated mRNAs, we show that the phosphorylation of eIF4E is required for translational up-regulation of several proteins implicated in tumorigenesis. Accordingly, increased phospho-eIF4E levels correlate with disease progression in patients with prostate cancer. Our findings establish eIF4E phosphorylation as a critical event in tumorigenesis. These findings raise the possibility that chemical compounds that prevent the phosphorylation of eIF4E could act as anticancer drugs.

PTEN | translational control

Aberrations in the control of mRNA translation initiation have been documented in many tumor types (1–4). Translation initiation is controlled in part by eIF4E, the mRNA 5' cap-binding protein. eIF4E is a proto-oncogene, inasmuch as its overexpression in immortalized rodent fibroblasts or human epithelial cells causes transformation (5, 6), and in mouse models its overexpression engenders tumor formation (7, 8). eIF4E is phosphorylated by the MNK1/2 serine/threonine kinases, which are activated in response to mitogenic and stress signaling downstream of ERK1/2 and p38 MAP kinase, respectively (9, 10). eIF4E phosphorylation at serine 209 by MNK1/2 promotes its transformation activity (11, 12). To study the role of eIF4E phosphorylation in tumorigenesis in the whole organism, we generated a knock-in (KI) mouse in which eIF4E serine 209 was mutated to alanine. Here, we show that mouse embryonic fibroblasts (MEFs) isolated from eIF4E^{S209A/S209A} embryos display a marked resistance to oncogene-induced transformation. Furthermore, the mutant mice are viable, but are resistant to development of *Pten* loss-induced prostate cancer, and this resistance is associated with a decrease in MMP3, CCL2, VEGFC, and BIRC2 proteins. Moreover, eIF4E is highly phosphorylated in hormone-refractory prostate cancer, which correlates with poor clinical outcome. These results demonstrate the importance of eIF4E phosphorylation in tumorigenesis and validate the eIF4E phosphorylation pathway as a potential therapeutic target for cancer.

Results

Ser209 Is the Only Phosphorylation Site in eIF4E. To address the role of eIF4E phosphorylation in tumorigenesis, a knock-in (KI) mouse in which serine 209 was replaced by an alanine residue was generated. The strategy and targeting vector construction for the generation, selection, and genotyping of the S209A mice is shown in

Fig. S1. The eIF4E^{S209A/S209A} mice (referred to as KI mice hereafter) showed no obvious phenotype. To determine whether S209 is the only phosphorylation site on eIF4E, orthophosphate labeling of MEFs isolated from WT and KI littermate embryos was performed. Phosphorous 32–radiolabeled eIF4E was detected by immunoprecipitation in only WT MEFs (Fig. 1A). As expected, TPA stimulation, which activates MNK (13), induced a twofold increase in eIF4E phosphorylation (Fig. 1A). Thus, mutating S209 abrogates eIF4E phosphorylation.

eIF4E-KI MEFs Are Resistant to RAS-Induced Transformation. RAS is an upstream activator of MNK1 and MNK2 through ERK-1 and -2 (9, 10). Therefore, it was pertinent to determine whether transformation by RAS is facilitated by eIF4E phosphorylation. To this end, the two-oncogene transformation assay was performed with retroviruses expressing RAS^{V12} (RAS containing the activating mutation G12V) together with c-MYC or E1A. Experiments were carried out in primary MEFs between passages three and five. Strikingly, KI MEFs infected with a combination of retroviruses expressing RAS^{V12} and c-MYC formed approximately fivefold fewer foci than WT MEFs (Fig. 1B). A similar difference in transformation efficiency was observed when MEFs were infected with retroviruses expressing the adenovirus E1A together with RAS^{V12} (Fig. 1B). WT and KI MEFs infected with an empty retrovirus failed to form foci (Fig. 1B). Anchorage-independent growth of WT and KI MEFs transduced with activated RAS^{V12} and c-MYC was determined by colony formation in soft agar. The assay was performed with three different pairs of MEFs, each isolated from embryos of a different pregnant mouse. KI MEFs formed four- to 10-fold fewer colonies than WT MEFs (Fig. 1C). These results demonstrate that eIF4E phosphorylation on serine 209 is required for efficient transformation by RAS. To further demonstrate the importance of eIF4E phosphorylation for cellular transformation, we performed transformation assays using immortalized WT or MNK1/2 DKO MEFs and HA-tagged eIF4E as the transforming oncogene. HA-tagged eIF4E was expressed at similar levels in WT and MNK1/2 DKO MEFs, and as expected no phosphorylation of endogenous or HA-tagged eIF4E was detected by Western blotting in MNK1/2 DKO MEFs (Fig. 1D). WT MEFs overexpressing HA-eIF4E formed foci after

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The authors declare no conflict of interest.

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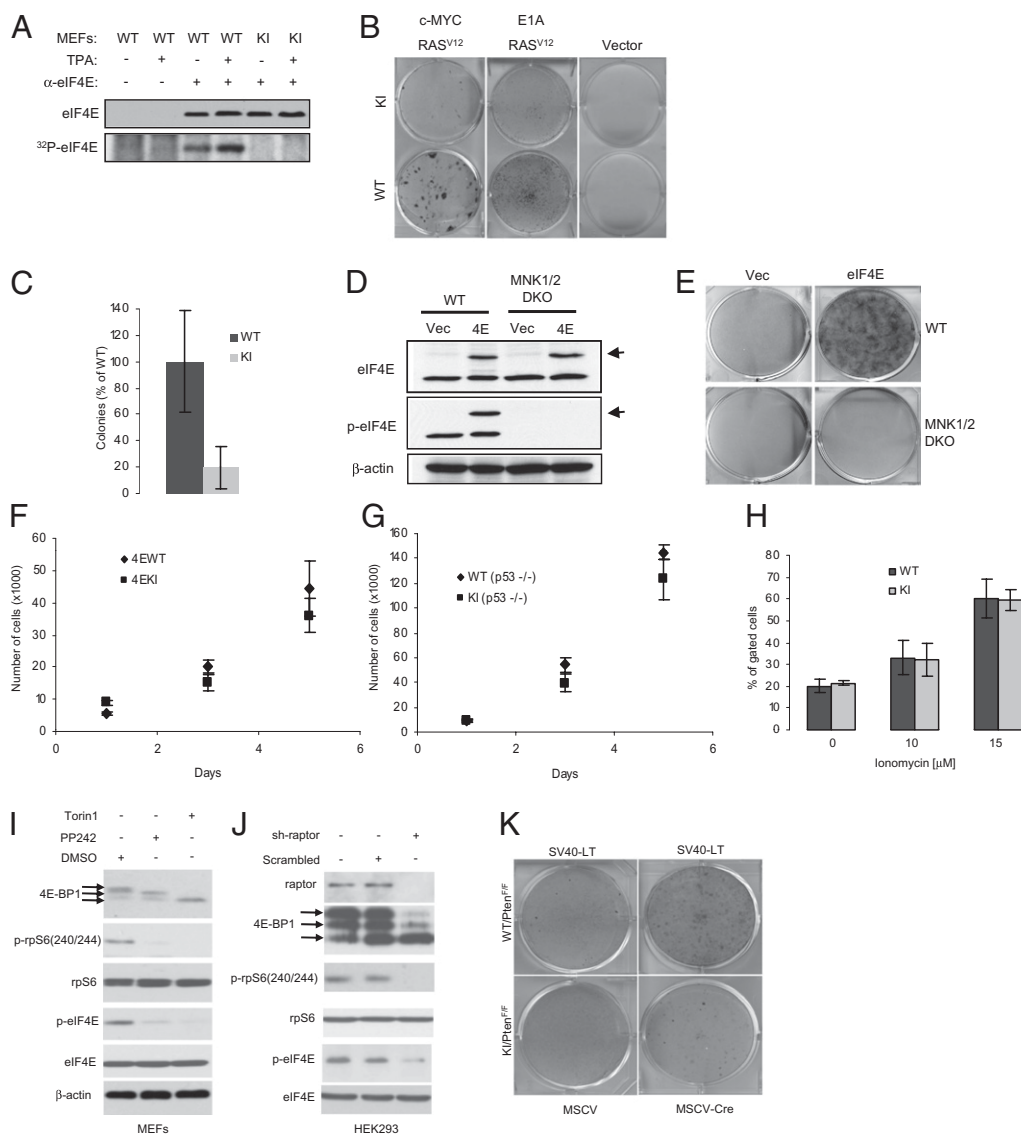
Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE17451).

See Commentary on page 13975.

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Fig. 1. KI MEFs are resistant to malignant transformation. (A) Untreated or TPA-treated (100 ng/mL) MEFs were labeled with 32 P-orthophosphate for 2 h as described in *Materials and Methods*. Cells were lysed and the supernatant was incubated with control (preimmune serum) or eIF4E antibody. Immunoprecipitated proteins were resolved by SDS/PAGE followed by autoradiography and Western blotting. (B) MEFs infected with the indicated retroviruses were grown on a monolayer and focus formation was determined after 10 d in culture by methylene blue staining. Similar results were obtained with eight independent pairs of MEFs. (C) WT and KI MEFs infected with c-MYC and RAS^{V12} expression vectors were grown in soft agar and the total number of colonies consisting of more than eight cells were counted (six wells for each pair of MEFs). WT MEFs formed significantly more colonies than KI MEFs (two-tailed Student *t* test, *P* = 0.037). (D) Cell lysates from WT and MNK1/2 DKO MEFs expressing HA-eIF4E were resolved by SDS/PAGE followed by Western blotting. Arrows indicate the slower migrating band corresponding to the HA-tagged eIF4E. (E) MEFs infected with the indicated retroviruses were grown on a monolayer and focus formation was determined after 10 d in culture by methylene blue staining. (F and G) Cells (1×10^5) were seeded on d 0 and counted on d 1, 3, and 5. Data points represent mean $\pm$ SD of three independent experiments. (H) Cells (1×10^6) were seeded and subjected to treatment for 24 h with the indicated concentrations of ionomycin. Floating and attached cells were collected, and the double-positive Annexin V/PI subpopulation of cells was gated. No significant differences were observed between WT and KI MEFs (two-tailed Student *t* test: not treated, *P* = 0.511; 10 μ M, *P* = 0.329; 15 μ M, *P* = 0.952). Similar results were obtained in three independent experiments. (I) MEFs were serum starved for 6 h in the presence of vehicle (DMSO), PP242 (2.5 μ M), or Torin1 (250 nM), serum-stimulated for 30 min, and the phosphorylation and levels of indicated proteins were determined by Western blotting. (J) Raptor was silenced in HEK293 cells by shRNA (sh-raptor). As a control, cells were infected with a lentivirus encoding a scrambled shRNA. Expression and the phosphorylation levels of the indicated proteins were determined by Western blotting. (K) WT/PTEN^{F/F} and KI/PTEN^{F/F} MEFs infected with the indicated retroviruses were grown as a monolayer and focus formation was determined after 10 d in culture by methylene blue staining. Similar results were obtained from two independent experiments each done in triplicate.



10 d in culture. Strikingly, MNK1/2 DKO MEFs were completely resistant to transformation by HA-eIF4E (Fig. 1E). These findings clearly demonstrate that eIF4E transforming activity is absolutely dependent on phosphorylation by the MNK1/2 kinases.

Abrogating eIF4E Phosphorylation Does Not Impair Cell Proliferation. The resistance of KI MEFs to transformation could be explained by an inherently reduced proliferative rate of KI MEFs compared with WT MEFs. To investigate this possibility, growth curves using primary MEFs at passage three were performed. No difference in growth between WT and KI MEFs over a 5-d period was detected (Fig. 1F). Primary MEFs are slow-growing cells and consequently a small effect on growth could have been missed. We therefore crossed the WT and the KI mice with p53-null mice to generate immortalized WT and KI MEFs, which grow faster than primary MEFs. Similar growth curves were obtained for both WT and KI p53-null MEFs (Fig. 1G). Another

possible explanation for the reduced transformation efficiency of KI MEFs is that they are more susceptible to apoptosis. To address this possibility, WT and KI primary MEFs were treated with the apoptosis inducing ionophore ionomycin. Apoptotic and necrotic cells were visualized by dual annexin V–propidium iodide staining. No difference in the percentage of apoptotic and necrotic cells was observed between WT and KI MEFs after ionomycin treatment (Fig. 1H). In addition, there was no difference in cell cycle progression or apoptosis between WT and KI cells transformed by c-MYC and RAS^{V12} (Fig. S2 A–C). These results indicate that the resistance of KI MEFs to Ras-induced transformation cannot be explained by decreased proliferation or increased cell death.

eIF4E Phosphorylation Is Regulated by Mammalian Target of Rapamycin Complex 1 and Contributes to Transformation via the PI3K Pathway. Another pathway that controls eIF4E activity is the PI3K pathway

via the phosphorylation of the suppressor 4E-BPs by mammalian target of rapamycin complex 1 (mTORC1) (1, 14). The loss of the tumor suppressor PTEN leads to AKT, and subsequent mTORC1 activation (15). To determine whether eIF4E phosphorylation is impacted by mTORC1, we treated immortalized MEFs with two active-site inhibitors of mTORC1, Torin1 (16) and PP242 (17). Inhibition of the mTORC1 kinase was confirmed by a decrease in the phosphorylation of the small ribosomal subunit protein S6 (p-rpS6) and 4E-BP1 (Fig. 1*I*). Interestingly, eIF4E phosphorylation was drastically diminished following mTORC1 inhibition (Fig. 1*I*). To confirm that the decrease in eIF4E phosphorylation was not a result of nonspecific effects of the two inhibitors, we used shRNA-mediated knockdown of RAPTOR as an alternative approach for inhibiting mTORC1 activity in 293 cells. Consistent with the results obtained with Torin1 and PP242, lowering the amount of RAPTOR caused a marked decrease in eIF4E phosphorylation (Fig. 1*I*). These results can be readily explained by the fact that dephos-

phorylation of 4E-BPs increases their binding to eIF4E, thus preventing eIF4E binding to eIF4G, on which MNK docks (18). We investigated further the role of eIF4E phosphorylation control downstream of mTORC1 by generating WT and KI MEFs in which both alleles of PTEN were deleted via CRE-mediated recombination (15). MEFs were immortalized with SV40 largeT and subsequently infected with a CRE-expressing retrovirus to delete *Pten*. Loss of PTEN in combination with SV40 large T expression caused malignant transformation as determined by focus formation in WT MEFs, whereas KI MEFs were resistant to transformation, as only a few foci were detected (Fig. 1K). These results demonstrate that eIF4E phosphorylation is involved in PTEN loss-driven cellular transformation.

eIF4E-KI Mice Are Resistant to PTEN Loss-Induced Prostate Cancer.

Because *Pten* deletion is expected to result in increased eIF4E phosphorylation, we investigated the importance of eIF4E phos-

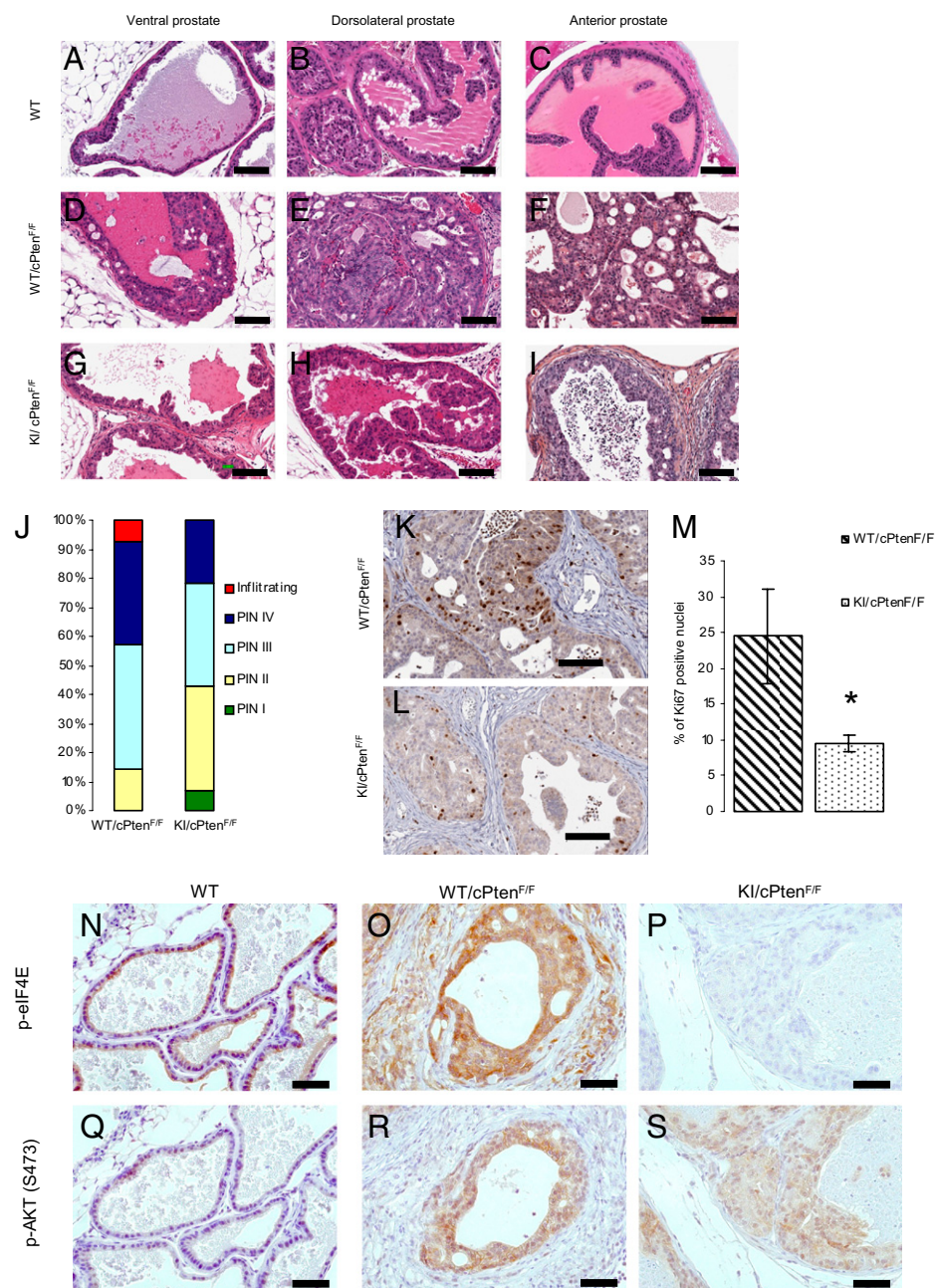


Fig. 2. KI mice are resistant to Pten loss-induced prostate cancer. (A–I) Representative H&E staining of sections from the anterior (AP), ventral (VP), and DLP lobes of the prostate of WT, WT/cPten^{F/F}, and KI/cPten^{F/F} from 5- to 6-mo-old mice (no histological difference was observed between WT and KI mice without Cre; Fig. S3). (Scale bar: 100 μm.) (J) Histograms represent percentage of predominant lesions in a cohort of seven WT/cPten^{F/F} and seven KI/cPten^{F/F}. (K and L) Representative images of Ki67 staining of DLP sections from WT/cPten^{F/F} and KI/cPten^{F/F} mice (5–6 mo old). (Scale bar: 100 μm.) (M) Slides were scanned with the Aperio Scanscope and nuclear staining was quantified with the Image Scope software nuclear algorithm. At least 4,000 nuclei per mouse were analyzed ($n = 4$ mice per group). Student *t* test (two-tailed) was performed. (N–S) Ventral prostate sections from WT, WT/cPten^{F/F}, and KI/cPten^{F/F} mice were used for IHC with the indicated antibodies. (Scale bar: 50 μm.)

phorylation in tumor formation in vivo in a mouse model of prostate cancer in which the tumor suppressor *Pten* is deleted in the prostate epithelium (*Pten*^{Flox/Flox}; PB-Cre4) (15). These mice develop invasive carcinoma by 5 to 8 mo of age with complete penetrance (15). KI mice were crossed with *Pten*-conditional KO mice to generate *eIF4E* WT/*Pten*^{Flox/Flox}/PB-Cre4 and *eIF4E* KI/*Pten*^{Flox/Flox}/PB-Cre4, hereafter referred to as WT/cPten^{F/F} and KI/cPten^{F/F}, respectively. Seven mice of each genotype were killed between the ages of 5 and 7 mo (Table S1). Cancerous lesions, mainly prostatic intraepithelial neoplasia (PIN), were graded from I to IV (Fig. S4 shows representative sections) (19, 20). Analysis of H&E-stained sections of all prostate lobes in 5- to 7-mo-old mice shows that KI/cPten^{F/F} mice had a lower incidence of high grade PIN (represented by PIN IV lesions) than WT/cPten^{F/F} mice (Fig. 2 A–I). There was a significant difference in the grade of lesions observed between WT/cPten^{F/F} and KI/cPten^{F/F} mice ($P = 0.038$, Mann–Whitney U test). The distribution of the lesions in WT/cPten^{F/F} and KI/cPten^{F/F} is shown in Fig. 2J. It is noteworthy that no infiltrating adenocarcinoma was observed in KI/cPten^{F/F}. It is unlikely that the differences seen in the severity of the lesions between WT/cPten^{F/F} and KI/cPten^{F/F} are caused by a small delay in tumor appearance, as the median age at sacrifice time was 25 wk for the KI/cPten^{F/F} compared with 20 wk for the WT/cPten^{F/F} mice (Table S1). The differences in the severity of the lesions between the WT and KI groups were also determined by the number of proliferative cells as detected by Ki67 staining in the dorsolateral prostate (DLP) of mice from each genotype (Fig. 2 K–L). DLP was chosen because it is histologically and functionally the most closely related to the peripheral zone of the human prostate (21), which is the region where most prostate cancers occur in men (22, 23). There was a significant decrease ($P = 0.013$) in the number of Ki67-positive nuclei between WT/cPten^{F/F} ($24.5 \pm 6.6\%$) and KI/cPten^{F/F} ($9.5 \pm 1.1\%$) mice (Fig. 2M). In addition, eIF4E was highly phosphorylated on Ser209 in PIN IV lesions from WT/cPten^{F/F} mice (Fig. 2 N–P). AKT^{Ser473} phosphorylation was increased to the same extent in WT/cPten^{F/F} and KI/cPten^{F/F} mice (Fig. 2 Q–S), suggesting that differences in lesion grade are not caused by a weaker activation of the PI3K pathway in the KI mice. Taken together, these results demonstrate that eIF4E phosphorylation plays an important role in *Pten* loss-induced tumorigenesis.

eIF4E Phosphorylation Increases the Translation Efficiency of a Subset of mRNAs Encoding Protumorigenic Factors. To study the molecular basis that underlies the eIF4E-KI resistance to tumorigenesis at the translational level, polysome profiling was performed on WT and KI immortalized MEFs. The changes in distribution of mRNAs along a sucrose density gradient between WT and KI MEFs were studied using DNA oligonucleotide microarrays. mRNAs in KI MEFs that shifted to lighter fractions relative to WT MEFs in the density gradient, after correcting for total cytoplasmic mRNA levels, are expected to be translated less efficiently in KI MEFs. A list of 35 mRNAs that exhibited the most pronounced shifts is shown in Table S2. Among these mRNAs are the chemokines *Ccl2* and *Ccl7* that are implicated in tumor progression. Targeting CCL2 with a neutralizing antibody caused prostate tumor regression (24–26). The list also includes mRNAs encoding the matrix metalloproteinases (MMPs) MMP3 and MMP9, which are overexpressed in PC3 prostate cancer cells (27), and promote invasion and metastasis by rearrangement of the ECM (28, 29). Other mRNAs encode the inhibitor of apoptosis, baculoviral IAP repeat-containing protein 2 (BIRC2), and the growth factor VEGFC. To determine whether the differences in polysome sedimentation between WT and KI MEFs are reflected in changes in the abundance of protein, we examined VEGFC, BIRC2, MMP3, and nuclear factor of κ -light polypeptide gene enhancer in B-cells inhibitor- α (NFKBIA; also known as I κ B α) proteins by Western blotting (Fig. 3A). A 1.7- to 4.8-fold reduction in the amount of these proteins was observed in KI MEFs compared with WT MEFs, whereas the amount of AKT, eIF4E, and β -actin remained unchanged. In addition,

inhibiting Mnk1/2 kinases in two prostate cancer cell lines with the inhibitor CGP57380 (30) led to a decrease in the amount of VEGFC, BIRC2, and NFKBIA proteins (Fig. S5A). We also examined the amount of MMP3 in DLP extracts isolated from KI/cPten^{F/F} and WT/cPten^{F/F} mice. A fourfold decrease in MMP3 protein in the DLP of KI/cPten^{F/F} compared with WT/cPten^{F/F} was detected, whereas the protein was barely detected in DLP from WT mice (Fig. 3B). Considering that the KI mice develop fewer PIN IV lesions, characterized by a loss of the fibromuscular layer around the prostatic ducts (20), as seen by staining for smooth muscle actin (SMA; Fig. 3 C–E), MMP3 staining by immunohistochemistry (IHC) was performed. Consistent with the Western blotting data, MMP3 staining intensity was weaker in the prostate of KI/cPten^{F/F} compared with WT/cPten^{F/F} mice (Fig. 3 F–H). In addition, CCL2 staining in the prostate of KI/cPten^{F/F} was weaker compared with WT/cPten^{F/F} mice (Fig. 3 I–K). These results demonstrate that several mRNAs important for tumor progression are differentially translated in WT versus KI MEFs. Impairment in the remodeling of the extracellular matrix or differences in paracrine signaling could be possible explanations for the resistance of KI mice to *Pten* loss-induced tumorigenesis.

eIF4E Phosphorylation Is Associated with High Gleason Score and Strongly Correlates with MMP3 Expression in a Cohort of Patients with Prostate Cancer. Next, we investigated the correlation between eIF4E phosphorylation in human prostate cancer and disease progression. A tissue microarray (TMA) constructed with human prostate cancer (PCa) samples including patients presenting primary and hormone-refractory PCa (HR PCa) was used (31, 32). Both eIF4E and phospho-eIF4E staining intensity in-

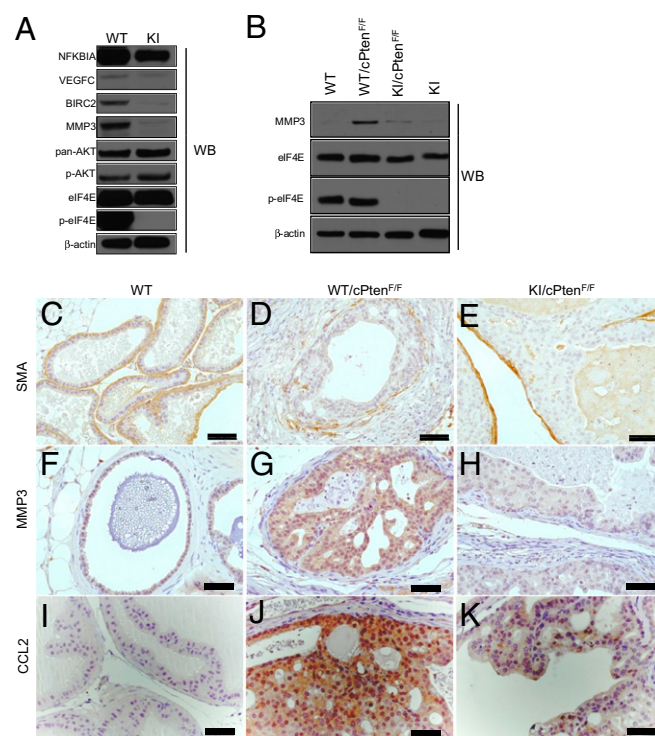


Fig. 3. Differential expression of selected proteins in prostate from WT versus KI mice. (A) Serum starved WT and KI MEFs were serum-stimulated for 2 h and cell lysates were resolved by SDS/PAGE followed by Western blotting with the indicated antibodies. (B) DLP extracts were resolved by SDS/PAGE and Western blotting with the indicated antibodies was performed. (C–K) Sections of the prostate from WT, WT/cPten^{F/F}, and KI/cPten^{F/F} mice were used for IHC with the indicated antibodies. VP sections were used for detection of SMA and MMP3. CCL2 was detected in the anterior prostate. (Scale bar: 50 μ m.)

creased gradually from normal to PIN, to hormone-sensitive (HS), and further to HR PCa tumors (Fig. 4A shows representative staining). Increased phospho-eIF4E and total eIF4E staining was significantly associated with HR tumors (Fig. 4B and C). Statistical analysis of the association between phospho-eIF4E staining and Gleason grading demonstrated that tumors presenting with a Gleason score greater than 7 displayed a statistically significant increase in staining compared with tumors with a Gleason score of 7 or lower (Fig. 4D). Thus, there is a strong association between the degree of eIF4E phosphorylation and progression of prostate cancer to its deadliest stage. To determine whether MMP3 could also serve as a marker of prostate cancer progression, we stained the same TMA for the presence of MMP3 (Fig. 4A shows representative staining). MMP3 expression was significantly higher in hormone-sensitive and HR tumors compared with PIN and normal tissue (Fig. 4E). Furthermore, analysis using Spearman rank correlation demonstrated that MMP3 is significantly associated with phospho-eIF4E ($\rho = 0.396$) and eIF4E ($\rho = 0.524$) in tumor tissues (Table S3). In addition, we performed staining for p-ERK and p-AKT to examine the association between upstream signaling activity and eIF4E phosphorylation. As expected, eIF4E phosphorylation correlated with p-ERK ($\rho = 0.266$) and p-AKT ($\rho = 0.321$). Strikingly, MMP3 was not significantly associated with p-ERK ($\rho = 0.006$) and p-AKT ($\rho = 0.110$), suggesting a stronger association between p-eIF4E and MMP3 than with upstream MAPK and PI3K signaling.

Discussion

We show that eIF4E phosphorylation promotes prostate tumor development and progression in mice. mRNAs that are less well translated in the absence of eIF4E phosphorylation include those that encode for proteins involved in the remodeling of the ECM, inhibition of apoptosis, and cellular growth and proliferation. The decrease in MMP3 and the chemokine CCL2 is consistent with the reduction in invasiveness observed in the KI mice prostate tumors. eIF4E phosphorylation is strongly associated with high Gleason score (>7) and HR prostate cancer in a patient cohort, which predicts poor survival (33, 34). It is well established that

eIF4E is overexpressed in many human tumors, including prostate cancer (35), and this was also shown here by using TMAs of patients with prostate cancer. However, an increase in the amount of eIF4E is not sufficient for transformation, as eIF4E must be phosphorylated by MNKs. The Oncomine database (36) documents that *MNK2* is overexpressed 1.5- to 4.4-fold in HR and metastatic prostate tumors (37–39). Moreover, MNK activity promotes proliferation in prostate cancer cells (40). This is expected to contribute to the increase in eIF4E phosphorylation seen in tumors with high Gleason scores, considering that MNK2 constitutively phosphorylates eIF4E (41).

Previous work has demonstrated that eIF4E phosphorylation is not required for translation under standard growth conditions (41). However, many studies documented a positive correlation between increased eIF4E phosphorylation and cell proliferation and enhanced translation (42, 43). Here, we identified mRNAs that are preferentially responsive to eIF4E phosphorylation for maximal translation. Future work will be needed to identify the mechanisms that render the translation of these mRNAs more sensitive to eIF4E phosphorylation. Several studies have demonstrated that p-eIF4E binds with lower affinity to the cap structure (44–46). Although this finding is counterintuitive, it was proposed that the decreased affinity could stimulate translation by releasing eIF4E from the cap, akin to the mechanism of promoter clearing during transcription initiation (47).

In a preclinical mouse model of prostate cancer, inhibition of mTOR and ERK signaling pathways using a combination therapy of rapamycin and PD0325901 impaired tumor growth (48). We demonstrated that the phosphorylation of eIF4E is regulated by these two signaling pathways; therefore, inhibition of eIF4E phosphorylation as a treatment for cancer is a very intriguing idea, considering that it does not have a conspicuous effect on normal proliferation.

Materials and Methods

IHC. IHC was performed using the Vectastain Elite ABC kit (Vector Laboratories) according to the manufacturer's instructions. Antibodies were used at the following dilutions: p-eIF4E 1:500 (Novus Biologicals), MMP3 1:250 (Abcam), CCL2 1:100 (Novus Biologicals), eIF4E 1:400 (CST), p-AKT 5473 1:50

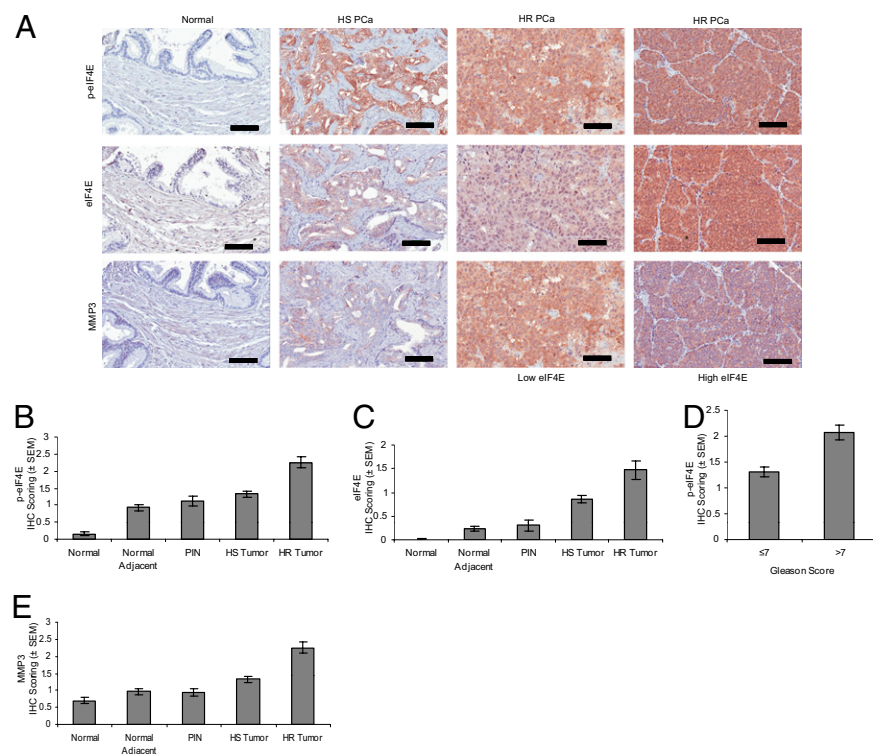


Fig. 4. p-eIF4E correlates with progression to HR PCa. eIF4E, phospho-eIF4E, and MMP3 were detected by IHC in human prostate cancer TMAs. The intensity of staining was scored from 0 to 4 for each core and data were analyzed as described in *Materials and Methods*. (A) Representative images of staining intensity obtained for eIF4E and p-eIF4E. (Scale bar: 100 μ m.) (B and C) eIF4E and phospho-eIF4E immunoreactivity shows a gradual increase in intensity from the normal, normal adjacent, PIN, HS tumor, and HR tumor tissues. Significant statistical differences were found between the different histopathological groups ($P < 0.001$, Kruskal-Wallis test). (D) The mean intensity difference of phospho-eIF4E in cases with Gleason score greater than 7 compared with Gleason scores of 7 or lower is statistically significant ($P < 0.05$, Mann-Whitney U test). (E) MMP3 immunoreactivity is gradually increased in intensity from PIN to HS tumor and HR tumor tissues. Significant statistical differences exist between the different histopathological groups ($P < 0.001$, Kruskal-Wallis test).

(CST), and Ki67 1:2,000 (Novocastra). SMA detection was performed with the Mouse-On-Mouse kit (Vector Laboratories) according to the manufacturer's instructions. SMA antibody (Dako) was used at 1:500.

Western Blotting. Primary antibodies were used at the following dilutions: pan-AKT 1:1,000 (CST), p-AKT S473 1:1,000 (CST), eIF4E 1:1,000 (CST), NFKBIA 1:1,000 (CST), BIRC2 1:1,000 (Novus Biologicals), p-eIF4E 1:5,000 (Novus Biologicals), MMP3 1:500 (Abcam), β -actin 1:10,000 (Sigma), VEGFC 1:250 (Abcam), RAPTOR 1:1,000 (CST), 4E-BP1 1:1,000 (CST), rpS6 1:5,000 (Santa Cruz Biotechnology), and p-rpS6[240/244] 1:1,000 (CST).

Transformation Assays. c-Myc-, E1A-, Ras^{V12}-, SV40 largeT-, CRE-, and HA-eIF4E-expressing retroviruses were generated by transfecting packaging Phoenix cells with the following plasmids: pBabe-c-Myc, pBabe-HA-eIF4E, pLpc-E1A/Ras^{V12}, pWZl-Ras^{V12}, pMSCV-CRE, and pSV40T (Myc-, E1A-, and Ras^{V12}-expressing plasmids were gifts of Scott Lowe, Cold Spring Harbor

Laboratory, NY; pSV40T was a gift of Julian Downward, London Research Institute, UK). MEFs were infected with retroviruses three times over a 48-h period and pools of infected cells were selected by two rounds of selection with puromycin for pBabe, hygromycin for pWZl, or G418 for pMSCV.

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Supporting Information

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SI Materials and Methods

Cloning of the eIF4E^{S209A} Targeting Vector. The KI DNA construct was built using amplified fragments of genomic DNA. The construct contained the serine 209 to alanine mutation in exon 8 and a Neo-TK selection cassette flanked by Frt sites within intron 7. The backbone vector, plox/frt, was previously described (1). The left flank was PCR amplified with the primers 5'-CCATTGTG-GAATGCCCTAGGCC-3' and 5'-CGTTATAGGCGCGCCAT-GGGCTCACAGCAATTGC-3' and then digested and ligated into the AvrII and AscI sites of the backbone vector. The right flank, which contains the point mutation, was amplified in three fragments. The first fragment (A) was amplified with primer pair 5'-GCCGCCGGTTTAAACTGATCGTGATTGCTTGTTAC-3' and 5'-CCTATTTTTAGTGGTGGCGCCGCTCTTTGTA-GC-3', digested with PmeI and NarI. The second fragment (B) was amplified with the primer pair 5'-GCTACAAAGAGCGG-CGCCACCACTAAAAATAGG-3' and 5'-CCACAATTATGT-TTAGGGATC-3' and then digested with NarI and HindIII. Fragments A and B were inserted into the PmeI/HindIII sites of the backbone vector by triple ligation. The third fragment (C) was amplified with the primer pair 5'-GCTCCACTCCATGCAG-GAGCGG-3' and 5'-CGCAGAGGTGACTTGCCCCATAA-TACTCCAC-3', digested with HindIII and SalI and ligated into the HindIII/SalI sites of the vector already containing fragments A and B. The entire right flank was digested with PmeI and SalI and ligated into PmeI/SalI sites of the left flank containing construct to generate p-eIF4E-KI S209A.

ES Cell Selection. p-eIF4E-KI S209A plasmid DNA was linearized with SalI and electroporated in 129Sv/J ES cells and selection of G418 resistant transformants was done as previously described (2). A total of 480 G418 resistant colonies were tested for recombination by Southern blotting.

Polysome Microarray Analysis. WT and KI MEFs (5×10^6 cells) were grown in DMEM supplemented with 10% FBS. Polysomal and total cytoplasmic RNA was isolated as described (3). Fractions containing mRNAs bound to more than three ribosomes were pooled and designated heavy polysomal RNA. RNA isolation was performed in two independent experiments from one pair of KI and WT MEFs. RNA quality was assessed using the Bioanalyzer Nano-Chip (Agilent), and all RNA samples were of good quality (integrity number, > 9.6). RNA (250 ng) was labeled using the one-cycle Illumina protocol and the resulting cRNA was hybridized with the "MouseRef-8_V2" BeadChip (Illumina). Data analysis was performed using the statistical environment R and the package lumi (4). Data were transformed using Variance Stabilizing Transformation and normalized using Robust Spline Normalization. Technical quality was validated and biological quality was assessed using principal components analysis, which showed good separation of the WT and the KI samples, for both the heavy polysome RNA data and the heavy polysome RNA data corrected for cytosolic RNA levels, in the first component. Correction for cytosolic RNA was performed by subtracting the log2 mean cytosolic RNA levels from the corresponding polysome RNA levels. Identification of differentially expressed genes was done using Significance Analysis of Microarrays algorithm (5). Genes that were significant ($q < 15$) before and after correction for the cytosolic RNA level were collected. Microarray data were deposited in the Gene Expression Omnibus database (accession no. GSE17451).

Immunoreactivity Quantification and Statistical Analysis of Human Prostate Tissue. The immunoreactivity scoring was performed by an experimenter blinded to the grade of the lesions. Scoring procedure was done as follows: each core was scored according to the staining intensity (value of 0 for absence, 1 for weak, 2 for moderate, 3 for strong staining, and 4 for very strong staining). The nonparametric Kruskal-Wallis test was used to evaluate the differences between the intensity means of the normal, normal adjacent, PIN, HS tumor, and HR tumor tissues. The mean intensity of Gleason scores (≤ 7 and > 7) was also compared by Mann-Whitney *U* test. The nonparametric Spearman rho correlation test was used to conduct correlation analysis. All statistical tests were performed using SPSS software, version 12 (SPSS).

Statistical Analysis of Mouse Prostate Tissue. Prostate tumor sections were stained with H&E and analyzed by two histopathologists blinded to the genotype of the mice. Lesions were graded from PIN I to PIN IV and invasive. A scoring procedure was done as follows: lesions were graded from 1 (PIN I) to 5 (invasive) and the weighted average was calculated for each section by taking into account the percentage of each lesion type on the section. The global score of each mouse was calculated by averaging the score of every slides corresponding to a single mouse. The nonparametric Mann-Whitney *U* test was used to evaluate the differences between the WT and KI cohorts.

Cell Cycle Analysis. Cells were lysed with NPE NIM-DAPI reagent (Beckman Coulter) and analyzed by using a Cell Lab Quanta SC (Beckman Coulter) flow cytometer.

Isolation of MEFs. MEFs were isolated from 14.5-d pregnant females as previously described (6). Animal protocols were approved by the McGill University Animal Care Committee and in compliance with McGill University guidelines.

Orthophosphate Labeling. eIF4E was immunoprecipitated from $H_3^{32}PO_4$ metabolically labeled MEFs as previously described (7).

Mouse Tissue Processing. Mouse urogenital system was isolated en bloc and fixed for 24 h in 10% buffered formalin. Alternatively, one half of the urogenital system was dissected to isolate the ventral, anterior, and dorsolateral lobes of the prostate. Tissues were paraffin-embedded, and 5- μ m sections were used for H&E staining and IHC.

shRNA-Mediated Knockdown of RAPTOR. shRNA vectors (RAPTOR 1857 and Scrambled 1864; 7 μ g; Addgene) were cotransfected into HEK293T cells in 100-mm dishes with lentivirus packaging plasmids PLP1, PLP2, and PLP-VSVG (7 μ g of each; Invitrogen) using Lipofectamine 2000 (Invitrogen). Supernatant was collected 48 h and 72 h after transfection, passed through a 0.45- μ m nitrocellulose filter, and applied on target cells with Polybrene (5 μ g/mL). Cells were reinfected the next day and selected with puromycin for 48 h (1 μ g/mL; Sigma).

Apoptosis Induction. Subconfluent layers of MEFs were treated with ionomycin for 24 h. Annexin V and PI staining was performed using Annexin-V-FLUOS staining kit (Roche Diagnostics) according to the manufacturer's instructions. The percentage of apoptotic and necrotic cells was determined as described (8).

Patient Cohort and TMA Construction. The TMA used for IHC was previously described (9, 10). Briefly, the specimen cohort, consisting

PIN I/II

PIN III

PIN IV

Infiltration

Fig. S4. Representative microphotographs of the lesions observed and their grading from PIN I to PIN IV and invasive.

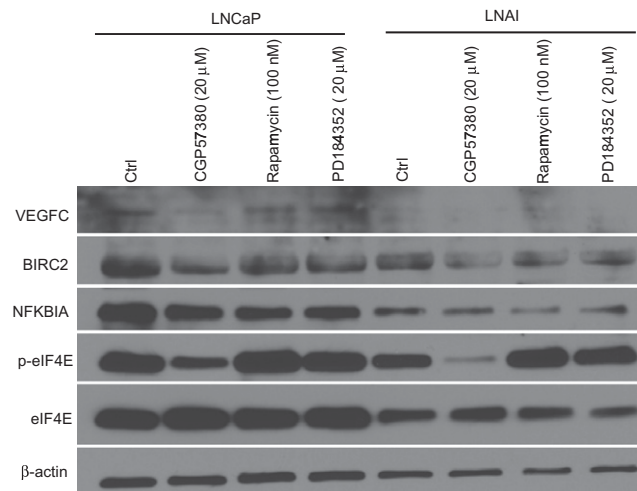


Fig. S5. LNCaP and LNAI prostate cancer cells were serum starved for 16 h. Cells were refed with 10% serum 1 h after the addition of the indicated inhibitors. Two hours after serum addition, cells were lysed and proteins were resolved by SDS/PAGE followed by Western blot analysis to detect the indicated proteins.

Table S1. Age of mice at the time they were euthanized

Type	5 mo	6 mo	7 mo	Median age, wk
WT/cPten ^{F/F}	5	2	0	20
KI/cPten ^{F/F}	2	3	2	25

Table S2. List of mRNAs more actively translated in WT compared with KI MEFs

Gene symbol	Fold change	Description
<i>8430410K20Rik</i>	5.09	RIKEN cDNA 8430410K20 gene
<i>Enpp2</i>	4.41	Ectonucleotide pyrophosphatase/phosphodiesterase 2
<i>Dcn</i>	4.05	Decorin
<i>Birc2</i>	3.54	Baculoviral IAP repeat-containing 2
<i>Casp4</i>	3.51	Caspase 4, apoptosis-related cysteine peptidase
<i>Ccl7</i>	3.36	Chemokine (C-C motif) ligand 7
<i>Upp1</i>	2.80	Uridine phosphorylase 1
<i>C3</i>	2.72	Complement component 3
<i>Cbr3</i>	2.51	Carbonyl reductase 3
<i>Mmp3</i>	2.46	Matrix metalloproteinase 3
<i>Ccl2</i>	2.42	Chemokine (C-C motif) ligand 2
<i>Slco4a1</i>	2.39	Solute carrier organic anion transporter family, member 4a1
<i>Cd82</i>	2.29	CD82 antigen
<i>Adh7</i>	2.16	Alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide
<i>Lxn</i>	2.15	Latexin
<i>Cyp1b1</i>	2.12	Cytochrome P450, family 1, subfamily b, polypeptide 1
<i>Serpina3g</i>	2.07	Serine (or cysteine) peptidase inhibitor, clade A, member 3G
<i>Sfrs3</i>	2.03	Splicing factor, arginine/serine-rich 3 (SRp20)
<i>Thbs2</i>	2.02	Thrombospondin 2
<i>Nfkb1a</i>	1.92	Nuclear factor of κ -light polypeptide gene enhancer in B-cells inhibitor, alpha
<i>Prl2c4</i>	1.92	Prolactin family 2, subfamily c, member 4
<i>Zfp36l1</i>	1.79	Zinc finger protein 36, C3H type-like 1
<i>Bxdc1</i>	1.74	Brix domain containing 1
<i>Vegfc</i>	1.73	Vascular endothelial growth factor C
<i>Sssca1</i>	1.71	Sjogren syndrome/scleroderma autoantigen 1 homologue (human)
<i>Plk2</i>	1.70	Polo-like kinase 2 (Drosophila)
<i>Prl2c3</i>	1.67	Prolactin family 2, subfamily c, member 3
<i>Arhgef3</i>	1.66	Rho guanine nucleotide exchange factor (GEF) 3
<i>Ccl9</i>	1.61	Chemokine (C-C motif) ligand 9
<i>Dcun1d5</i>	1.58	DCN1, defective in cullin neddylation 1, domain containing 5 (<i>Saccharomyces cerevisiae</i>)
<i>Pdgfra</i>	1.57	Platelet derived growth factor receptor, alpha polypeptide
<i>Slc24a3</i>	1.56	Solute carrier family 24 (sodium/potassium/calcium exchanger), member 3
<i>Mtvr2</i>	1.55	Mammary tumor virus receptor 2
<i>Mmp9</i>	1.53	Matrix metalloproteinase 9
<i>RbmX</i>	1.51	RNA binding motif protein, X chromosome

8. Supporting Data

Fig. 1

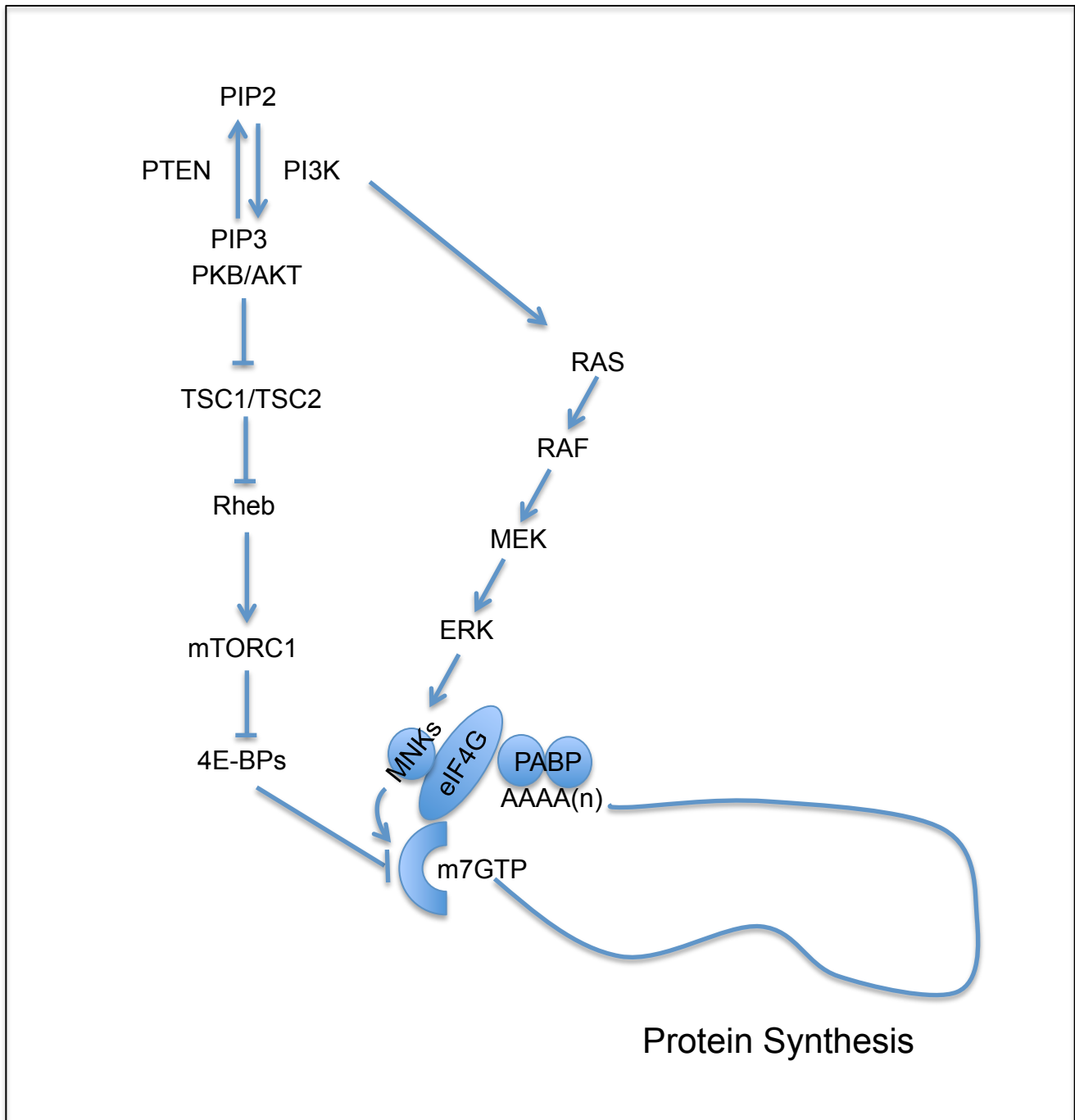
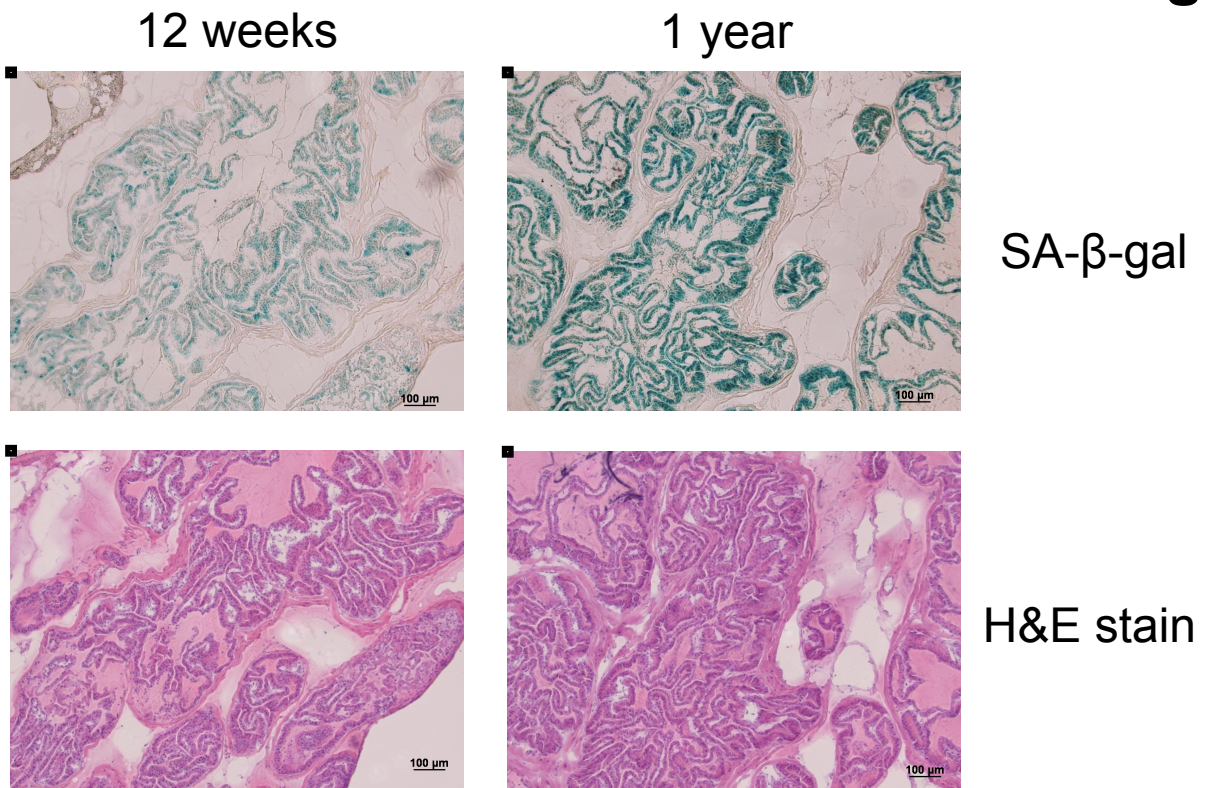


Fig. 1. Schematic representation of signaling pathways upstream of eIF4E. PI3K/AKT/mTORC1 signaling pathway regulates phosphorylation of 4E-BPs and their binding to eIF4E. RAS/RAF/MEK/ERK/MNKs pathway phosphorylate Ser²⁰⁹ on eIF4E to regulate eIF4E cap-binding and prostate cancer development.

Fig. 2

A



B

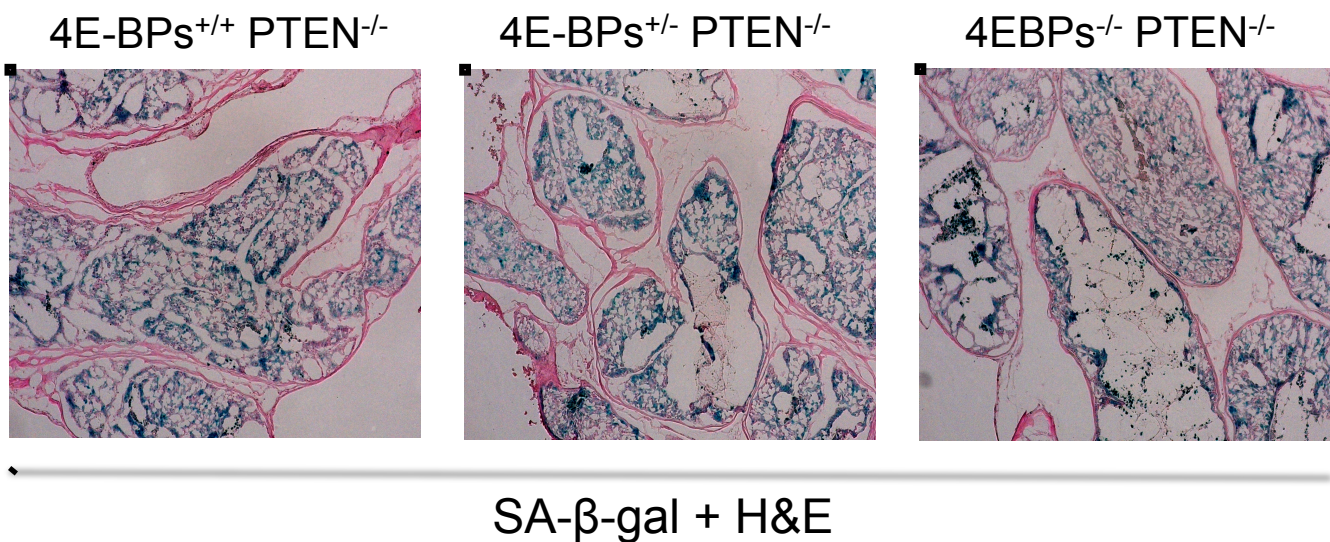


Fig. 2. SA- β -gal and H&E staining of mouse prostate sections. (A) Prostate sections of 12-week old and 1-year old wildtype mice were stained for SA- β -gal and H&E. **(B)** SA- β -gal and H&E staining of prostate sections of wildtype 4E-BP1/4E-BP2, heterozygous 4E-BP1/4E-BP2 and 4E-BP1/4E-BP2 null mice (in a PTEN-null background).

Fig. 3

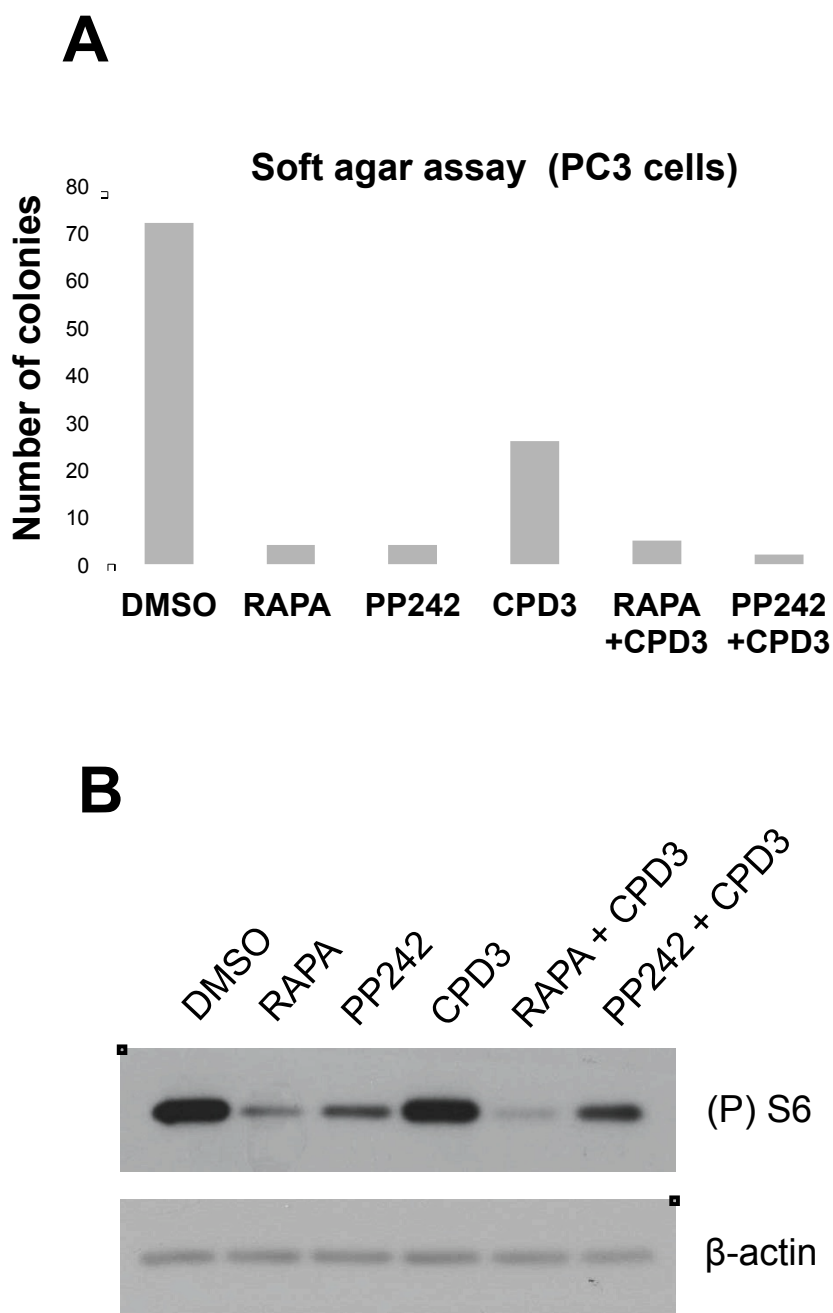


Fig. 3. Anchorage-independent growth of human prostate cancer (PC-3) cell line treated with mTOR and Mnk inhibitors. (A) PC-3 cells were incubated with 10 nM rapamycin, 2.5 μ M PP242 and/or 10 μ M compound 3. Anchorage-independent growth using the soft-colony formation assay. **(B)** Samples of lysates were analysed for mTORC1 activation by Western blot with the antisera indicated.