eIF4E/4E-BP Ratio Predicts the Efficacy of mTOR Targeted Therapies

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Abstract

Active-site mTOR inhibitors (asTORi) hold great promise for targeting dysregulated mTOR signaling in cancer. Because of the multifaceted nature of mTORC1 signaling, identification of reliable biomarkers for the sensitivity of tumors to asTORi is imperative for their clinical implementation. Here, we show that cancer cells acquire resistance to asTORi by downregulating eukaryotic translation initiation factor (eIF4E)-binding proteins (4E-BPs—E|IF4E|BP1, E|IF4E|BP2). Loss of 4E-BPs or overexpression of eIF4E renders neoplastic growth and translation of tumor-promoting mRNAs refractory to mTOR inhibition. Conversely, moderate depletion of eIF4E augments the anti-neoplastic effects of asTORi. The anti-proliferative effect of asTORi in vitro and in vivo is therefore significantly influenced by perturbations in eIF4E/4E-BP stoichiometry, whereby an increase in the eIF4E/4E-BP ratio dramatically limits the sensitivity of cancer cells to asTORi. We propose that the eIF4E/4E-BP ratio, rather than their individual protein levels or solely their phosphorylation status, should be considered as a paramount predictive marker for forecasting the clinical therapeutic response to mTOR inhibitors. Cancer Res; 72(24); 6468–76. ©2012 AACR

Introduction

mTOR is a multifunctional serine/threonine kinase, which exists in 2 distinct complexes, mTOR complex 1 and 2 (mTORC1 and 2; ref. 1). mTORC1 governs many cellular processes including mRNA translation, cell growth, and proliferation, by phosphorylating downstream targets such as 4E-BPs and S6Ks (1). mTORC2 controls cell survival and cytoskeleton organization by modulating the activity of AGC kinases (e.g., Akt and SGK1) and regulates nascent polypeptide stability (2). Hyperactivation of mTOR signaling frequently occurs in cancer (in more than 70% of patients; ref. 3). Therefore, targeting mTOR represents one of the most attractive anti-cancer therapeutic strategies. Rapamycin, a naturally occurring allosteric inhibitor of mTORC1, and its analogues (rapalogs), are clinically approved for treatment of renal cell carcinomas, mantle cell lymphomas, and pancreatic neuroendocrine tumors (4, 5). Nonetheless, the overall success of rapalog monotherapies is limited. This has been attributed to the incomplete inhibition of mTORC1-mediated phosphorylation of 4E-BPs, and the activation of Akt via the loss of a negative feedback mechanism (4, 6, 7). Recently, asTORi (also referred to as TORKin or dual mTORC1/mTORC2 inhibitors) were developed to overcome these issues. asTORi abolish phosphorylation of 4E-BPs via the inhibition of mTORC1, suppress Akt signaling via the inhibition of mTORC2, and exhibit stronger anti-proliferative and anti-tumorigenic effects than rapamycin (8–11). However, mTOR regulates various cancer-related processes via a multitude of substrates, and this complexity of mTOR signaling represents a significant challenge for identifying surrogate biomarkers that could serve to predict the efficacy of asTORi in the clinic (12).

eIF4E is the 5′ mRNA cap-binding subunit of the eIF4F complex, which recruits mRNA to the ribosome. eIF4F also includes the large scaffolding protein eIF4G and the DEAD-box RNA helicase eIF4A (13, 14). 4E-BPs (in mammals 4E-BP1, 2, and 3) are small-molecular-weight translational repressors, which impair the assembly of the eIF4F complex by competing with eIF4G for binding to eIF4E (15). 4E-BPs in mammals (4E-BP1, 2, and 3) are small-molecular-weight translational repressors, which impair the assembly of the eIF4F complex by competing with eIF4G for binding to eIF4E. This increases the amount of eIF4E available to engage in the eIF4F complex assembly (15–17). Ectopic expression of eIF4E leads...
to transformation of immortalized rodent and human cells (18, 19) and is tumorigenic in vivo (20). About 30% of cancers exhibit elevated eIF4E levels, which correlate with poor prognosis (21). eIF4E overexpression induces cell transformation by selectively augmenting translation of mRNAs referred to as eIF4E-sensitive mRNAs, which encode proliferation- and survival-promoting proteins (e.g., cyclins, c-myc, and Bcl-xL; refs. 18, 22). Multiple factors can induce the overexpression of eIF4E in cancer cells including gene amplification (23), transcriptional upregulation by c-myc (24), and increase in eIF4E mRNA stability by HuR (25). Dysregulated expression and/or increased phosphorylation of 4E-BPs in cancer have also been linked to poor patient outcomes (21, 26). 4E-BPs mediate the effects of mTOR signaling on translation of mRNAs that encode proteins that are associated with cancer progression, invasion, and metastasis (e.g., Y-box protein 1, vimentin, and CD44; ref. 11). 4E-BP1 expression is controlled at the transcriptional and protein stability levels by ATF4 (27) and the KLH25-CUL3 complex (28).

In this study, we show that cancer cells acquire resistance to asTORi by increasing eIF4E availability via downregulation of 4E-BP1 and 2. Accordingly, we show that an increase in eIF4E, or reduction in 4E-BP levels by RNA interference, strongly attenuates the anti-neoplastic effects of the asTORi PP242, Torin1, and INK1341 (an analogue of the recently characterized INK128 compound; ref. 11) Conversely, a decrease in the eIF4E/4E-BP ratio ameliorates the inhibitory effects of asTORi on translation of eIF4E-sensitive cyclin D3 and E1 mRNAs. Thus, the eIF4E/4E-BP ratio in the tumor could be a significant predictive marker of the efficacy of asTORi that should be considered in devising personalized asTORi treatments.

Materials and Methods

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

E1A/Ras-transformed WT (p53+/−/−) and 4E-BP1/2 DKO (p53−/−/−) mouse embryonic fibroblasts (MEF), referred to as WT+E1A/Ras and 4E-BP1/2 DKO+E1A/Ras MEFs respectively, were previously described (7). HeLa, SK-HEP-1, and HepG2 cells were directly purchased from and authenticated by American Type Culture Collection. Cells were maintained in Dulbecco’s modified Eagle Medium (DMEM; Invitrogen), supplemented with 10% FBS (Invitrogen), 2 mmol/L L-glutamine, and 100 units/mL penicillin/streptomycin (Invitrogen) indicated in the manufacturer’s protocol (Bio Vision). Cell lines that stably express 4E-BP1 were generated as described previously (31). Briefly, pBABE constructs were transiently transfected into the Ecotropic Phoenix-293T packaging cell line. After 48 hours, virus-containing medium was filtered (0.45 μm), collected, and used to infect MEFs in the presence of 5 μg/mL polybrene (Sigma-Aldrich). Infection was repeated the next day. Twenty-four hours after the second infection, medium supplemented with puromycin (1 μg/mL, Sigma-Aldrich) was added, and cells were subjected to selection for 1 week, after which time colonies were pooled.

pcDNA3-FLAG-eIF4EWT and eIF4EW56A (cap-binding mutant) were constructed by cloning the entire coding sequence of mouse eIF4E into pcDNA3-FLAG using a PCR (32). These vectors were transfected and cells selected in G418 for 2 weeks. Lentiviral vectors were from Sigma. Short hairpin RNA (shRNA) vector accession numbers are: mouse eIF4E (Sigma: TRCN0000077474), the Non-Target shRNA Control (Sigma: SHC002). 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 posttransfection, 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 (1 μg/mL, Sigma). PP242 and INK1341 were provided by Intellikine. Torin1 was from N. Gray and D. Sabatini.

Cell-cycle analysis and apoptosis

Cells were seeded in 100-mm dishes, grown overnight, and treated as indicated in figure legends. Twenty-four or 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 μL of NPE Nuclear Isolation Media (NIM)-DAPI (Beckman Coulter) and analyzed using a Cell Lab Quanta SC (Beckman Coulter) flow cytometer. For apoptosis, cells were analyzed by flow cytometry using the Annexin V-FTTC Apoptosis Detection Kit according to the manufacturer’s protocol (Bio Vision).

Cell proliferation, soft agar, and anchorage-dependent foci formation assay

For the bromodeoxyuridine (BrdU) incorporation assay (Cell Proliferation ELISA BrdU Kit from Roche), cells were seeded in 96-well plates (1,000 cells/well) and maintained as indicated in the figure legends. Absorbance at 370 nm (reference wavelength = 492 nm) was measured using a Varioskan microplate reader (Thermo Electron Corporation). For Trypan blue exclusion, cells were seeded in 6-well plates (50,000 cells/well) overnight and maintained under conditions outlined in the figure legends. Cell proliferation was determined by direct counting (dead cells were excluded by Trypan blue staining). For soft agar assays, experiments were carried out in 6-well plates coated with a base layer of 0.5% agarose (Agar Noble, Difco). WT+E1A/Ras and 4E-BP1/2 DKO+E1A/Ras MEFs were seeded in triplicates at a density of 5,000 cells/well in 0.33% agarose containing either vehicle [dimethyl sulfoxide (DMSO)], PP242 (1 μmol/L), or INK1341 (250 nmol/L). Cells were overlaid with DMEM supplemented with 10% FBS, 2 mmol/L L-glutamine, and 100 units/mL penicillin/streptomycin (Invitrogen) containing either DMSO, PP242 (1 μmol/L), or INK1341 (1 nmol/L), and incubated for up to 10 to 14 days. Media containing vehicle, PP242, or INK1341 were changed every 3 to 4 days during the course of the experiment. Colonies were counted using a light

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microscope. For focus formation assays, cells were seeded at a density of 10,000 cells/10-cm dish and allowed to grow in the presence of either vehicle (DMSO) or PP242 (1 μmol/L) or INK1341 (250 nmol/L). Media containing drugs were changed as above every 3 to 4 days.

**Western blot analysis and cap (m′GDP) pull-down assay**

Cell lysates were prepared, and Western blotting was carried out as described (33). Antibodies against 4E-BP1, 4E-BP2, phospho-4E-BP (Thr47/46, Ser65, Thr70), rpS6, phospho-rpS6 (Ser24/244), Akt, phospho-Akt (S473), eIF4G1, and cyclin D3 were from Cell Signaling Technology (Danvers, MA). Antibody against cyclin E1 was from Santa Cruz Biotechnology. Antibodies against elf4E and β-actin were from Sigma. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-mouse IgG were from Amersham Biosciences. For cap-binding affinity assay, cells were lysed in the lysis buffer [50 mmol/L HEPES-KOH (pH 7.5), 150 mmol/L KCl, 1 mmol/L EDTA, 2 mmol/L dithiothreitol (DTT), and 0.2% Tween] containing protease inhibitors. Cell lysates were incubated with m′GDP-agarose and washed 4 times using the lysis buffer. m′GDP-bound proteins were determined by Western blotting.

**Polysome analysis, RNA isolation, and sqRT-PCR**

Polysome profile analysis was carried out as previously described (34). Briefly, cells were cultured in 15-cm dishes and treated with PP242 (1 μmol/L), INK1341 (250 nmol/L), or vehicle (DMSO) for 8 hours. Cells were washed with cold PBS containing 100 μg/mL cycloheximide, collected, and lysed in a hypotonic lysis buffer [5 mmol/L Tris-HCl (pH 7.5), 2.5 mmol/L MgCl2, 1.5 mmol/L KCl, 100 μg/mL cycloheximide, 2 mmol/L DTT, 0.5% Triton X-100, and 0.5% sodium deoxycholate]. Lysates were loaded onto 10% to 50% sucrose density gradients [20 mmol/L HEPES-KOH (pH 7.6), 100 mmol/L KCl, 5 mmol/L MgCl2] and centrifuged at 36,000 rpm for 2 hours at 4°C. Gradients were fractionated, and the optical density (OD) at 254 nm was continuously recorded using an ISCO fractionator (Teledyne ISCO). RNA from each fraction was isolated using TRIzol (Invitrogen) and treated with DNaseTurbo (Ambion) (Supplementary Fig. S2C). Moreover, depletion of 4E-BP1 and 2 rendered neoplastic growth of SK-HEP-1 and HepG2 cells partially resistant to PP242 (Supplementary Figs. S1A—S1C and S2D—S2F). These results show that loss of 4E-BPs attenuates the anti-neoplastic efficacy of asTORi.

**Increased elf4E availability renders cells resistant to asTORi**

When mTOR signaling is inhibited, 4E-BPs limit the fraction of elf4E available for the assembly of the elf4F complex (15–17). Thus, we investigated whether the sequestration of elf4E by 4E-BPs is required for the anti-neoplastic activity of INK1341 by overexpressing 4E-BP3WT and 4E-BP1ME, which lacks the elf4E-binding motif and thus cannot bind elf4E (ref. 35; Fig. 1H). Expression of 4E-BP3WT, but not 4E-BP1ME, augmented the anti-neoplastic effect of INK1341 (Figs. 1I and J). Moreover, forced expression of 4E-BP1WT markedly reduced the association between elf4E and elf4G1 as compared with a control (Supplementary Fig. S2G and S2H). Taken together, these results show that 4E-BPs mediate the anti-tumorigenic activity of asTORi by limiting the availability of elf4E to associate with elf4G1, thereby impeding the elf4F complex assembly.

Statistical analysis

Error bars for all data represent SDs from the mean. P values were calculated using Student's t tests.

Results

**Acquired resistance to asTORi coincides with downregulation of 4E-BP1 and 2 expression**

To investigate the potential mechanism of acquired resistance to asTORi in cancer cells, we cultivated E1A/Ras-transformed p53−/− MEFs (WT4E-BP1/2) and 2 liver cancer cell lines (HepG2 and SK-HEP-1) in the presence of the asTORi PP242 (1 μmol/L for 8 weeks). Strikingly, all 3 cell lines acquired resistance to PP242, which correlated with a downregulation of 4E-BP1 and 2 (Figs. 1A–C; Supplementary Fig. S1A–S1F), but not with a loss of mTORC1 or 2 inhibition, inasmuch as asTORi induced similar suppression of ribosomal protein S6 (S6) and Akt phosphorylation in resistant and control cells (Supplementary Fig. S1G and S1H).

To ascertain that 4E-BPs are responsible for the anti-neoplastic activity of asTORi, we investigated the effects of PP242 on the neoplastic growth of WT4E-BP1/2 and E1A/Ras-transformed p53−/−/4E-BP1/2−/− MEFs (4E-BP DKO4E-BP1/2/). 4E-BP DKO4E-BP1/2 MEFs are devoid of all 4E-BPs, as MEFs do not express 4E-BP3 (7). WT4E-BP1/2 and 4E-BP DKO4E-BP1/2 MEFs proliferated at the same rate in full growth medium (Fig. 1D) where mTOR activity is high and 4E-BP1 and S6 are hyperphosphorylated (Fig. 1E). However, when PP242 inhibits mTORC1 signaling to a similar extent in these cells, as illustrated by a comparable reduction in the phosphorylation of S6 (Fig. 1E), the effects of PP242 on neoplastic growth were significantly less pronounced in 4E-BP DKO4E-BP1/2 MEFs (~45% reduction in number of colonies and foci relative to control) than in WT4E-BP1/2 MEFs (~70% Figs. 1F and G; Supplementary Fig. S2A–S2C). Moreover, depletion of 4E-BP1 and 2 rendered neoplastic growth of SK-HEP-1 and HepG2 cells partially resistant to PP242 (Supplementary Figs. S1A–S1C and S2D–S2F). These results show that loss of 4E-BPs attenuates the anti-neoplastic efficacy of asTORi.
eIF4E is frequently overexpressed in cancer (21). We examined whether overexpression of eIF4E, akin to loss of 4E-BPs, alleviates the anti-proliferative effects of the asTORi, Torin1 (9). Torin1 equally inhibited mTORC1 signaling in vector-transfected NIH3T3 cells and NIH3T3 cells that stably overexpress eIF4E (NIH3T3/4E; ref. 18), as shown by

Figure 1. 4E-BP1 and 2 downregulation underlies acquired resistance to asTORi. A, Western blot analysis of the indicated proteins in WT E1A/Ras MEFs maintained in the presence of DMSO (control) or PP242 (1 μmol/L) for a period of 8 weeks. β-Actin served as a loading control. B, resistant and control WT E1A/Ras MEFs were treated with PP242 (1 μmol/L) for 7 days, and cell proliferation was determined by Trypan blue exclusion. Results represent the mean cell number relative to a DMSO control (set to 100%) ± SD (n = 3). C, cells described in B were stained with crystal violet. D, phase contrast images of WT E1A/Ras or 4E-BP DKO E1A/Ras MEFs (top). Proliferation rates of WT E1A/Ras or 4E-BP DKO E1A/Ras MEFs in full growth medium were determined by Trypan blue exclusion (bottom). Results are presented as mean numbers of cells ± SD (n = 3). E, WT E1A/Ras or 4E-BP DKO E1A/Ras MEFs were treated with DMSO or PP242 (1 μmol/L) for 3 hours. Levels and the phosphorylation status of the indicated proteins were determined by Western blot analysis. β-Actin served as a loading control. Arrows indicate the hyperphosphorylated (hyper-P) and hypophosphorylated (hypo-P) forms of 4E-BP1. F, effects of DMSO and PP242 (1 μmol/L) on anchorage-independent growth of WT E1A/Ras and 4E-BP DKO E1A/Ras MEFs were monitored using a soft agar assay. Colonies were counted after 10 days of treatment. Results are presented as a mean number of colonies relative to a DMSO control (set to 100%) ± SD (n = 3). G, representative photographs of colonies formed by the cells described in F. H, WT E1A/Ras MEFs were transduced with an empty vector (pBABE) or vector expressing hemagglutinin (HA)-tagged wild-type 4E-BP1 (4E-BP1WT) or 4E-BP1D4E mutant, and expression of the indicated proteins was determined by Western blot analysis. β-Actin served as a loading control. I, effects of INK1341 (250 nmol/L) on anchorage-independent growth of WT E1A/Ras and 4E-BP DKO E1A/Ras MEFs were monitored using a soft agar assay. Colonies in soft agar were counted after 10 days. Results are presented as a mean number of colonies relative to DMSO-treated cells (set to 100%) ± SD (n = 3). J, representative photographs of the colonies formed by the cells described in H.

eIF4E is frequently overexpressed in cancer (21). We examined whether overexpression of eIF4E, akin to loss of 4E-BPs, alleviates the anti-proliferative effects of the asTORi, Torin1 (9). Torin1 equally inhibited mTORC1 signaling in vector-transfected NIH3T3 cells and NIH3T3 cells that stably overexpress eIF4E (NIH3T3/4E; ref. 18), as shown by
comparable reduction in the phosphorylation of 4E-BP1 and S6 (Supplementary Fig. S3A). In contrast, Torin1 inhibited proliferation and G1 to S-phase cell-cycle progression in NIH3T3/4E cells to a dramatically lesser extent (~15%) as compared with control cells (~85%; Supplementary Fig. S3B and S3C).

Figure 2. Elevated eIF4E/4E-BP ratio diminishes the anti-proliferative effects of asTORi. A, phase contrast images of WTE1A/Ras MEFs transduced with scrambled (Scr) or eIF4E shRNA (eIF4E KD; top). Proliferation rates of the indicated cells in full growth medium were determined by Trypan blue exclusion (bottom). Results are presented as mean numbers of cells ± SD (n = 3). B, cells described in A were treated with DMSO or PP242 (1 μmol/L) for 3 hours, and the levels and the phosphorylation status of indicated proteins were determined by Western blot analysis. β-Actin served as a loading control. C, effects of DMSO or PP242 (1 μmol/L) on anchorage-independent growth of cells described in A were monitored using a soft agar assay. Colonies in soft agar were counted after 10 days. Results are presented as a mean number of colonies relative to DMSO-treated cells (set to 100%) ± SD (n = 3). D, representative photographs of colonies formed by cells described in A. E, WTE1A/Ras MEFs transduced with scrambled (WT14/14) or eIF4E shRNA (WT14/14 + eIF4E KD) and 4E-BP DKO14/14 MEFs were treated with PP242 (1 μmol/L) or INK1341 (250 nmol/L) for 3 hours. Levels of the indicated proteins were determined by Western blot analysis. β-Actin served as a loading control. F, effects of DMSO and INK1341 (250 nmol/L) on anchorage-independent growth of cells described in E were monitored using a soft agar assay. Colonies were counted after 10 days of treatment. Results represent the mean colony number relative to a DMSO control (set to 100%) ± SD (n = 3). G, representative photographs of colonies formed by cells described in E. H, WTE1A/Ras MEFs were transfected with an empty vector (pcDNA3.1) or vector expressing Flag-tagged wild type (eIF4EWT) or a cap-binding mutant of eIF4E (eIF4EWT). Levels of the indicated proteins were determined by Western blot analysis. β-Actin served as a loading control. Arrows indicate exogenous Flag-tagged (Flag-eIF4E) and endogenous eIF4E. I, effect of DMSO or PP242 (1 μmol/L) on anchorage-independent growth of cells described in H was monitored using soft agar assay. Colonies were counted after 10 days of treatment. Results represent the mean cell number relative to a DMSO control (set to 100%) ± SD (n = 3). J, representative photographs of colonies formed by cells described in H.

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To further establish that the alterations in eIF4E availability determine the anti-neoplastic effects of asTORi, we depleted eIF4E in WT^E1A/Ras^ MEFs and compared the anti-neoplastic effects of PP242 with those observed in control cells. As significant eIF4E downregulation inhibits proliferation and survival (36), we selected cells in which eIF4E depletion (~50% of control) does not affect cell proliferation under optimal nutrient conditions wherein mTOR is active (Fig. 2A and B). Nonetheless, an approximate 50% decrease in eIF4E levels strongly augmented the sensitivity of WT^E1A/Ras^ MEFs to PP242 (~85% reduction in colony and foci formation), as compared with control (~65%; Figs. 2C and D; Supplementary Fig. S3D–S3F). Similar results were obtained using INK1341 (Figs. 2E–G; Supplementary Fig. S3G and S3H). Suppression of neoplastic growth by asTORi was caused by the inhibition of cell-cycle progression from G1 to S-phase, whereas asTORi did not exert a major effect on cell survival (Supplementary Fig. S4A–S4C). Importantly, the asTORi-induced inhibition of G1–S progression was alleviated in cells with high eIF4E/4E-BP ratio (Supplementary Fig. S4A). These results support a model whereby asTORi suppress neoplastic growth by limiting eIF4E availability and cell-cycle progression.

**Persistent translation of eIF4E-sensitive mRNAs renders cells refractory to asTORi**

The eIF4E/4E-BP ratio dictates cap-dependent mRNA translation rates (15). Thus, we investigated whether translational activity of eIF4E is required for attenuation of the anti-neoplastic effects of asTORi by overexpressing wild-type (eIF4E^WT^) and a translationally inactive W56A eIF4E mutant (eIF4E^W56A^; refs. 37, 38) in WT^E1A/Ras^ MEFs. Both proteins were expressed to a comparable level (Fig. 2H). Cells overexpressing...
eIF4EWT exhibited significantly lower sensitivity (~50% inhibition of proliferation) to the anti-neoplastic and anti-proliferative effects of PP242 than control cells (~70%), whereas suppression of proliferation and neoplastic growth by PP242 in cells overexpressing eIF4Eε56A was similar to that observed in control cells (Figs. 2I and J; Supplementary Fig. S4D and S4E). Similar results were obtained in HeLa cells (Supplementary control cells (Figs. 2I and J; Supplementary Fig. S4D and S4E).

Next, we examined the effect of PP242 and INK1341 on global mRNA translation in WTE1A/Ras and 4E-BP1DKOE1A/Ras MEFs, as well as in WT1A/Ras MEFs depleted of eIF4E, by studying polysome formation. The fraction of ribosomes engaged in polysomes is directly proportional to the translation initiation rate (39). Consistent with previous reports that PP242 suppresses initiation of cap-dependent mRNA translation (8), a decrease in the number of ribosomes engaged in polysomes, and a concomitant increase in 80S monosome peak was detected in all cells treated with PP242 as compared with control (Fig. 3A). However, inhibition of polysome formation by PP242 was most pronounced in WTE1A/Ras MEFs in which eIF4E was depleted, intermediate in control WTE1A/Ras MEFs, and weakest in 4E-BP1DKOE1A/Ras MEFs (Fig. 3A). Similar results were obtained using INK1341 (Fig. 3B).

While our data show that an elevated eIF4E/4E-BP ratio mitigates the inhibition of global mRNA translation by PP242 or INK1341, the lack of a complete dissociation of polysomes indicates that the majority of mRNAs remain translationally active in asTORi-treated cells. This suggests that the resistance to asTORi is caused by the resistance of eIF4E-sensitive mRNAs to inhibition in cancer cells with elevated eIF4E/4E-BP ratio (21, 40). Thus, we investigated the effects of PP242 and INK1341 on translation of the prototypical eIF4E-sensitive mRNAs cyclin D3, and cyclin E1, as well as β-actin mRNA, which is only marginally sensitive to changes in eIF4E (7, 22). In vehicle-treated cells, in which the sequestration of eIF4E by 4E-BPs is minimal due to the hyperphosphorylation of 4E-BPs, loss of 4E-BPs or approximately 50% depletion of eIF4E had no major impact on translation of eIF4E-sensitive mRNAs (Figs. 3C–E). PP242 and INK1341 abolished the phosphorylation of 4E-BPs (Figs. 1E, 2B, and 3E) and strongly suppressed translation of the eIF4E-sensitive mRNAs cyclin D3 and E1, as well as the expression of corresponding proteins in control

Figure 4. The eIF4E/4E-BP ratio determines the anti-neoplastic effects of PP242 in vivo. A and B, nude/C24 mice were subcutaneously implanted with 5 × 10⁶ WTE1A/Ras and 4E-BP1/2 DKO1A/Ras MEFs. When tumors reached palpable size (~20 mm²), mice were injected intraperitoneally with vehicle or PP242 (60 mg/kg) daily for 10 days. Tumor growth was followed every second day, and 2-dimensional measurements were taken using a caliper. C and D, nude/C24 mice were subcutaneously implanted with 5 × 10⁶ WTE1A/Ras MEFs infected with scrambled (Scr) or eIF4E shRNA (eIF4E KD) and treated as in A and B. E, photographs of representative tumors isolated from each group of mice. F, levels and the phosphorylation status of S6 and 4E-BP1 proteins at indicated time points in tumors described in A were determined by Western blot analysis. S6 served as loading control. Arrows indicate the hyperphosphorylated (hyper-P) and hypophosphorylated (hypo-P) forms of 4E-BP1. G, proposed model of cancer cell sensitivity to asTORi. AsTORi induce sufficient sequestration of eIF4E to inhibit translation of eIF4E-sensitive mRNAs and neoplastic growth of cancer cells with low (left circle) but not high (right circle) eIF4E/4E-BP ratio.
WT E1A/Ras MEFs, which was further potentiated by the depletion of elf4E (Figs. 3C–E). In stark contrast, translation of cyclin D3 and E1 mRNAs and expression of cyclin D3 and E1 proteins in 4E-BP DKO E1A/Ras MEFs were largely insensitive to PP242 and INK1341 (Figs. 3C–E). As expected, the translation of β-actin mRNA or expression of β-actin protein were not influenced by asTORi treatment in any of the cell lines (Figs. 3C–E). Finally, Torin1 strongly reduced cyclin D3 expression in control NIH3T3 cells, but not in NIH3T3 cells over-expressing elf4E (Supplementary Fig. S3A). Collectively, these results show that the resistance to the anti-neoplastic effects of asTORi of cancer cells with high elf4E/4E-BP ratio stems from the failure of asTORi to efficiently inhibit translation of elf4E-sensitive mRNAs.

elf4E/4E-BP ratio determines sensitivity to asTORi in vivo

Next, we studied the impact of perturbations in the elf4E/4E-BP ratio on the anti-neoplastic activity of asTORi in vivo. Mice bearing subcutaneous tumors formed by WT E1A/Ras or 4E-BP DKO E1A/Ras MEFs were treated daily with PP242 or a vehicle. Remarkably, whereas PP242 inhibited the growth of tumors formed by WT E1A/Ras MEFs, tumors lacking 4E-BPs were completely resistant to PP242. Moreover, depletion of elf4E in WT E1A/Ras tumors further increased their sensitivity to PP242 (Figs. 4A–E). PP242 strongly inhibited mTOR signaling in vivo, as illustrated by inhibition of S6 phosphorylation 30 minutes and 24 hours postinjection from tumor tissues (Fig. 4F). Thus, consistent with the in vitro findings, the anti-neoplastic activity of asTORi in vivo is predominantly established by the elf4E/4E-BP ratio in the tumor.

Discussion

mTOR signaling is frequently dysregulated in cancer and is being targeted in clinical trials using asTORi (1, 41). However, there are currently no reliable markers that can predict the therapeutic efficacy of asTORi. Recently, several models have been proposed to explain resistance to asTORi as well as dual PI3K/mTOR kinase inhibitors in tumors. These include activation of alternative signaling pathways, such as the extracellular signal–regulated kinase pathway that can render 4E-BP1 persistently inactive despite treatment with mTOR inhibitors (42), or pathways that activate cap-independent translation of survival-promoting mRNAs (43). We have unraveled a different mechanism to explain the acquired resistance to mTOR inhibitors, whereby cancer cells become insensitive to asTORi by downregulating expression of 4E-BP1 and 2. This leads to an increase in the elf4E/4E-BP ratio, thereby attenuating the anti-neoplastic effects of asTORi as it limits their inhibitory effect on the translation of elf4E-sensitive mRNAs. Our model explains recent findings showing that resistance to BEZ235, a dual PI3K/mTOR inhibitor, can be acquired through amplification of the elf4E gene (44). Thus, our results support a model whereby an elevated elf4E/4E-BP ratio renders tumors resistant not only to asTORi (Fig. 4G) but also to dual PI3K/mTOR inhibitors.

Recent studies have proposed that the combination of mitogen-activated protein kinase with mTOR inhibitors could overcome resistance to mTOR inhibitors (42, 45). However, data presented here raise the possibility that using therapies that target elf4E in the tumor (21, 36) may be more beneficial in cases where elevated elf4E/4E-BP ratio is present. Moreover, our findings strongly suggest that the elf4E/4E-BP ratio could serve as a predictive marker to tailor personalized treatments using asTORi in the clinic.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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