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Research Article **Oncology**

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Therapeutic suppression of translation initiation modulates chemosensitivity in a mouse lymphoma model

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Disablement of cell death programs in cancer cells contributes to drug resistance and in some cases has been associated with altered translational control. As eukaryotic translation initiation factor 4E (eIF4E) cooperates with c-Myc during lymphomagenesis, induces drug resistance, and is a genetic modifier of the rapamycin response, we have investigated the effect of dysregulation of the ribosome recruitment phase of translation initiation on tumor progression and chemosensitivity. eIF4E is a subunit of eIF4F, a complex that stimulates ribosome recruitment during translation initiation by delivering the DEAD-box RNA helicase eIF4A to the 5′ end of mRNAs. eIF4A is thought to prepare a ribosome landing pad on mRNA templates for incoming 40S ribosomes (and associated factors). Using small molecule screening, we found that cyclopenta[*b*]benzofuran flavaglines, a class of natural products, modulate eIF4A activity and inhibit translation initiation. One member of this class of compounds, silvestrol, was able to enhance chemosensitivity in a mouse lymphoma model in which carcinogenesis is driven by phosphatase and tensin homolog (PTEN) inactivation or elevated eIF4E levels. These results establish that targeting translation initiation can restore drug sensitivity in vivo and provide an approach to modulating chemosensitivity.

Introduction

Dysregulation of protein synthesis occurs in many cancer cells harboring lesions in the PI3K/Akt pathway (1). This signaling cascade regulates mammalian target of rapamycin (mTOR) activity, which in turn controls translation initiation — the rate-limiting step of general protein synthesis (1). mTOR achieves this by controlling assembly of eukaryotic translation initiation factor (eIF) 4F, a complex that stimulates ribosome recruitment to mRNA templates. eIF4F is composed of 3 subunits: eIF4E, which binds to the cap structure present at the 5′ end of mRNAs; eIF4A, a DEAD-box RNA helicase implicated in preparing a ribosome landing pad for 43S preinitiation complexes (40S ribosomal subunit and associated factors) by unwinding 5′ mRNA structure; and eIF4G, a large scaffolding protein involved in recruiting the 43S preinitiation complex via its interaction with 40S-associated eIF3 (2). Ancillary factors, such as eIF4B and eIF4H, facilitate ribosome recruitment by stimulating eIF4A helicase activity (1).

mTOR regulates the availability of eIF4E and eIF4A for incorporation into the eIF4F complex (1, 3). The association of eIF4E with either eIF4G or with one of 3 negative regulatory proteins, known as the eIF4E-binding proteins (4E-BPs), is under mTOR control (1). 4E-BPs and eIF4G occupy mutually exclusive binding sites on the surface of eIF4E. In their hypophosphorylated form, the 4E-BPs bind to and sequester eIF4E, leading to suppression of translation initiation (1). mTOR-mediated phosphorylation of 4E-BPs liberates eIF4E from this inhibitory complex and allows it to enter the eIF4F complex (1). The activity of eIF4A is regulated by binding to a tumor suppressor gene product, Pdcd4, which inhibits its helicase activity and its interaction with the C-terminal domain of eIF4G (4). In response to mitogens, Pdcd4 is phosphorylated by S6K1, a downstream target of mTOR, and subsequently degraded, leading to the release of eIF4A and its assembly into eIF4F (3).

Many lines of evidence suggest a direct link between deregulation of the ribosome recruitment phase of translation initiation and transformation. Increased eIF4F activity (5) and ectopic expression of eIF4E, eIF4G, or some of the eIF3 subunits can transform cells in culture (6–8). Overexpression of eIF4E cooperates with c-Myc during lymphomagenesis (9–11) by antagonizing its proapoptotic activities (9, 12–14), induces drug resistance, and leads to rapamycin resistance in vivo (9, 10). Altering translation initiation rates by inhibition of mTOR activity or ectopic overexpression of eIF4E remolds the oncoproteome by influencing the expression of a subset of cellular mRNAs — several of which have been implicated in cellular transformation (15–17). Strategies aimed at lowering eIF4E (18–20) or eIF4A (21) levels in transformed cells reduce malignant progression. Herein, we report that cyclopenta[*b*]benzofuran flavaglines (CBFs), compounds isolated from species of the *Aglaia* genus of the Meliaceae plant family, are novel inhibitors of translation initiation that act as chemical inducers of dimerization (CIDs),

Nonstandard abbreviations used: CBF, cyclopenta[*b*]benzofuran flavagline; 4E-BP, eIF4E-binding protein; eIF4E, eukaryotic translation initiation factor 4E; FA, 1-*O*-formylaglafoline; FF, firefly luciferase; IRES, internal ribosome entry site; mTOR, mammalian target of rapamycin; mTORC2, rictor/mTOR protein complex; PTEN, phosphatase and tensin homolog; Ren, *Renilla* luciferase.

Conflict of interest: The authors have declared that no conflict of interest exists.

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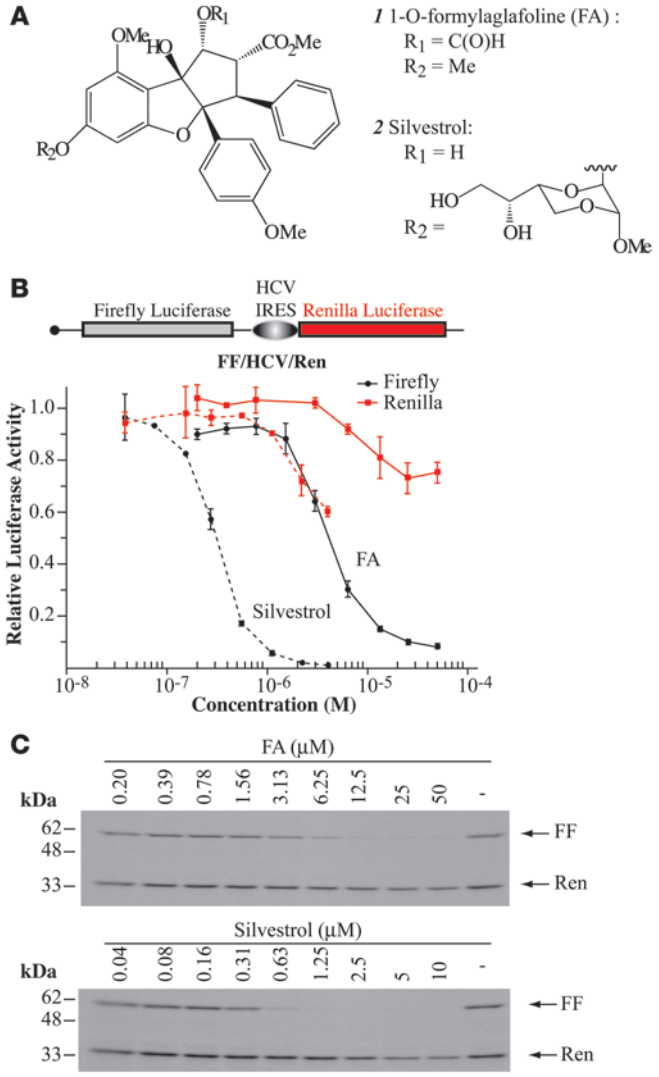

Figure 1
CBFs inhibit cap-dependent translation. (**A**) Chemical structure of FA and silvestrol. (**B**) FA and silvestrol inhibit cap-dependent translation. Top: Schematic representation of the FF/HCV/Ren mRNA. Bottom: Dose-dependent inhibition of cap-dependent protein synthesis by FA and silvestrol in Krebs-2 translation extracts programmed with FF/HCV/Ren mRNA (10 μg/ml). Luciferase activity (RLU) results are expressed relative to values obtained in the presence of vehicle (MeOH) control. Results are expressed as mean ± SEM of 2 experiments. (**C**) A representative autoradiograph from in vitro translations performed in Krebs-2 extracts with [^{35}S]methionine and programmed with FF/HCV/Ren mRNA. The position of migration of FF and Ren proteins is indicated on the right.

forcing an engagement between eIF4A and RNA. One member of this class of compounds, silvestrol (22), was able to alter chemosensitivity in Eμ-myc tumors harboring either a phosphatase and tensin homolog (PTEN) lesion or overexpressing eIF4E. These results suggest a strategy aimed at altering drug sensitivity in cancers by targeting translation initiation.

Results

CBFs are novel inhibitors of cap-dependent translation. During the course of a small molecule screening campaign designed to identify novel inhibitors of eukaryotic translation (23), a class of natural products, CBFs (also known as rocaglates), were found to exert potent inhibitory activity. Members of this family are known to be cytotoxic (22, 24–28) and inhibit protein synthesis (25, 29), but their molecular target(s) and mode of action are unknown. Two CBFs, 1-*O*-formylaglafoline (FA; also known as 1-*O*-formyl methyl rocaglate) and silvestrol (22) (Figure 1A), were identified and demonstrated a dose-dependent inhibition of cap-dependent firefly luciferase (FF) translation, but only a modest effect (~2-fold decrease) on HCV internal ribosome entry site–mediated (HCV IRES–mediated) translation of *Renilla* luciferase (Ren) in an in vitro translation assay (Figure 1, B and C; IC_{50} for FF inhibition, ~4 μM and 0.3 μM for FA and silvestrol, respectively) (see Discussion for an explanation of the HCV IRES–mediated translation effect). Rocaglates are active in several different eukaryotic cell–free translation systems (Figure 1 and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI34753DS1), and silvestrol was approximately 10-fold more potent than FA (Figure 1, B and C). These results suggest that CBFs preferentially target cap-dependent translation.

CBFs target eIF4A and inhibit translation initiation. To identify the step of translation targeted by CBFs, we measured the effects of these compounds on the ribosome recruitment phase of translation initiation. In the presence of the elongation inhibitor cycloheximide, 80S complexes were trapped at the AUG codon on radiolabeled mRNA templates and could be resolved by sedimentation velocity centrifugation (Figure 2A). FA inhibited 80S initiation complex formation and caused a concomitant increase in free mRNA, indicating an impairment of ribosome recruitment (Figure 2A).

Proper recruitment of the 43S preinitiation complex to mRNA 5′ ends is dependent on eIF4F, whose activity can be probed using a chemical cross-linking assay (30). Here, initiation factors (IFs) were cross-linked to the cap structure of radiolabeled mRNA and resolved by SDS-PAGE (Figure 2B). Cross-linking of eIF4E to the cap structure is ATP independent, whereas the cross-linking of eIF4A and eIF4B is ATP dependent (Figure 2B, compare lane 2 with lane 1), as previously reported (30). The binding of eIF4A, eIF4B, and eIF4E to mRNA is cap specific, since m^7GDP, but not GDP, causes a reduction in their cross-linking (Figure 2B, compare lanes 3 and 4 with lane 1). FA stimulated the cross-linking of eIF4A, but not of eIF4B and eIF4E, to mRNA (Figure 2B, compare lane 6 with lane 5). The increased cross-linking of eIF4A caused by silvestrol is cap specific and ATP dependent (data not shown).

eIF4A exists as a free form ($eIF4A_f$) or as part of the eIF4F complex ($eIF4A_c$) and is thought to recycle through eIF4F during translation initiation (31, 32). To assess the effects of CBFs on the RNA-binding activity of $eIF4A_f$ and $eIF4A_c$, cross-linking experiments were performed using recombinant $eIF4AI_f$ (Figure 2C) or purified eIF4F (Figure 2D). FA and silvestrol stimulated the cross-linking of $eIF4AI_f$ (Figure 2C, lanes 1–3) and $eIF4A_c$ (Figure 2D, compare lane 2 with lane 1) to mRNA. Consistent with the ability of CBFs to perturb $eIF4A_f$ activity, FA inhibited translation driven by the eIF4A-dependent encephalomyocarditis virus (EMCV) IRES (Supplemental Figure 2) (2). One possible mechanism by which CBFs might inhibit translation could be by inducing sequestration of $eIF4A_f$ on RNA, such that less eIF4A is available for assembly into the eIF4F complex.

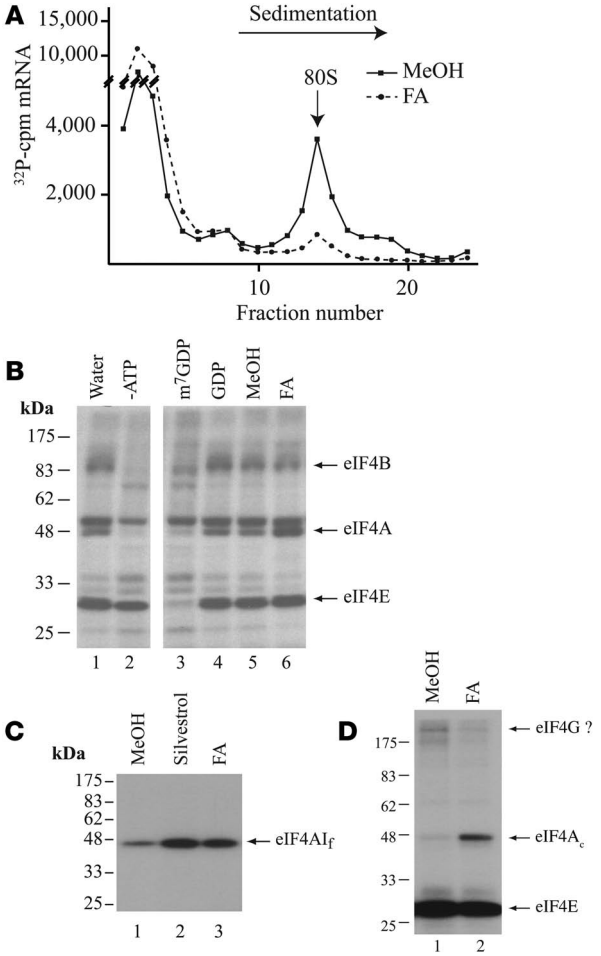

Figure 2
CBFs inhibit translation initiation and stimulate eIF4A RNA-binding activity. (**A**) CBFs prevent 80S complex formation. [^{32}P]-labeled CAT mRNA (200,000 cpm) was incubated with cycloheximide (CHX) in the presence of FA or vehicle (MeOH) in rabbit reticulocyte lysates. Reactions were resolved by centrifugation through glycerol gradients. Following centrifugation, fractions were collected and the amount of radioactivity determined by liquid scintillation counting. The total counts recovered from each gradient and the percentages of mRNA bound in 80S complexes were: CAT mRNA/CHX + MeOH: 35,178 cpm, 19% binding; and CAT mRNA/CHX + FA: 37,655 cpm, 2% binding. (**B**) CBFs stimulate cross-linking of eIF4A to mRNA. Initiation factor preparations were cross-linked to [^{32}P]-cap-labeled mRNA in the absence (lane 2) or presence of ATP (lanes 1 and 3–6), 0.6 mM m^{7}GDP (lane 3), 0.6 mM GDP (lane 4), MeOH (lane 5), or 50 μM FA (lane 6). Following nuclease digestion, samples were resolved by SDS-PAGE, and the gel was subjected to autoradiography. The position of migration of eIF4E, eIF4A, and eIF4B is indicated and is based on their known behavior in this assay (30). (**C**) CBFs stimulate the RNA-binding activity of eIF4AI$_f$. [^{32}P]-cap-labeled mRNA was cross-linked to recombinant eIF4AI$_f$ in the presence of MeOH (lane 1), 50 μM FA (lane 3), or 50 μM silvestrol (lane 2). Following nuclease digestion, samples were resolved by SDS-PAGE, and the gel was subjected to autoradiography. (**D**) CBFs stimulate the RNA-binding activity of eIF4A$_c$. [^{32}P]-cap-labeled mRNA was cross-linked to eIF4F in the presence of MeOH (lane 1) or 50 μM FA (lane 2). Following nuclease digestion, samples were resolved by SDS-PAGE, and the gel was subjected to autoradiography. Cross-linking of a higher-molecular-mass species (>175 kDa) is indicated by "eIF4G ?" since it may reflect RNA-bound eIF4G.

eIF4A is a member of the DEAD-box protein family comprising more than 35 members sharing 9 conserved amino acid motifs (33). We probed the selectivity of CBFs by testing the effects of FA on the activity of other DEAD-box proteins. FA did not stimulate cross-linking of Ded1 to mRNA (Supplemental Figure 3A, compare lane 4 with lane 3). We also tested the effect of FA on mRNA splicing, a process known to require the activity of more than 7 DEXD/H-box proteins (34) and found that in vitro splicing was not affected by FA (Supplemental Figure 3B, compare lane 4 with lane 3). These results, in addition to the findings that CBFs do not affect the RNA-binding activity of eIF4B and eIF4E (Figure 2B) or YB-1 (data not shown), indicate that these compounds show selectivity toward eIF4A.

CBFs inhibit translation in vivo and trigger apoptosis. To determine the potency of CBFs on protein synthesis in vivo, we performed metabolic labeling studies (Figure 3A). FA and silvestrol efficiently inhibited [^{35}S]methionine incorporation into proteins in a dose-dependent manner, with silvestrol being more potent than FA in vivo (Figure 3A, IC_{50} for FA, ~0.1 μM; IC_{50} for silvestrol, 0.02 μM, for a 1-hour incubation). The potency of these compounds was much higher in vivo than in vitro (Figure 1A), and this may reflect differences in target accessibility; intracellular accumulation of the drug in vivo such that the concentration of drug in the media does not accurately reflect the concentration of drug inside the cell; or nonspecific sequestration of the drug in vitro by nontranslational components of cell-free extracts. Nonetheless, these results indicate that CBFs are potent translation inhibitors in vivo. At FA and silvestrol concentrations that completely blocked protein synthesis, RNA ([^{3}H]uridine incorporation) and DNA ([^{3}H]thymidine incorporation) synthesis were not affected (Figure 3B), indicating that CBFs primarily target protein synthesis in vivo (25). To investigate whether the inhibition exerted by CBFs is reversible, the rate of [^{35}S]methionine incorporation was monitored in cells recovering from a translational block imposed by FA or silvestrol (Figure 3C). Translation rates were completely restored 8 hours following FA and silvestrol removal, although the rate of recovery from silvestrol inhibition was significantly slower (Figure 3C). These experiments do not address whether this is a consequence of differences in compound metabolism or binding affinity but do indicate that silvestrol is a more potent inhibitor of translation in vivo than FA.

The ability of CBFs to inhibit cap-dependent translation in vivo was assessed by monitoring luciferase production in cells transfected with the bicistronic reporter pcDNA/Ren/HCV/FF in the presence of FA (Figure 3D). Cap-dependent translation was impaired by CBFs, whereas HCV IRES–mediated translation was slightly stimulated in vivo (Figure 3D). The effects of CBFs on translation in vivo were not due to mRNA degradation or activation of a cryptic promoter within the IRES, since mRNA integrity was not altered in cells following exposure to compound (Supple-

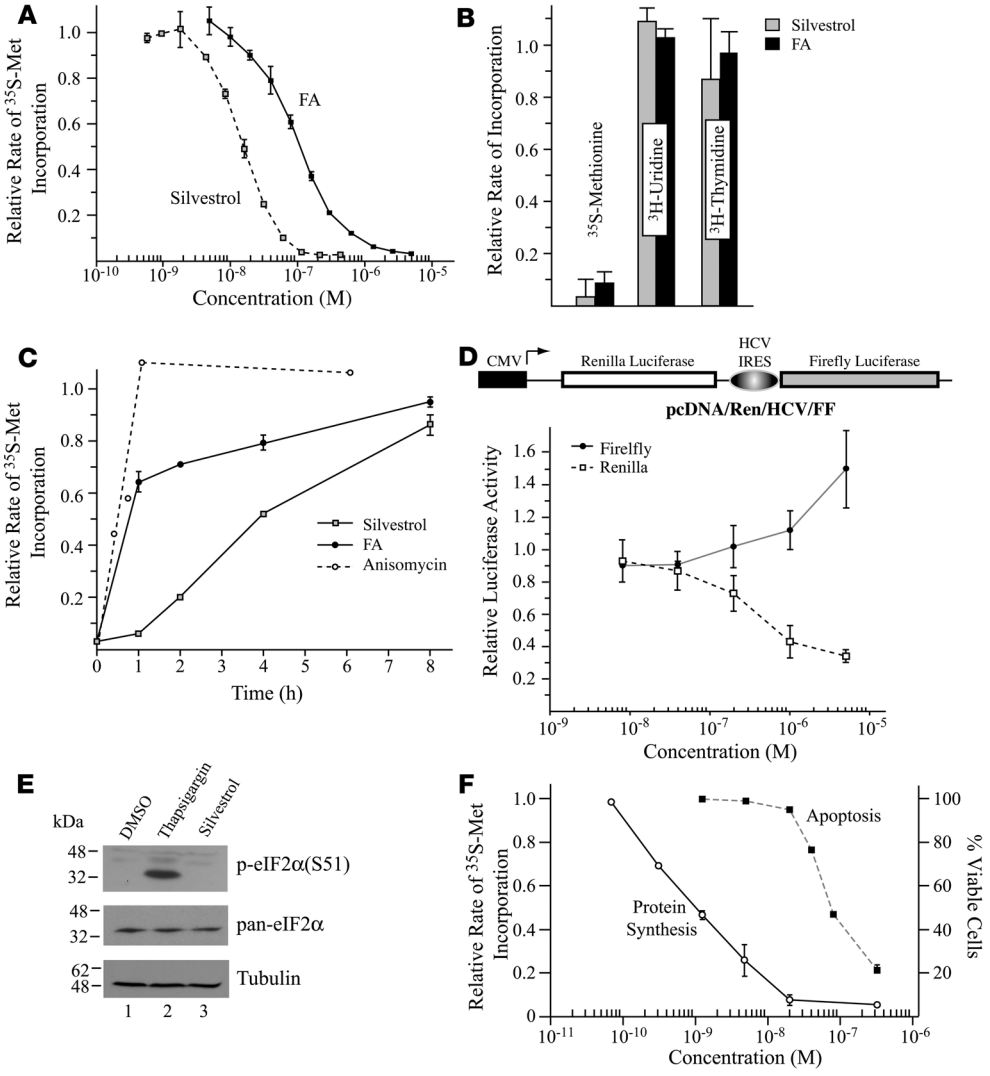

Figure 3

Effects of CBFs on protein, RNA, and DNA synthesis in vivo. (**A**) Dose-dependent inhibition of protein synthesis in vivo by CBFs. HeLa cells were incubated with the indicated concentrations of FA and silvestrol for 1 hour, with [^{35}S]methionine added 10 minutes before the end of the incubation. The rate of [^{35}S]methionine (^{35}S-Met) incorporation is expressed relative to that of cells treated with vehicle (MeOH). Results are expressed as mean ± SEM of 2 experiments. (**B**) CBFs primarily impact protein synthesis in vivo. HeLa cells were incubated with 5 μM FA, 0.4 μM silvestrol, or vehicle (MeOH) for 1 hour. The rate of incorporation of each radioisotope tracer into TCA-insoluble material is expressed relative to that in MeOH-treated cells. Results are expressed as mean ± SEM of 2 experiments. (**C**) Inhibition of translation by CBFs is reversible. HeLa cells were incubated for 1 hour with 10 μM anisomycin, 5 μM FA, 0.4 μM silvestrol, or MeOH. Cells were then washed with PBS and incubated with medium lacking compound for the indicated times. Ten minutes before harvesting, [^{35}S]methionine was added to the culture. The rate of [^{35}S]methionine incorporation into TCA-insoluble material is expressed relative to that in MeOH-treated cells. Results are expressed as mean ± SEM of 3 experiments. Anisomycin acts a positive control, since recovery of protein synthesis from inhibition with this compound occurs within an hour of its removal from cells (65). (**D**) CBFs inhibit cap-dependent translation in vivo. Top: Schematic representation of pcDNA/Ren/HCV/FF expression vector. Bottom: Effect of FA on cap-dependent and HCV IRES–mediated translation in 293 cells transfected with pcDNA/Ren/HCV/FF. Luciferase activity is expressed relative to that in MeOH-treated cells and is the mean ± SEM of 2 experiments. (**E**) Silvestrol does not induce eIF2α phosphorylation. HeLa cells were incubated for 2 hours in the presence of vehicle (DMSO), thapsigargin (2 μg/ml), or silvestrol (400 nM), after which extracts were analyzed by Western blotting. (**F**) Silvestrol induces apoptosis at concentrations higher than those required to inhibit protein synthesis. Jurkat cells were incubated with the indicated silvestrol concentrations for 13 hours, after which the rate of [^{35}S]methionine incorporation or the percentage of living cells was measured. Results are expressed as mean ± SEM of 2 experiments.

mental Figure 4). In addition, since compounds that induce or sustain eIF2α phosphorylation strongly inhibit protein synthesis, we assessed the ability of silvestrol to induced this event in vivo. Treatment of HeLa cells with thapsigargin (an ER stress inducer) or silvestrol strongly suppressed protein synthesis (Supplemental Figure 5). Whereas thapsigargin induced eIF2α phosphorylation (Figure 3E, compare lane 2 with lane 1), silvestrol did not (compare lane 3 with lane 2) at concentrations sufficient to inhibit protein synthesis. These results indicate that the inhibition of protein synthesis associated with silvestrol in vivo is not a consequence of eIF2α phosphorylation.

CBFs have been previously shown to be capable of inducing apoptosis (24). To assess the dose required for this event versus that required for translation inhibition, Jurkat cells were exposed to various concentrations of silvestrol, and translation and cell death were monitored. At 80 nM, silvestrol induced apoptosis in approximately 50% of Jurkat cells following a 13-hour exposure (Figure 3F). However, a much lower concentration of silvestrol was sufficient to inhibit protein synthesis over this extended time period (IC_{50}, 1 nM) (Figure 3F). These results indicate that the effects of CBFs on translation in vivo are not an indirect consequence of triggering apoptosis.

CBFs cause RNA-dependent sequestration of eIF4A in vivo. Given the ability of silvestrol to stimulate the RNA-binding activity of eIF4A in vitro, we wished to determine whether a similar mechanism was at work in vivo. Consequently, the position of migration of eIF4A was monitored in polysome profiles generated from Jurkat cells treated with silvestrol or vehicle (Figure 4). The profiles obtained in the presence of silvestrol were consistent with this compound inhibiting translation initiation

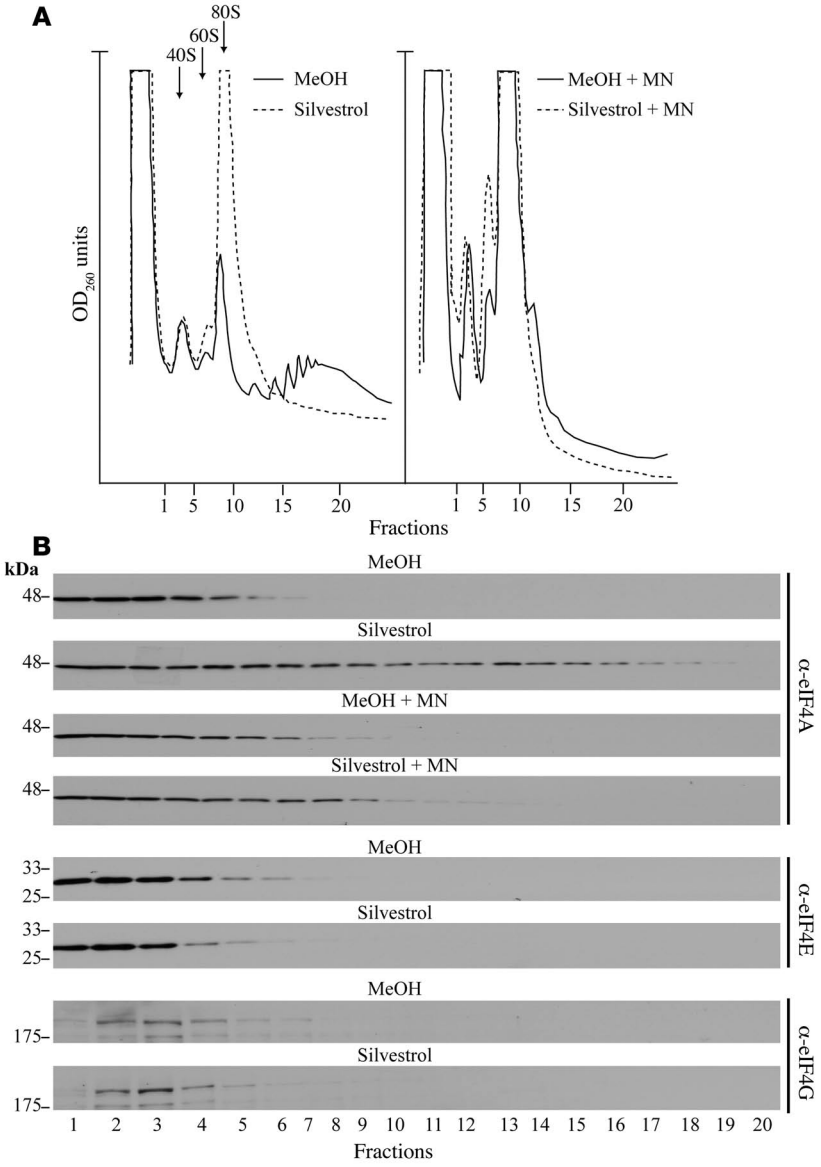

Figure 4
Silvestrol induces eIF4A association into RNAse-sensitive heavy sedimenting complexes in vivo. (**A**) Effect of silvestrol on Jurkat cell polyribosomes. Jurkat cells were exposed to vehicle (MeOH) or 0.2 μM silvestrol for 60 minutes. Cell extracts were prepared and treated with micrococcal nuclease (MN) where indicated. Reactions were resolved on 10%–50% sucrose gradients by centrifugation in an SW40 rotor at 150,000 *g* for 2 hours. Fractions were collected from the gradients and monitored with an ISCO UA-6 UV detector. (**B**) Western blots demonstrating the position of migration of eIF4A, eIF4E, and eIF4G1 in sucrose fractions collected from untreated or MN-treated lysates prepared from cells exposed to MeOH or 0.2 μM silvestrol.

and allowing ribosome run-off (Figure 4A). Immunoblotting for eIF4A in fractions from these gradients revealed that silvestrol caused eIF4A to shift into heavier sedimenting fractions (Figure 4B). This shift was not observed with the eIF4E or eIF4G1 subunits of eIF4F (Figure 4B). Treatment of cell extracts with micrococcal nuclease prior to centrifugation prevented the shift of eIF4A toward heavier fractions (Figure 4B). These results suggest that silvestrol stimulates the RNA-binding activity of eIF4A in vivo.

Silvestrol reverses chemoresistance mediated by PTEN inactivation or eIF4E overexpression in Eμ-Myc lymphomas. The Eμ-Myc murine lymphoma model is a powerful, genetically defined system for studying drug action in vivo. Activation of Akt signaling in this preclinical model accelerates tumorigenesis and promotes chemoresistance (9). The mTOR inhibitor rapamycin is capable of modulating sensitivity to doxorubicin and cyclophosphamide in *Eμ-Myc/myr-Akt* and *Pten*$^{+/-}$*Eμ-Myc*, but not in *Eμ-Myc/eIF4E*, lymphomas, suggesting that a significant proportion of Akt survival signaling is mediated by mTOR and eIF4E (9, 10). To investigate the consequences of inhibiting ribosome recruitment on chemosensitivity, silvestrol was tested in mice bearing lymphomas of different genotypes. We found that daily injections of silvestrol over a course of 8 days was well tolerated by mice and did not cause immunosuppression, weight loss, or impaired liver function (R. Cencic and J. Pelletier, unpublished observations). Doxorubicin and rapamycin synergized in mice bearing *Pten*$^{+/-}$*Eμ-Myc* tumors and extended tumor-free survival to 12–16 days (Figure 5A; $P < 0.0001$ for rapamycin plus doxorubicin versus rapamycin or doxorubicin alone). As a single agent, silvestrol showed no activity against *Pten*$^{+/-}$*Eμ-Myc* lymphomas but did synergize with doxorubicin, with all animals achieving remissions that lasted up to 16 days (Figure 5A; $P < 0.001$ for silvestrol plus doxorubicin versus silvestrol or doxorubicin alone). No synergy between silvestrol and doxorubicin was observed in *Eμ-Myc/Bcl-2* lymphomas, implying that the observed effect may be specific to tumors in which translation is deregulated (Figure 5B). Since *Eμ-Myc/eIF4E* lymphomas are refractory to the combination of rapamycin and doxorubicin treatment (9) (Figure 5C), we tested the ability of silvestrol to sensitize tumors of this genotype to doxorubicin. The *Eμ-Myc/eIF4E* lymphomas did not respond to silvestrol alone (Figure 5C). Doxorubicin or the combination of doxorubicin/rapamycin induced remissions in approximately 60% of the animals that lasted on average only approximately 4 days (Figure 5C). The combination of silvestrol and doxorubicin produced a longer tumor-free survival than either single agent, with all animals achieving remissions lasting up to 25 days (Figure 5C; $P < 0.001$ for silvestrol plus doxorubicin versus silvestrol or doxorubicin alone). These results clearly indicate that silvestrol plus doxorubicin treatment is more effective than rapamycin plus doxorubicin in this tumor type.

As previously reported (10), *Pten*$^{+/-}$*Eμ-Myc* tumors harbored elevated levels of phosphorylated Akt and S6 (Figure 5D, compare lane 2 with lane 1). Rapamycin treatment of *Pten*$^{+/-}$*Eμ-Myc* tumors inhibited mTOR activity, as judged by a reduction in p-S6 levels (Figure 5D, compare lane 3 with lane 2). Exposure of *Pten*$^{+/-}$*Eμ-Myc* lymphomas to doxorubicin or silvestrol had no detectable effect on p-S6 or p-Akt levels (Figure 5D, compare lanes 5 and 6 with lane 4).

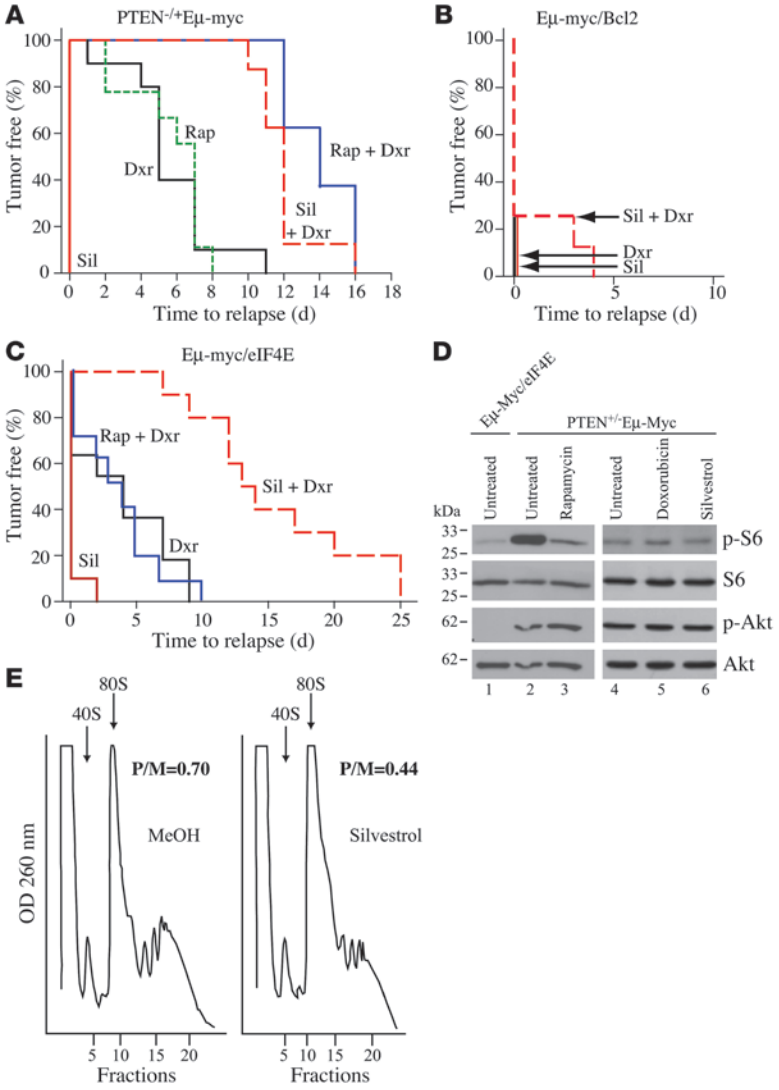

Figure 5
Silvestrol alters chemosensitivity in *Pten*$^{+/-}$*Eμ-Myc* and *Eμ-Myc/eIF4E* tumors in vivo. (**A**) Silvestrol sensitizes *Pten*$^{+/-}$*Eμ-Myc* tumors to the effects of doxorubicin in vivo. Kaplan-Meier plot showing tumor-free survival of mice bearing *Pten*$^{+/-}$*Eμ-Myc* tumors following treatment with doxorubicin (Dxr, solid black line; $n = 10$), rapamycin (Rap, dashed green line; $n = 9$), rapamycin and doxorubicin (Rap + Dxr; solid blue line; $n = 8$), silvestrol (Sil, solid red line; $n = 10$), or silvestrol and doxorubicin (Sil + Dxr, dashed red line; $n = 8$). (**B**) Silvestrol does not alter drug response in *Eμ-Myc/Bcl-2* tumors in vivo. Kaplan-Meier plot showing tumor-free survival of mice bearing *Eμ-Myc/Bcl-2* tumors following treatment with Dxr ($n = 9$), Sil ($n = 7$), or Sil + Dxr ($n = 8$). (**C**) Silvestrol sensitizes *Eμ-Myc/eIF4E* tumors to the effects of Dxr in vivo. Kaplan-Meier plot showing tumor-free survival of mice bearing *Eμ-Myc/eIF4E* tumors following treatment with Rap + Dxr ($n = 10$), Dxr ($n = 11$), Sil ($n = 10$), or Sil + Dxr ($n = 10$). (**D**) Western blot analysis of *Eμ-myc/eIF4E* (lane 1) and *Pten*$^{+/-}$*Eμ-Myc* lymphomas (lanes 2–6). Lysates prepared from *Eμ-myc/eIF4E* or *Pten*$^{+/-}$*Eμ-Myc* lymphomas from untreated (lanes 1, 2, and 4) and rapamycin- (lane 3), doxorubicin- (lane 5), and silvestrol-treated (lane 6) animals were subjected to immunoblotting for analysis of phosphorylated and total ribosomal S6 protein (p-S6 and S6) and Akt (p-Akt and Akt). (**E**) Silvestrol inhibits translation in *Pten*$^{+/-}$*Eμ-Myc* tumors in vivo. Mice bearing *Pten*$^{+/-}$*Eμ-Myc* tumors were injected with MeOH or silvestrol (0.2 mg/kg). Cytoplasmic extracts were prepared from tumors 4 hours later and resolved on 10%–50% sucrose gradients by centrifugation in an SW40 rotor at 150,000 *g* for 2 hours. Fractions were collected and monitored using an ISCO UA-6 UV detector. Plotted are results of 1 representative experiment of 3 that showed similar results. The positions in the gradients of 40S and 80S ribosomes are labeled, and the polysome/monosome (P/M) ratios are indicated.

To determine whether inhibition of translation was associated with this increased sensitivity to doxorubicin, we analyzed the polysome profile of tumors extracted from animals that had been treated with silvestrol (Figure 5E). As expected for an inhibitor of translation initiation, we found that the polysome to monosome ratio (P/M) in tumors treated with silvestrol decreased approximately 50% compared with that in nontreated tumors among 3 independent experiments (Figure 5E and data not shown). We blotted for eIF4E and eIF4A across the fractions from these polysomes to determine whether the reduction in P/M ratio induced in vivo was also associated with a shift of eIF4A to heavier sedimenting RNA complexes (Supplemental Figure 6). As documented for silvestrol-treated cell lines (Figure 4B), eIF4A was also found in heavier sedimenting complexes in polysomes from *Pten*$^{+/-}$*Eμ-Myc* tumors isolated from silvestrol-treated mice (Supplemental Figure 6). The lower amount of heavier sedimenting eIF4A-containing complexes observed for silvestrol-treated lymphomas compared with silvestrol-treated cells (Figure 4B) likely reflects the smaller effects of silvestrol on tumor polysomes (i.e., 50% reduction in polysomes in tumor samples compared with cell lines [compare Figure 5E with Figure 4A]).

We investigated the mechanism by which silvestrol was altering chemoresponsiveness of *Pten*$^{+/-}$*Eμ-Myc* tumors by analyzing a series of tumor samples 3 and 6 hours after treatment (Figure 6). TUNEL staining revealed an increase in apoptotic cells for the silvestrol/doxorubicin and rapamycin/doxorubicin combinations, compared with single-agent treatments with doxorubicin, silvestrol, or rapamycin (Figure 6, A and B). Inhibitors of apoptosis (IAPs) are known to block cell death and are regulated at the translational level, especially during stress (35). We did not detect changes in expression of XIAP, CIAP1, or CIAP2 in silvestrol-treated cells (data not shown) but did observe that treatments causing increased apoptosis also stimulated poly(ADP-ribose) polymerase (PARP) cleavage (Figure 6C, compare lanes 4 and 6 with lanes 1–3 and 5). These results demonstrate the effectiveness of targeting translation initiation to modulate chemoresponsiveness.

Discussion

Our results demonstrate that inhibiting translation initiation can modulate the response of transformed cells to chemotherapeutic insult. Inhibiting translation initiation induces apoptosis (36, 37), although the precise mechanism by which this event is triggered is

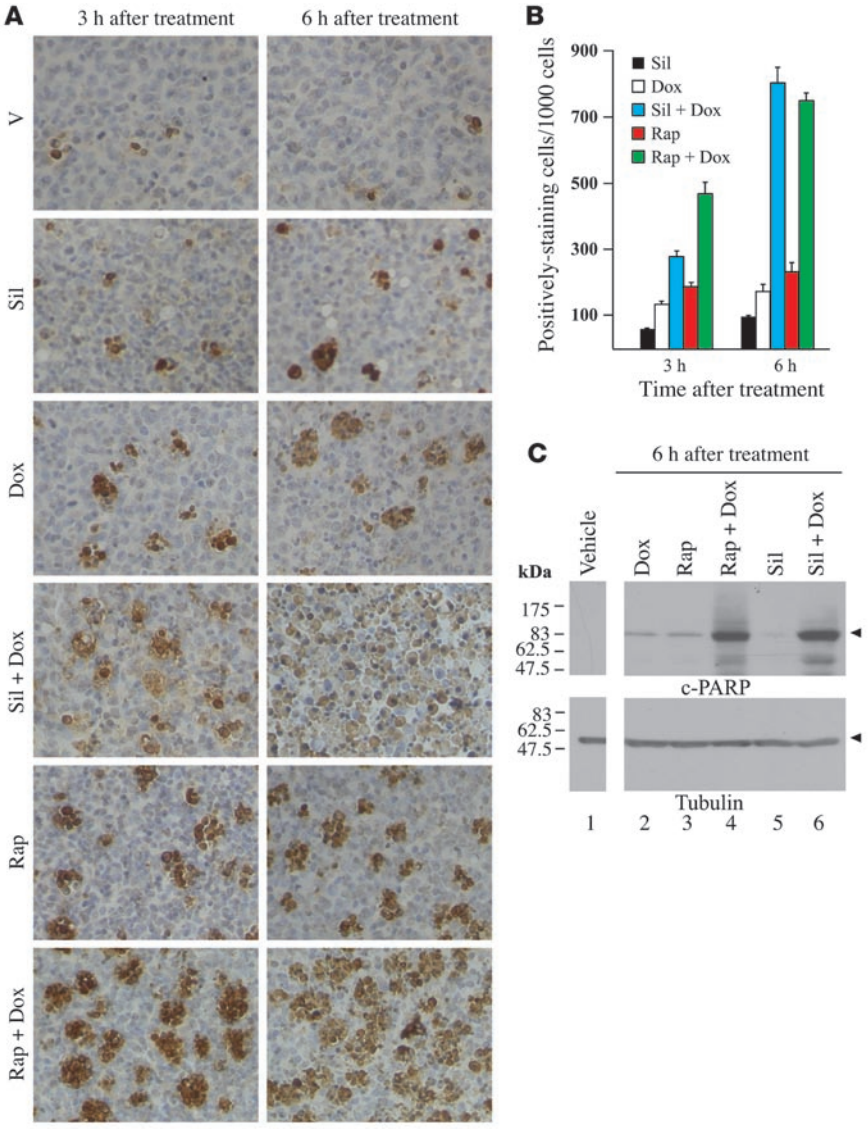

Figure 6
Silvestrol synergizes with doxorubicin to induce apoptosis in *Pten*$^{+/-}$*E*μ-*Myc* lymphoma cells in vivo. (**A**) Representative micrographs (original magnification, ×200) of *Pten*$^{+/-}$*E*μ-*Myc* lymphoma sections stained with H&E and TUNEL. C57BL/6 mice bearing well-palpable tumors were injected with vehicle, silvestrol, or rapamycin. Twenty-four hours later, the mice were injected again with silvestrol or rapamycin alone or in combination with doxorubicin. Three and 6 hours after treatment, tumors were extracted and stained. (**B**) Amount of tumor cells positive for TUNEL staining per 1,000 cells following the treatments described in **A**. The cell count was obtained from 4 different fields taken from 2 different sections. Results are expressed as mean ± SD. In vehicle-treated cells, there were 36 ± 1 TUNEL-positive cells/1,000 cells. (**C**) Western blot analysis of *Pten*$^{+/-}$*E*μ-*Myc* lymphomas treated as described in **A**. Tumor cells were extracted and lysed and the amount of cleaved poly(ADP-ribose) polymerase (c-PARP) and tubulin determined by Western blotting.

not fully understood. One hypothesis is that changes in the levels or activity of eIF4F mediate the translational regulation of specific genes involved in survival and apoptosis (38). Gene-specific translational control is thought to be mediated by the amount of secondary structure within the 5′ untranslated region of mRNAs, which dictates the eIF4A$_c$ requirement for ribosome recruitment (39). This is supported by the observation that altering eIF4F activity by ectopic overexpression of eIF4E (17, 40) or rapamycin treatment (16) alters expression of a subset of mRNA transcripts involved in cell proliferation and apoptosis. Consistent with this hypothesis is the finding that a partial collapse of polysomes is observed in *Pten*$^{+/-}$*E*μ-*myc* tumors exposed to silvestrol in vivo (Figure 5D).

The genotype-selective efficacy of silvestrol (in conjunction with doxorubicin) correlates with the expected occurrence of deregulated translation in these tumors. Since alteration of mTOR signaling in *Pten*$^{+/-}$*E*μ-*myc* lymphomas results in prosurvival signaling mediated by eIF4E (10), inhibition of translation initiation is expected to curtail this event and render tumors sensitive to DNA-damaging agents. The finding that silvestrol (in conjunction with doxorubicin) is effective against *Pten*$^{+/-}$*E*μ-*myc* and *E*μ-*myc/eIF4E* tumors, but not against *E*μ-*myc/Bcl2* lymphomas, is consistent with this hypothesis and suggests that the chemosensitizing effects of silvestrol are mediated by translation inhibition.

The effects of CBFs on translation reported herein are similar to those reported for pateamine, another small molecule inhibitor of translation initiation that also forces an engagement between eIF4A and RNA and prevents incorporation of eIF4A$_f$ into the eIF4F complex (41, 42). Although both pateamine and CBFs stimulate eIF4A$_f$ RNA-binding activity, only CBFs are capable of stimulating eIF4A$_c$ RNA-binding activity (Figure 2D) (41). In addition, pateamine is an extremely toxic compound, most likely due to the irreversible nature of its effects on translation (41), whereas the effects of CBFs on translation are reversible (Figure 3C). CBFs may thus act as chemical inducers of dimerization — forcing an interaction between eIF4A and RNA — suggesting that these compounds may impair the recycling of eIF4A$_f$ into the eIF4F complex (32). CBFs have been reported to inhibit mdm2, nuclear factor of activated T cells (NF-AT), and NF-κB activity (43–45), and some

of these other activities may be an indirect consequence of CBFs' effect on translation.

The mechanism of action of CBFs may explain the partial inhibition of HCV IRES–mediated translation (an eIF4A-independent process) observed at high CBF concentrations in vitro (Figure 1) (2), since CBF-mediated nonspecific binding of eIF4A$_F$ to the IRES could interfere with its function. In contrast, stimulation of HCV IRES–driven translation is observed in vivo in the presence of much lower concentrations of CBFs and may reflect the fact that 43S preinitiation complexes are limiting for HCV IRES–mediated translation and become available upon inhibition of cap-dependent translation (Figure 3D). This phenomenon has been previously reported for 2 other small molecule modulators of eIF4A activity (42, 46). Inhibition of translation initiation by RNA-mediated sequestration of eIF4A$_F$ is not unique to pateamine and CBFs, since this mechanism has been reported for BC1, a small, brain-specific noncoding RNA that represses translation at synaptodendritic microdomains (47, 48).

Deregulated expression of the eIF4F subunits has been implicated in the transformation process and drug resistance. The eIF4A subunit was shown to be overexpressed in human cancers, such as melanomas (49) and hepatocellular carcinomas (50). The availability of the eIF4A$_F$ subunit for incorporation into the eIF4F complex is regulated by its association with the tumor suppressor gene product Pdcd4, a protein whose levels are reduced in human lung, renal, and glial tumors (51). Moreover, overexpression of Pdcd4 in the epidermis delays tumor onset and progression in a chemically induced murine skin tumor model (21). Ectopic overexpression of eIF4G and eIF4E is also oncogenic (6, 7), and the levels of the eIF4E subunit are elevated in many human cancers and have been proposed as an independent prognostic tumor marker for breast cancer (52, 53). Reciprocally, the levels of 4E-BP1, a negative regulator of eIF4E, inversely correlate with progression of colon cancers (54). eIF4E is also a genetic modifier of the rapamycin response in vivo (10). Rapamycin resistance is an emerging complex and important clinical problem (55) that is thought to result in part from weakening by rapamycin of a negative feedback loop from S6K to IRS-1, leading to activation of IGF-1 (and Akt) signaling. This would have the undesired consequence of reducing the antitumor effects of mTOR inhibitors (56). The prevalence of this mechanism in human cancers is not known. Long-term exposure to rapamycin also targets the rictor/mTOR protein complex (mTORC2) and leads to inhibition of Akt S473 in primary acute myeloid leukemias (57–59). Exposure of *Pten*$^{+/-}$*Eμ-myc* lymphomas to rapamycin did not increase p-Akt levels, indicating that the S6K/IRS-1 loop is not operative in *Pten*$^{+/-}$*Eμ-myc* lymphomas (Figure 5D and unpublished observations). Our experiments do not address the ability of CBFs to reverse general rapamycin resistance, but they do suggest that in situations where this is a consequence of elevated eIF4E expression, CBFs may be beneficial. Moreover, direct targeting of eIF4A by CBFs did not directly impact on the S6K/IRS-1 negative feedback loop or mTORC2-directed Akt phosphorylation (Figure 5D), potentially conferring an advantage to CBFs over rapamycin in sensitizing cells to chemotherapy. CBFs prevented growth of human Jurkat (Figure 3F) and LNCaP cells in culture (27, 60) and have shown encouraging effects in xenograft tumor models (25), but their ability to synergize with other cytotoxic agents has not previously been tested. As a single agent, silvestrol, was ineffective against Eμ-myc lymphomas harboring PTEN lesions or overexpressing eIF4E (Figure 5, A and C). This may be due to the fact that only partial inhibition of protein synthesis was observed upon silvestrol administration in vivo compared with in vitro experiments (Figure 5E). This level of inhibition may be sufficient to curtail prosurvival signaling, but below the threshold required to halt cellular proliferation. However, in combination with doxorubicin, silvestrol was effective against *Pten*$^{+/-}$*Eμ-myc* and *Eμ-Myc/eIF4E* tumors, the latter being refractory to rapamycin/doxorubicin treatment. We do not believe that an inherent feature of Eμ-myc lymphomas makes them particularly sensitive to the effects of silvestrol/doxorubicin combination, since *Eμ-myc/Bcl2* lymphomas were not sensitive to this drug combination (Figure 5B). Our results support the idea that curtailing translation initiation by modulating eIF4A activity is a promising approach to altering drug resistance associated with PI3K/mTOR activation.

Methods

Compound isolation, synthesis, and storage. FA was isolated from the roots of *Aglaia australiensis* C. M. Pannell (HG 662) collected in Atherton Tablelands, Queensland, Australia. The methanolic extract was partitioned between water and chloroform, and the lipophilic fraction was separated by preparative medium pressure liquid chromatography and thin-layer chromatography as described previously (61). FA was also chemically synthesized using a previously described synthetic route (62). The synthetic derivative and natural product showed equivalent potency in translation inhibition assays. Silvestrol was a kind gift from Murray Tait (Cerylid Biosciences Ltd., Richmond, Victoria, Australia) (63). FA and silvestrol were resuspended in methanol and stored at –70°C. Doxorubicin (Sigma-Aldrich) was dissolved in water and stored at 4°C. Rapamycin (LC Laboratories) was resuspended in 100% ethanol and stored at –70°C.

In vitro transcription and translation. For in vitro transcriptions, plasmids pKS/FF/Ren, pKS/FF/EMCV/Ren, and pKS/FF/HCV/Ren were linearized with *Bam*HI and transcribed with T3 RNA polymerase to generate mRNA. In vitro translations were performed using Krebs-2 extracts at final mRNA and K^+ concentration of 5 μg/ml and 100 mM, respectively (23). FF and Ren activities (RLU) were measured on a Lumat LB 9507 luminometer (Berthold Technologies). Following in vitro translations in the presence of [^{35}S]methionine, protein products were separated on 10% polyacrylamide/SDS gels that were treated with EN^{3}HANCE (PerkinElmer), dried, and exposed to X-Omat (Kodak) film.

Cell culture, transfections, luciferase and apoptosis assays. For in vivo luciferase assays, 293 cells were maintained in DMEM medium supplemented with 10% FCS. The day before calcium phosphate transfection, cells were seeded at 3×10^6 cells/10-cm dish. Following transfection with pcDNA/Ren/HCV/FF, 293 cells were incubated for 10 hours with vehicle (MeOH) or CBF before harvesting and were collected 48 hours after transfection. Luciferase assays were performed with the Dual-Luciferase assay kit according to the manufacturer's instructions (Promega). Probes for Northern blots were produced using the Rediprime kit (Amersham).

For annexin V binding assays, Jurkat cells were maintained in RPMI media supplemented with 10% FCS. Cells (1×10^6) were plated in 12-well plates and incubated with vehicle (MeOH) or silvestrol for 13 hours. Cells were harvested by centrifugation, washed with PBS, and resuspended in 500 μl annexin V binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$). Five microliters of annexin V–FITC (BD Biosciences — Pharmingen) was added, and the reactions were incubated for 5 minutes at room temperature in the dark. Propidium iodide (PI) was then added (1 μg/μl), and the reactions were incubated for another 5 minutes. The percentage of living cells (negative for annexin V and PI staining) was measured by flow cytometry and expressed relative to MeOH-treated cells.

In vivo metabolic labeling studies. Briefly, 6×10^4 HeLa cells were seeded into 24-well plates 1 day prior to the experiment. Cells were incubated in the presence of compound or vehicle for the indicated periods of time. [^{35}S]methionine (150–225 μCi/ml) and [^{3}H]uridine (24 μCi/ml) were added to cells 10 minutes before harvesting, whereas [^{3}H]thymidine (48 μCi/ml) was added to cells 20 minutes before harvesting. For protein labeling, [^{35}S]methionine was added in methionine-free medium supplemented with 10% dialyzed FCS. For [^{3}H]uridine and [^{3}H]thymidine labeling, the isotopes were added in DMEM supplemented with 10% dialyzed FCS. Cells where then collected and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). Radiolabeled proteins were isolated by TCA precipitation on Whatman 3 MM paper. Radiolabeled nucleic acids were isolated by filtration through Whatman GF/C glass fiber filters. The amount of radioactivity was determined by scintillation counting, and the counts were normalized to protein concentration, which had been determined using a modified Lowry assay (DC Protein Assay; Bio-Rad).

Ribosome binding and chemical cross-linking. Ribosome binding assays were performed by incubating [^{32}P]-labeled CAT mRNA in rabbit reticulocyte lysate in the presence of 600 μM cycloheximide (CHX), vehicle (MeOH), or 100 μM FA at 30°C for 10 minutes (23). Following centrifugation through 10%–30% glycerol gradients (SW40; 187,000 *g*/3.5 hours), fractions from each gradient were collected using a Brandel Tube Piercer connected to an ISCO fraction collector. Fractions of 500 μl were collected, and radioactivity was determined by scintillation counting.

Chemical cross-linking of initiation factor preparations to [^{32}P]-cap-labeled oxidized CAT mRNA was performed under standard reaction conditions (30) containing 0.9 mM ATP. For chemical cross-linking with individual factors, 1 μg of recombinant eIF4AI$_{fs}$, 1 μg Ded1, or 0.7 μg eIF4F was used. After cross-linking, samples were treated with RNase A and separated on 10%–15% SDS-PAGE gradient gels (for ribosomal salt wash preparations) or 10% SDS-PAGE gels (for eIF4A$_f$ or eIF4F). The gels were dried and exposed to X-Omat (Kodak) film at –70°C with an intensifying screen.

Polysome profiling and Western blotting. Polysome profiles were generated as previously described (42). Briefly, 2×10^7 Jurkat cells were incubated with vehicle (MeOH) or 0.2 μM silvestrol for 1 hour. For polysome profiling performed with the *Pten*$^{+/-}$*Eμ-Myc* tumors, lymphomas were extracted from the animals following a 4-hour treatment with vehicle (MeOH) or 1.0 mg/kg silvestrol. In both cases, cells were then harvested and lysed in buffer A (5 mM Tris, pH 7.5, 2.5 mM $MgCl_2$, 1.5 mM KCl, 2 mM DTT, 1% Triton X-100, 0.5% sodium deoxycholate) in the presence of 100 μg/ml cycloheximide. Where indicated, the supernatants were treated with micrococcal nuclease by incubation at room temperature with 1 mM $CaCl_2$ and 100 U/ml of micrococcal nuclease for 20 minutes, followed by termination of the reaction by addition of 2 mM EGTA. The lysates were loaded onto 10%–50% sucrose gradients and centrifuged in an SW40 rotor at 150,000 *g* for 2 hours. Fractions were collected, and absorbance was monitored during the process. The fractions were then resolved on 10% SDS-PAGE gels and proteins visualized by Western blotting using anti-eIF4A(5D3) (64), anti-eIF4E (BD Biosciences Inc.), and anti-eIF4G1 (Bethyl Laboratories Inc.) antibodies.

Treatment studies. The generation of *Pten*$^{+/-}$*Eμ-Myc*, *Eμ-Myc/Bcl-2*, and *Eμ-Myc/eIF4E* lymphomas has been described elsewhere (9, 10). A total of 2×10^6 secondary lymphoma cells was injected into the tail vein of 6- to 8-week-old female C57BL/6 mice. Upon development of well-palpable tumors (auxiliary and inguinal lymph nodes), mice were injected intraperitoneally with doxorubicin (once at 10 mg/kg), rapamycin (4 mg/kg daily for 5 days), silvestrol (0.2 mg/kg daily for 5 days). Rapamycin, doxorubicin, and silvestrol were diluted in 5.2% PEG400/5.2% Tween-80 immediately prior to intraperitoneal injection. In combination studies, silvestrol or rapamycin were administered once daily for 5 consecutive days, while doxorubicin was administered on day 2. Tumor-free survival is defined as the time between disappearance and reappearance of a palpable lymphoma following treatment. All animal studies were approved by the McGill University Faculty of Medicine Animal Care Committee.

Statistics. The data were analyzed in the Kaplan-Meier format using the log-rank (Mantel-Cox) test for statistical significance (SigmaStat software). *P* values of less than 0.001 were considered significant.

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