Primary research

Expression of constitutively active 4EBP-1 enhances p27Kip1 expression and inhibits proliferation of MCF7 breast cancer cells

Hong Jiang, Jennifer Coleman, Robin Miskimins and W Keith Miskimins*

Address: Division of Basic Biomedical Sciences, University of South Dakota School of Medicine, Vermillion, South Dakota, 57069, USA

Email: Hong Jiang - hjiang@usd.edu; Jennifer Coleman - jcoleman@usd.edu; Robin Miskimins - rmiskim@usd.edu; W Keith Miskimins* - kmiskimi@usd.edu

* Corresponding author

Abstract

Background: Eukaryotic initiation factor 4E (eIF4E) is essential for cap-dependent initiation of translation. Cell proliferation is associated with increased activity of eIF4E and elevated expression of eIF4E leads to tumorigenic transformation. Many tumors express very high levels of eIF4E and this may be a critical factor in progression of the disease. In contrast, overexpression of 4EBP, an inhibitor of eIF4E, leads to cell cycle arrest and phenotypic reversion of some transformed cells.

Results: A constitutively active form of 4EBP-1 was inducibly expressed in the human breast cancer cell line MCF7. Induction of constitutively active 4EBP-1 led to cell cycle arrest. This was not associated with a general inhibition of protein synthesis but rather with changes in specific cell cycle regulatory proteins. Cyclin D1 was downregulated while levels of the CDK inhibitor p27Kip1 were increased. The levels of cyclin E and CDK2 were unaffected but the activity of CDK2 was significantly reduced due to increased association with p27Kip1. The increase in p27Kip1 did not reflect changes in p27Kip1 mRNA or degradation rates. Rather, it was associated with enhanced synthesis of the protein, even though 4EBP-1 is expected to inhibit translation. This could be explained, at least in part, by the ability of the p27Kip1 5'-UTR to mediate cap-independent translation, which was also enhanced by expression of constitutively active 4EBP-1.

Conclusions: Expression of active 4EBP-1 in MCF7 leads to cell cycle arrest which is associated with downregulation of cyclin D1 and upregulation of p27Kip1. Upregulation of p27Kip1 reflects increased synthesis which corresponds to enhanced cap-independent translation through the 5'-UTR of the p27Kip1 mRNA.

Background

Most eukaryotic mRNAs are translated through a cap-dependent mechanism of initiation. In this process, eIF4E, which functions by binding the 7-methylguanosine cap, is the rate-limiting factor due to its low abundance [1,2]. In normal cells, elevated eIF4E activity is associated with proliferation and is regulated by multiple mechanisms. eIF4E gene transcription is enhanced in response to mitogenic stimulation and may be mediated by c-myc [3]. Phosphorylation of eIF4E by the protein kinase Mnk1 increases its affinity for the cap, and thus increases cap-dependent initiation of translation [4–6]. An inhibitor of eIF4E, 4E binding protein (4EBP), is also regulated by phosphorylation. Phosphorylation of 4EBP disrupts its ability to bind eIF4E, freeing eIF4E to bind eIF4G, which functions to recruit additional components of the initiation complex [7,8]. The net result is that phosphorylation of 4EBP enhances cap-dependent translation initiation. 4EBP
phosphorylation is thought to be mediated by mammalian target of rapamycin (mTOR) in mitogen stimulated cells [9–11]. Numerous studies have implicated elf4E in formation and progression of tumors. Overexpression of elf4E leads to deregulated growth and malignant transformation of a variety of cultured cell lines [12–14]. Moreover, elevated levels of elf4E are commonly found in solid tumors, especially in breast, colon, and head and neck tumors [15]. Clinical studies indicate that elf4E gene amplification and protein overexpression is associated with malignant progression in these tumors [16]. High elf4E levels are also associated with a higher rate of cancer recurrence and cancer-related death [17]. In contrast to elf4E, overexpression of 4EBP inhibits cell proliferation [18] and 4EBP-1 expression levels are inversely correlated with the progression of certain types of tumors [19].

The findings summarized above indicate that elevated elf4E activity plays a fundamental role in cancer formation and progression and suggest the possibility that elf4E could be exploited as a therapeutic target. This is supported by several recent investigations. Inhibiting the expression of elf4E by expressing antisense RNA reverses the mediated transformation of cultured cells as indicated by decreased efficiency of growth in soft agar and tumor formation in nude mice [20]. Similarly, antisense-mediated reduction of elf4E in breast cancer cells inhibits both their tumorigenic and angiogenic properties [21]. On the other hand, overexpression of 4EBP reverses transformation mediated by v-src [18].

Rapamycin and its analogs are potent inhibitors of mTOR and lead to cell cycle arrest. Thus there is interest in using this class of inhibitors for chemotherapeutic treatment of cancer and several phase I trials have been carried out [22]. Treatment of mammalian cells with rapamycin leads to dephosphorylation and activation of 4EBP, which in turn binds to and inhibits elf4E. However, several other targets downstream of mTOR are also affected by rapamycin. These include effects on other translational control proteins including p70 S6 kinase (p70S6K), elf4G, elf4B, and eEF2 (reviewed in [23]). At present neither the targets that are essential for inhibition of cell proliferation by rapamycin nor the mechanism by which these targets mediate cell cycle arrest are completely understood.

In the present study an MCF7 breast cancer cell line was developed which can be induced to express a mutant form of 4EBP-1 in which five amino acids that are targets for phosphorylation have been changed to alanines [24]. Since the protein cannot be inactivated by phosphorylation it constitutively binds to and inhibits elf4E. Thus expression of the mutant mimics the effects of rapamycin on 4EBP but does not affect the other targets of mTOR. Induction of the constitutively active 4EBP-1 leads to cell cycle arrest which correlates with loss of cyclin D1 expression and increased levels of the cyclin dependent kinase inhibitor p27Kip1. The increase in p27Kip1 is mediated, at least in part, by enhanced synthesis of the protein and corresponds to activation of the p27Kip1 mRNA 5'-untranslated region (UTR) and its ability to mediate cap-independent initiation of translation.

**Results**

Expression of constitutively active 4EBP-1 inhibits the proliferation of MCF7 cells

To test the effect of 4EBP-1 on the proliferation of human breast cancer cells, an expression vector encoding the constitutively active 4EBP-1 mutant (4EBP-1-5A) was stably transfected into MCF7 cells. The 4EBP-1-5A cDNA was placed under the control of an ecdysone analog. In the absence of pon A an ecdysone receptor VP16 fusion protein actively represses the promoter leading to very low basal levels of expression. In the presence of pon A the ecdysone receptor recruits coactivators that lead to elevated expression. The stably transfected MCF7 line (MCF7-4EBP-1-5A) was treated with pon A and at various times cells were harvested for analysis of 4EBP-1 expression by Western blotting (Fig. 1A). Several forms of 4EBP-1 are present in the stably transfected MCF7 cells. The slower migrating bands represent endogenous 4EBP-1 that is phosphorylated at various sites. The fastest migrating band corresponds to hypophosphorylated 4EBP-1. Treatment with pon A leads to a rapid increase in the intensity of the fastest migrating band, which corresponds to the 4EBP-1-5A mutated form of the protein which cannot be phosphorylated. In cells treated with pon A, the fastest migrating form of 4EBP-1 is the predominant band at all time points. Expression of this form of 4EBP-1 continues to increase for at least five days after treatment with pon A. In the control cells, which were not treated with pon A, phosphorylated, inactive 4EBP-1 is the predominant form of the protein (Fig. 1A). Treatment of untransfected MCF7 cells with pon A had no effect on expression or phosphorylation status of 4EBP-1 (data not shown). The expressed 4EBP-1-5A protein is active as determined by a large increase in the amount of 4EBP-1 bound to elf4E in pon A treated MCF7-4EBP-1-5A cells (Fig. 1B).

Expression of constitutively active 4EBP-1, through inhibition of elf4E activity, would be expected to slow the rate of proliferation of MCF7 cells. To test this, the growth of MCF7-4EBP-1-5A breast cancer cells was determined by counting cell number in cultures treated with or without pon A (Fig. 1C). In the absence of pon A, the cells continued to proliferate for at least five days. However, induction of 4EBP-1-5A by the addition of pon A almost
completely blocked cell proliferation. There was no indication of cell death in the pon A treated cultures and pon A had no effect on the untransfected parental MCF7 cell line (Fig. 1C). These results indicate that expression of constitutively active 4EBP-1 leads to cell cycle arrest of MCF7 cells.

Expression of 4EBP-1-5A inhibits proliferation of MCF-7 cells. (A) MCF7-4EBP-1-5A cells were treated with or without pon A for up to 5 days as indicated. Cell extracts representing $2 \times 10^5$ cells were used for Western blotting with anti-4EBP-1. The lower band (arrow) represents endogenous, hypophosphorylated 4EBP-1 and 4EBP-1-5A induced by pon A. (B) MCF7-4EBP-1-5A cells were cultured in the presence or absence of pon A for 2 days. Cell extracts were prepared and equal amounts of protein were incubated with 7-methyl-GTP-Sepharose as described in Experimental Procedures. Bound eIF4E and associated 4EBP-1 were detected by Western blotting. (C) MCF7-4EBP-1-5A (left) or MCF7 (right) cells were grown in the presence (open circles) or absence (closed circles) of pon A. Cell numbers were counted on days 1, 3 and 5 as indicated. Each point represents the mean (± standard error) of either 4 (left panel) or 3 (right panel) independent determinations of cell number.

Expression of 4EBP-1-5A enhances expression of p27Kip1 and inhibits expression of cyclin D1. (A) MCF7-4EBP-1-5A cells were treated with or without pon A and harvested 1, 2 or 3 days after treatment for analysis of cellular proteins by Western blotting. Each lane represents proteins from $2 \times 10^5$ cells. The same membrane was used to detect p27Kip1, cyclin D1, CDK2, CDK4 and β-actin. (B) MCF7-4EBP-1-5A (left) or MCF7-pERV3 (right) cells treated with ponA (gray bars) or left untreated (open bars) for 3 days were analyzed as described in A. After Western blotting, protein levels were estimated by densitometry and normalized using β-actin as a standard.

Expression of 4EBP-1-5A results in enhanced p27Kip1 expression and downregulation of cyclin D1
Elevated levels of eIF4E are associated with tumorigenic transformation and enhanced expression of specific cell cycle regulatory proteins such as cyclin D1. Expression of constitutively active 4EBP-1 should lead to a global decrease in cap-dependent translation which could lead to cell cycle arrest. However, it is also possible that constitutively active 4EBP-1 inhibits proliferation through specific effects on cell cycle regulatory proteins. Therefore, a number of G1 cell cycle regulatory molecules were examined in MCF7-4EBP-1-5A cells that had been treated with...
or without pon A. Addition of pon A altered the levels of two critical G1 regulatory proteins. There was a significant increase in the level of p27Kip1 observed at every time point up to three days after addition of pon A (Fig. 2A and 2B). At the same time there was a substantial decrease in the level of cyclin D1. No difference was observed in the protein levels of CDK4 or CDK2 between pon A treated and untreated cells. Treatment of MCF7-pERV3 cells, which express the ecdysone receptor/VP16 fusion protein but not the inducible constitutively active 4EBP-1 (see Methods), with pon A had no effect on the expression of any of these proteins (Fig. 2B).

Constitutively active 4EBP-1 does not alter p27Kip1 and cyclin D1 turnover rates

The results above suggest that cell cycle arrest of MCF7 cells in response to 4EBP-1-5A expression involves specific effects on expression of cyclin D1 and p27Kip1. The most plausible explanation is that the observed changes are mediated by altered rates of translation. However, others have shown that elf4E overexpression enhances cyclin D1 mRNA transport from the nucleus to the cytosol [25] and it is possible that 4EBP-1 affects this process. Also, p27Kip1 expression is actually enhanced while 4EBP-1 would be expected to inhibit translation rates. Therefore it was of interest to examine the mechanism by which 4EBP-1-5A expression modulates p27Kip1 and cyclin D1 levels.

First, to determine if 4EBP-1 led to a change in degradation of either p27Kip1 or cyclin D1, turnover rates were estimated in MCF7-4EBP-1-5A cells that had been treated with or without pon A for 24 hours. Cycloheximide was added for various times and p27Kip1 and cyclin D1 levels were estimated by western blotting (Fig. 3). Neither the rate of p27Kip1 turnover nor that of cyclin D1 was significantly altered by pon A-induced expression of 4EBP-1-5A. Thus, modulation of p27Kip1 and cyclin D1 levels in response to expression of constitutively active 4EBP-1 does not appear to be mediated by changes in protein degradation.

Expression of constitutively active 4EBP-1 does not alter p27Kip1 mRNA levels but decreases cyclin D1 mRNA levels

To examine whether 4EBP-1 could regulate p27Kip1 or cyclin D1 at the transcriptional level or through changes in message stability, the levels of p27Kip1 and cyclin D1 mRNA levels were examined by Northern blotting (Fig. 4A and 4B). There was no difference in mRNA levels encoding p27Kip1 after treatment with pon A. However, pon A treatment led to an approximately 50% decrease in the levels of cyclin D1 mRNA (Fig. 4B). This may contribute to the downregulation of cyclin D1 protein and is consistent with a previous report showing an increase in cyclin D1 mRNA in cells overexpressing elf4E [26].

Expression of 4EBP-1-5A enhances de novo p27Kip1 protein synthesis

The observation that expression of constitutively active 4EBP-1 leads to increased p27Kip1, but does not significantly affect protein turnover or mRNA levels suggests that synthesis of p27Kip1 is affected. Therefore, the effect of 4EBP-1-5A on the de novo synthesis of p27Kip1 was tested by metabolic labeling with [35S]-labeled amino acids. After treatment of MCF7-4EBP-1-5A cells with or without pon A for 48 hours the cells were pulse labeled for 1.5 hours. Half of the cellular extract was used to immunoprecipitate cyclin D1 and the other half to immunoprecipitate p27Kip1 (Fig. 5A and 5B). The results demonstrate an increase in p27Kip1 protein synthesis of approximately two-fold in cells expressing 4EBP-1-5A. Since no changes in p27Kip1 mRNA levels or protein turnover were observed, the increased synthesis of p27Kip1 most likely reflects increased rates of translation. In the same experiment a substantial decrease in de novo synthesis of cyclin D1 was observed, which could be due, in part, to lower cyclin D1 mRNA levels (see Fig. 4). Interestingly, total protein synthesis, as determined by tetrachloroacetic acid (TCA) precipitation of total cellular proteins following pulse labeling, was decreased only about 15% by expression of constitutively active 4EBP-1 (Fig. 5B). This is consistent with reports that changes in the levels of active
eIF4E have a much greater effect on the translation of specific classes of mRNA than on general translation rates [15,27].

Expression of 4EBP-1-5A leads to inhibition of CDK2 activity
A critical factor in p27Kip1-mediated cell cycle arrest is inhibition of CDK2. 4EBP-1-5A expression did not significantly change CDK2 protein levels (see Fig. 2A). However, there was a substantial loss of CDK2 activity as determined by an immunoprecipitation kinase assay (Fig. 6). This loss of CDK2 activity correlated with an increase in the amount of p27Kip1 that co-immunoprecipitated with CDK2 (Fig. 6). At the same time, the association of p27Kip1 with CDK4 was dramatically decreased (Fig. 6). These results suggest that 4EBP-dependent cell cycle arrest involves p27Kip1-mediated inhibition of CDK2 kinase activity.

Expression of constitutively active 4EBP-1-5A enhances cap-independent translation through the p27Kip1 5’-UTR
A novel finding of the experiments above is that synthesis of endogenous p27Kip1 is enhanced under conditions that are expected to reduce cap-dependent translation. One possibility is that the p27Kip1 mRNA is translated through a cap-independent mechanism that does not require
elf4E. It was recently demonstrated that elements within a 217 nucleotide sequence from the 5’-UTR of the mouse p27Kip1 mRNA is able to mediate cap-independent translation of a reporter gene in NIH3T3 and D6P2T cells [28]. There is a high level of sequence homology between the mouse and human 5’-UTRs and the major transcriptional start site is also conserved [29]. Therefore it is likely the 5’-UTR of the human p27Kip1 message is also able to mediate cap-independent translation initiation. To test this, a 472 nucleotide fragment, representing the full-length human p27Kip1 5’-UTR derived from transcription initiation at the major start site, was inserted into the bicistronic expression vector pGL2CAT/Luc [30]. In this vector, the first cistron, encoding chloramphenicol acetyltransferase (CAT), is proximal to the mRNA’s cap structure and therefore expected to be translated by the conventional cap-dependent translation mode. The second cistron, encoding luciferase, is downstream of the CAT stop codon and therefore must be translated by a cap-independent mechanism mediated by sequence elements inserted between the two cistrons. Bicistronic constructs without an insert or with the human p27Kip1 5’-UTR inserted in either the correct or reverse orientation were transiently expressed in MCF7 cells. The cells were harvested after one day and both CAT and luciferase activities were analyzed (Fig. 7A and 7B). Expression of CAT was nearly identical with all three constructs and was therefore independent of an insert between the two coding regions. In contrast, only the construct carrying the p27Kip1 5’-UTR insert in the correct orientation expressed a significant level of luciferase. The ratio of luciferase/CAT was enhanced approximately 12-fold by the human 5’-UTR (Fig. 7B). Thus the full-length human p27Kip1 5’-UTR is able to mediate cap-independent translation in a manner similar to that previously shown for the 217 nucleotide sequence of the mouse 5’-UTR [28].

The effect of constitutively active 4EBP-1 on the activity of the p27Kip1 5’-UTR was determined by cotransfecting the bicistronic construct together with an expression vector encoding 4EBP-1-5A into MCF7 cells. Expression of 4EBP-1-5A led to a two-fold increase in the luciferase/CAT ratio (Fig. 7C). Interestingly, this is approximately the same level of increase that is observed in synthesis of endogenous p27Kip1 protein (see Fig. 5B).

Discussion

In normal mitogen-activated cells elf4E activity is enhanced due to elevated transcription of the elf4E gene, a phosphorylation-dependent increase in affinity for the 7-methylguanosine cap, and phosphorylation-dependent inactivation of the inhibitor protein 4EBP. In many tumor cells elf4E levels are elevated and overexpression of the protein in normal cultured cells causes transformation. Elevated elf4E levels enhance translation of specific mRNAs. Many of these mRNAs are involved in cell cycle progression. They have long GC-rich 5’-UTRs that can form extensive secondary structure and have been classified as “weak” mRNAs because they are translated inefficiently when elf4E levels are limiting (reviewed in [15]). It has been proposed that when elf4E levels are elevated these mRNAs can more effectively recruit elf4E and its associated factors including elf4G, a scaffold protein essential in recruiting the small ribosomal subunit, and elf4A, a helicase essential for scanning through secondary structures in the 5’-UTR [15,27].

In the experiments described here we have inducibly expressed a constitutively active form of 4EBP-1 in the human breast cancer cell line MCF7. This leads to nearly complete inhibition of cell proliferation which is associated with an increase in binding of 4EBP-1 to elf4E. This does not lead to a major drop in total protein synthesis as indicated by metabolic labeling with 35S-amino acids. However, there are major changes in expression of two cell cycle regulatory proteins, cyclin D1 and p27Kip1. Cyclin D1 levels decline to about 30% of that observed in uninduced cells and this appears to be due to a reduction in cyclin D1 mRNA levels and protein synthesis, since there was no evidence for changes in cyclin D1 turnover. The observed decline in cyclin D1 is expected since constitutively active 4EBP should reverse cyclin D1 expression mediated by elf4E overexpression. Interestingly, others have demonstrated that elf4E has an additional role in the nucleus. A portion of the cellular elf4E is imported into the nucleus through a mechanism that requires the protein 4E-T [31]. In the nucleus elf4E is localized to specific nuclear bodies and associates with promyelocytic leukemia protein (PML, [32]). Nuclear elf4E appears to be involved in export of cyclin D1 mRNA to the cytosol [25]. Both 4E-T and PML interact with the same domain of elf4E that binds 4EBP [32]. Thus expression of constitutively active 4EBP may block nuclear functions of elf4E, one consequence being a reduction in cyclin D1 mRNA that can be translated in the cytoplasm.

In contrast to cyclin D1, p27Kip1 levels increase when 4EBP-1-5A is expressed. An interesting note is that the p27Kip1 mRNA has a long 5’-UTR that is 65% GC and is predicted to form stable secondary structures [29]. Thus, even though it shares these characteristics with the putative “weak” mRNAs that require elevated elf4E activity for efficient translation, p27Kip1 responds in an opposite manner. Part of this can be explained by the fact that the p27Kip1 5’-UTR is able to promote cap-independent translation. When placed between the CAT and luciferase coding regions in a bicistronic mRNA, the 5’-UTR stimulates high level expression of the downstream coding region (luciferase). This property is shared by the highly homologous mouse p27Kip1 5’-UTR [28]. Coexpression of
4EBP-1-5A with the bicistronic construct containing the p27^Kip1 5'-UTR leads to an approximately two-fold increase in cap-independent expression. This increase is similar to that observed for endogenous p27^Kip1 in cells induced to express 4EBP-1-5A. The mechanism by which 4EBP-1-5A could mediate enhanced translation through the p27^Kip1 5'-UTR is not presently known. One possibility is that binding of 4EBP-1 to eIF4E releases other initiation factors that are in limited supply but also required for cap-independent initiation.

Inhibition of cell proliferation by 4EBP-1-5A correlates with a large decrease in CDK2 activity. This is not due to a decline in CDK2 or cyclin E protein levels. Rather it corresponds to enhanced binding of p27^Kip1 to the cyclin E/CDK2 complex. This is probably mediated by two separate mechanisms. First, downregulation of cyclin D1 leads to release of p27^Kip1 sequestered by the cyclin D1/CDK4 complex. This is indicated by a large decrease in co-immunoprecipitation of p27^Kip1 with CDK4 upon induction of 4EBP-1-5A. Second, de novo synthesis contributes to the pool of cellular p27^Kip1 that is available to bind the cyclin E/CDK2 complex.

Given its role in control of cell proliferation and in tumorigenesis, there is interest in targeting the pathways that control eIF4E activity for cancer chemotherapy. Clinical trials using the rapamycin analog CCI-779 show promise for this strategy [22]. Rapamycin and its synthetic analogs block mTOR kinase activity leading to dephosphorylation of 4EBP and other mTOR substrates. MCF7 cells are sensitive to rapamycin analogs in the low nM range [33]. A potential problem with the use of rapamycin and its analogs is that some tumors are intrinsically resistant to its effects [33,34]. Resistance in mammalian cells has been associated with decreased expression of 4EBP-1 [35], mutations in the ATM protein [36], and low level expression of p27^Kip1 due to enhanced turnover of the protein [37]. In addition, it has been demonstrated that fibroblasts from p27^Kip1 knockout mice are more resistant to the effects of rapamycin than normal cells [37]. Thus p27^Kip1 is an important mediator of cell growth inhibition in response to rapamycin. Many tumor cells express very low levels of p27^Kip1 and this is correlated with poor prognosis. If rapamycin and its analogs are to be developed as useful antitumor agents, it will be important to determine how p27^Kip1 levels affect responsiveness to these compounds.

Rapamycin and its analogs affect all of the downstream targets of mTOR, including p70 S6 kinase and other components of the translation machinery. The data presented here indicate that direct inhibition of eIF4E may be more effective and a more specific means of targeting tumor cells than treatment with rapamycin. It will be important to determine if direct inhibition of eIF4E is able to arrest the cell cycle in rapamycin resistant tumors. If so, it may be possible to develop reagents that target eIF4E activity, thereby bypassing the upstream signaling events in the pathway.

**Methods**

**Cell culture**

MCF7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin. The inducible MCF7 cell line expressing 4EBP-1-5A was established using the Complete Control™ Inducible Mammalian Expression System (Stratagene). First, MCF7 cells were transfected with the pERV3 vector encoding both the RXR protein and an ecdysone/glucocorticoid receptor fusion protein using GenePorter (Gene Therapy Systems). Stably transfected colonies were selected using G418 (400 µg/ml). These colonies were further screened for the ability to inducibly express luciferase from the pEGSH-luc construct in response to pon A in transient transfection assays. A responsive cell line derived in this manner (designated MCF7-pERV3) was further transfected with pEGSH-4EBP-1-5A, a construct in which a constitutively active form of 4EBP-1 is inserted into the pon A-inducible expression cassette of pEGSH. Cell clones were selected using hygromycin B (100 µg/ml). The resulting colonies were isolated, expanded, and tested for pon A-inducible expression of 4EBP-1-5A. A single stably transfected cell line designated MCF7-4EBP-1-5A was used in the experiments described.

**Transfections and reporter assays**

Cells growing in 35 mm plates were transfected using GenePorter. One day after transfection cells were lysed using reporter lysis buffer (Promega) and scraped from the dishes. Luciferase activity was determined using the Steady-Glo substrate (Promega) according to the manufacturer’s protocol, using 50 µl of cellular extract. CAT enzyme activity was determined using 14C-labeled chloramphenicol as described previously [38].

**Plasmid constructs**

The human p27^Kip1 5’UTR was amplified by PCR using primers CCCCAGGTCTCTCCGGGTTCTGACGACCGCGCCTCT and CCCAACGTTTCTGTACGCCCTCCCTTCAC. The PCR product was digested with Hind III and ligated into the Hind III site of pGL2CAT/Luc (a gift of R.E. Rhoads). To subclone 4EBP-1-5A into pEGSH the plasmid pcDNA3.1-4EBP-1-5A was digested with Hind III, the ends were filled using Klenow fragment, and then the insert was released by digesting with Xba I. The resulting fragment was ligated into pEGSH that had been digested with Eco RV and Xba I.
Cell proliferation assay
MCF7 and MCF7-4EBP-1-5A cells were treated with pon A (10 µM) or the same volume of ethanol for 5 days. The medium was changed every day. After 1, 3, or 5 days incubation, cells were harvested using trypsin and the cell number was determined using a hemacytometer.

Western blotting
Cells were harvested by trypsinization and the cell number was determined using a hemacytometer. The cells were pelleted and washed with phosphate-buffered saline (PBS). The cell pellets were resuspended in sodium dodecylsulfate (SDS)-sample buffer at 1 × 10^4 cells per µl and then sonicated to shear nucleic acids. Samples representing 2 × 10^5 cells were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon P membranes. Membranes were probed with the appropriate antibodies. The p27^Kip1 and eIF4E monoclonal antibodies were purchased from Transduction Laboratories. Anti-PHAS1 (4EBP-1) polyclonal antibody was purchased from Zymed. Anti-β-actin monoclonal antibody was purchased from Sigma. Antibodies against cyclin D1, CDK2, and CDK4 were purchased from Santa Cruz Biotechnology. Secondary horseradish peroxidase-linked anti-mouse or anti-rabbit IgG antibodies were purchased from Santa Cruz Biotechnology. Bands were visualized using the SuperSignal West Pico chemiluminescence detection system (Pierce).

Protein turnover analysis
MCF7-4EBP-1-5A cells were cultured in the presence or absence of pon A (10 µM) for 24 hours. The medium was then changed with readdition of pon A or an equal volume of ethanol and the protein synthesis inhibitor cycloheximide (100 µg/ml, Sigma) was added. Cells were incubated for the indicated times and then harvested in SDS-sample buffer as described above for Western blotting. Cyclin D1 and p27^Kip1 proteins were detected by Western blotting and the levels of each protein were estimated by densitometry using a Chemilager system (Alpha Innotech).

Ccap-binding assay
MCF7-4EBP-1-5A cells were cultured in the presence or absence of pon A (10 mM) for 48 hours. Cell monolayers were washed with PBS three times and then lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 10 µg/ml leupeptin, 2 µg/ml Aprotinin, and 0.1 mM PMSF) by subjecting the cells to 3 cycles of freezing in liquid nitrogen and thawing in a 37°C water bath. The lysate was then centrifuged at full speed in a microcentrifuge at 4°C for 15 min. Protein concentration was determined using the Bio-Rad protein assay solution.

For pulse-labeling, the cells were washed three times with DMEM lacking Met and Cys and then incubated in the same medium containing 80 µCi/ml of 35S-labeled Met and Cys (Tran35S-label, ICN) and the proteasome inhibitor MG-132 (10 µM, Calbiochem) for 1.5 hours. The cells were washed three times with ice-cold PBS and lysed as above. A portion of the supernatant was precipitated with TCA to estimate the level of total protein synthesis. The re-
For CDK2 kinase assays, lysates (100 µg protein) prepared as described above were used to immunoprecipitate CDK2 with a polyclonal antibody. The beads with the bound proteins were washed and then resuspended in 30 μl of kinase buffer [50 mM HEPES, pH 7.4, 2.5 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM vanadate, 1 mM NaF, 1 mM DTT, 10 mM MgCl2, 20 µM ATP, 50 µCi γ-32P-ATP (7000 Ci/mmol, ICN), and 1 µg histone H1]. Reaction mixtures were incubated for 15 min at 30°C and stopped by adding the same volume of 2X SDS-sample buffer. The phosphorylated histone H1 was resolved by SDS-PAGE and detected by autoradiography using Kodak XAR-5 film.

For co-immunoprecipitation of p27Kip1 with CDK4 or CDK2, immunoprecipitations were performed as described above with either anti-CDK4 or anti-CDK2. The precipitated proteins were dissolved in SDS-sample buffer, separated by SDS-PAGE and then p27Kip1 and CDK2 were detected by Western blotting.

**Authors’ Contributions**

HJ participated in manuscript preparation and design and performance of the experiments. JC constructed and characterized the bicistronic reporter constructs and aided with manuscript preparation. RM and WKM conceived of the project, directed the performance of the experiments, and prepared the final manuscript. All authors read and approved the final manuscript.

**Acknowledgements**

This work was supported by NIH grant CA84325.

**References**

1. Hiremath LS, Webb NR and Rhoads RE Immunological detection of the messenger RNA cap-binding protein. J Biol Chem 1985, 260:7843-7849
2. Duncan A, Milburn SC and Hershey JW Regulated phosphorylation and low abundance of HeLa cell initiation factor elf-4F suggest a role in translational control. Heat shock effects on elf-4F. J Biol Chem 1987, 262:380-388
3. Jones RM, Branda J, Johnston KA, Polymenis M, Gadd M, Rustgi A, Callanan L and Schmidt EV An essential E box in the promoter of the gene encoding the mRNA cap-binding protein (eukaryotic initiation factor 4E) is a target for activation by c-myc. Mol Cell Biol 1996, 16:4754-4764
4. Pyronnet S, Imataka H, Gingras AC, Fukunaga R, Hunter T and Sonenberg N Human eukaryotic translation initiation factor 4G (elf-4G) recruits Mnk1 to phosphorylate elf-4E. EMBO J 1999, 18:270-279
5. Waskiewicz AJ, Johnson JC, Penn B, Mahalingam M, Kimball SR and Cooper JA Phosphorylation of the cap-binding protein eukaryotic translation initiation factor 4E by protein kinase Mnk1 in vivo. Mol Cell Biol 1999, 19:1871-1880
6. Wang X, Flynn A, Waskiewicz AJ, Webb BL, Vries RG, Baines IA, Cooper JA and Proud CG The phosphorylation of eukaryotic initiation factor elf-4E in response to phorbol esters, cell stresses, and cytokines is mediated by distinct MAP kinase pathways. J Biol Chem 1998, 273:9373-9377
7. Haghighat A, Mader S, Pause A and Sonenberg N Repression of cap-dependent translation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor-4E. EMBO J 1995, 14:5701-5709
8. Pause A, Belshaw GJ, Gingras AC, Donzé O, Lin TA, Lawrence JC Jr and Sonenberg N  Inhibition-dependent stimulation of protein synthesis by phosphorylation of a regulator of S'-cap function. Nature 1994, 371:762-767
9. Hara K, Yonezawa K, Kozlowski MT, Sugimoto T, Andرابi K, Weng QP, Kasuga M, Nishimoto I and Avruch J Regulation of eIF-4E BP1 phosphorylation by mTOR. J Biol Chem 1997, 272:26457-26463
10. von Manteuffel SR, Dennis PB, Pullen N, Gingras AC, Sonenberg N and Thomas G The insulin-induced signalling pathway leading to S6 and initiation factor 4E binding protein 1 phosphorylation bifurcates at a rapamycin-sensitive point immediately upstream of p70S6k. Mol Cell Biol 1997, 17:5426-5436
11. Brunn GJ, Hudson CC, Sekulic A, Williams JM, Hosoi H, Houghton PJ, Lawrence JC Jr and Abraham RT Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. Science 1997, 277:99-101
12. Lazaris-Karatzas A, Montine KS and Sonenberg N Malignant transformation of primary rodent fibroblasts. Mol Cell Biol 1992, 12:2234-2246
13. Lazaris-Karatzas A and Sonenberg N The mRNA S' cap-binding protein, eIF-4E, cooperates with v-myc or E1A in the transformation of primary rodent fibroblasts. Mol Cell Biol, 1992, 12:2234-2246
14. De Benedetti A, Joshi B, Graff JR and Zimmer SG CHO cells transformed by the translation factor eIF-4E display increased c-myc expression but require overexpression of Max for tumorigenicity. Mol Cell Biol 1994, 2:247-271
15. De Benedetti A and Harris AL eIF-4E expression in tumors: its possible role in progression of malignancies. Int J Biochem Cell Biol 1999, 31:59-72
16. Sorrells DL, Meschonac C, Black D and Li BD Pattern of amplification and overexpression of the eukaryotic initiation factor 4E gene in solid tumor. J Surg Res 1999, 85:37-42
17. Li BD, McDonald JC, Nassar R and De Benedetti A Clinical outcome in stage I to III breast carcinoma and eIF4E overexpression. Ann Surg 1998, 227:756-766discussion 761-763.
18. Rousseau D, Gingras AC, Pause A and Sonenberg N The eIF4E-binding proteins 1 and 2 are negative regulators of cell growth. Oncogene 1996, 13:2415-2420
19. Martin ME, Perez MI, Redondo C, Alvarez T, Weng QP, Kasuga M, Nishimoto I and Avruch J Regulation of eIF-4E BP1 phosphorylation by mTOR. J Biol Chem 1997, 272:26457-26463
20. Rinker-Schaeffer CW, Graff JR, De Benedetti A, Zimmer SG and Rhoads RE Decreasing the level of translation initiation factor 4E with antisense RNA causes reversal of ras-mediated transformation and tumorigenesis of cloned rat embryo fibroblasts. Int J Cancer 1993, 55:841-847
21. Nathan CA, Carter P, Liu L, Li BD, Abreo F, Tudor A, Zimmer SG and De Benedetti A Elevated expression of eIF4E and FGF-2 isoforms during vascularization of breast carcinomas. Oncogene 1997, 15:1087-1094
22. Hidalgo M and Rowinsky EK The rapamycin-sensitive signal transduction pathway as a target for cancer therapy. Oncogene 1996, 13:6860-6866
23. Raught B, Gingras AC and Sonenberg N The target of rapamycin (TOR) proteins. Proc Natl Acad Sci U S A 2001, 98:7037-7044
24. Mothe-Satney I, Yang D, Fadden P, Haystead TA and Lawrence JC Jr Multiple mechanisms control phosphorylation of PHAS-I in five (S/T/P) sites that govern translational repression. Mol Cell Biol 2000, 20:3558-3567
25. Rousseau D, Kaspar R, Rosenwald I, Gehryk L and Sonenberg N Translation initiation of ornithine decarboxylase and nucleo-cytoplasmic transport of cyclin D1 mRNA are increased in cells overexpressing eukaryotic initiation factor 4E. Proc Natl Acad Sci U S A 1996, 93:1065-1070
26. Long E, Lazaris-Karatzas A, Karatzas C and Zhao X Overexpression eukaryotic translation initiation factor 4E stimulates bovine mammary epithelial cell proliferation. Int J Biochem Cell Biol 2001, 33:133-141
27. Sonenberg N mRNA S' cap-binding protein eIF4E and control of cell growth. Cold Spring Harbor Cold Spring Harbor Laboratory Press 1996,
28. Miskimins WK, Wang G, Hawkison M and Miskimins R Control of cyclin-dependent kinase inhibitor p27 expression by cap-independent translation. Mol Cell Biol 2001, 21:4960-4967
29. Coleman J, Hawkinson M, Miskimins R and Miskimins WK The major transcription initiation site of the p27Kip1 gene is conserved in human and mouse and produces a long 5'-UTR. BMC Mol Biol 2001, 2:12
30. Gan W, Celle ML and Rhoads RE Functional characterization of the internal ribosome entry site of eIF4G mRNA. J Biol Chem 1998, 273:5006-5012
31. Dostie J, Ferriauolo M, Pause A, Adam SA and Sonenberg N A novel shuttling protein, 4E-T, mediates the nuclear import of the mRNA S' cap-binding protein, eIF4E. EMBO J 2000, 19:3142-3156
32. Cohen N, Sharma M, Kentsis A, Perez JM, Strudwick S and Borden KL PML RING suppresses oncogenic transformation by reducing the affinity of eIF4E for mRNA. EMBO J 2001, 20:4547-4559
33. Yu K, Toral-Barza L, Discafani C, Zhang WG, Skotnicki J, Frost P and Gibbons JJ mTOR, a novel target in breast cancer: the effect of CCI-779, an mTOR inhibitor, in preclinical models of breast cancer. Endocr Relat Cancer 2001, 8:249-258
34. Hosoi H, Dilling MB, Liu LN, Danks MK, Shikata T, Sekulic A, Abrah- am RT, Lawrence JC Jr and Houghton PJ Studies on the mechanism of resistance to rapamycin in human cancer cells. Mol Pharmacol 1998, 54:815-824
35. Dilling MB, Germain GS, Duchin L, Jaryaraman AL, Zhang X, Harwood FC and Houghton PJ eIF4E-binding proteins, the suppressors of eukaryotic initiation factor 4E, are down-regulated in cells with acquired or intrinsic resistance to rapamycin. J Biol Chem 2002, 277:13907-13917
36. Beamish H, Williams R, Chen P, Khanna KK, Hobison K, Watters D, Shiloh Y and Lavin M Rapamycin resistance in ataxia-tel-angiectasia. Oncogene 1996, 13:963-970
37. Luo Y, Marx SO, Kiyokawa H, Koff A, Massague J and Marks AR Rapamycin resistance tied to defective regulation of p27Kip1. Nat Cell Biol 1996, 16:744-751
38. Ouyang Q, Bommakanti M and Miskimins WK A mitogen-responsive promoter region that is synergistically activated through multiple signalling pathways. Mol Cell Biol 1993, 13:1796-1804

Publish with BioMed Central and every scientist can read your work for free of charge

"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime.*
Sir Paul Nurse, Cancer Research UK

Your research papers will be:
• available free of charge to the entire biomedical community
• peer reviewed and published immediately upon acceptance
• cited in PubMed and archived on PubMed Central
• yours — you keep the copyright

Submit your manuscript here:
http://www.biomedcentral.com/info/publishing_adv.asp