Repulsion of Cap-Dependent Translation Attenuates the Transformed Phenotype in Non–Small Cell Lung Cancer Both In vitro and In vivo


Departments of Medicine and Surgery, University of Minnesota; The Research Service, Minneapolis Veterans Affairs Medical Center; and The Biostatistics Core of the University of Minnesota Cancer Center, Minneapolis, Minnesota

Abstract

Aberrant hyperactivation of the cap-dependent protein synthesis apparatus has been documented in a wide range of solid tumors, including epithelial carcinomas, but causal linkage has only been established in breast carcinoma. In this report, we sought to determine if targeted disruption of deregulated cap-dependent translation abrogates tumorigenicity and enhances cell death in non–small cell lung cancer (NSCLC). NSCLC cell lines were stably transfected with either wild-type 4E-BP1 (HA-4E-BP1) or the dominant-active mutant 4E-BP1A37/A46 (HA-TTAA). Transfected NSCLC cells with enhanced translational repression showed pronounced cell death following treatment with gemcitabine. In addition, transfected HA-TTAA and HA-4E-BP1mt proteins suppressed growth in a cloning efficiency assay. NSCLC cells transduced with HA-TTAA also show decreased tumorigenicity in xenograft models. Xenograft tumors expressing HA-TTAA and HA-4E-BP1mt proteins exhibit decreased potential, and mortality have also been associated with increased eIF4E level (26, 27). Furthermore, in transformed mammary cell lines, 4E-BP1 is known to be hyperphosphorylated, resulting in decreased oncogenic role of eIF4E is the reversion of the neoplastic phenotype following overexpression of 4E-BP1 that consequently interferes with eIF4E binding to eIF4G (13, 14). It is believed that when overexpressed, eIF4E exerts its oncogenic effect through the enhanced translation of malignancy-associated proteins (15–17). The level of eIF4E is critical for assembly of the translation initiation complex. In vivo models demonstrate that 4E-BP1 and eIF4E share a common binding motif for their binding is both competitive and mutually exclusive (3). Phosphorylation of 4E-BP1 occurs at a series of threonine and serine residues (T37, T46, S65, T70, S83, and S112) with requisite mTOR-mediated phosphorylation of T37 and T46 occurring before phosphorylation at S65 and T70 (4–6). mTOR thus controls protein translation, which in turn is crucial for proper control of cell growth and cell cycle progression. Two known partners, raptor and GβL, interact with mTOR to promote the activation of the translational activator S6K and the inactivation of 4E-BPs, enhancing protein translation via two independent mechanisms (7).

Overexpression of exogenous eIF4E in immortalized fibroblasts causes malignant transformation (8–10). Additionally, in human mammary epithelial cells, ectopic expression of eIF4E decreases apoptosis, enhances clonogenic potential, and engenders anchor-age-independent growth (10). These effects induced by deregulated cap-dependent translation are abrogated by overexpression of 4E-BP1. Enhanced levels of eIF4E also promote solid tumor formation and cooperate with deregulated c-myc in lymphogenesis in transgenic mice (11, 12). Further evidence for the oncogenic role of eIF4E is the reversion of the neoplastic phenotype following overexpression of 4E-BP1 that consequently interferes with eIF4E binding to eIF4G (13, 14). It is believed that when overexpressed, eIF4E exerts its oncogenic effect through the enhanced translation of malignancy-associated proteins (15–17). The level of eIF4E is critical for assembly of the translation initiation complex eIF4E, and elevated levels are common in a wide variety of human carcinomas (17–25). Cancer recurrence, metastatic potential, and mortality have also been associated with increased eIF4E level (26, 27). Furthermore, in transformed mammary cell lines, 4E-BP1 is known to be hyperphosphorylated, resulting in an increase in the active concentration of eIF4E following disassociation from 4E-BP1 (10). Despite this accumulating body of evidence linking aberrant translational control with malignancy, the only data establishing causality are in a single epithelial neoplasm, human breast cancer (10).

In this study, we sought to determine if translational control is aberrant in lung cancer, and if so, whether targeted disruption of deregulated cap-dependent translation reverses the malignant phenotype. Currently, mTOR inhibitors, such as CCI-779 and RAD001, are undergoing clinical testing in lung cancer and other malignancies (28, 29). Direct inhibition of mTOR has been shown to enhance cis-platinum chemosensitivity in lung cancer cells and could be an effective adjunct to current treatments (30). However, mTOR inhibition may have a variety of effects not limited to inhibition of 4E-BP1 phosphorylation (31). Alternatively, therapies...
directed at 4E-BP1 regulation of the cap-mediated complex, a downstream target of mTOR, could be a novel treatment strategy for non–small-cell lung cancer (NSCLC) and other cancers.

**Materials and Methods**

**NSCLC cell lines and tumors.** NSCLC cell lines H441, H522, H661, H838, H1155, H2009, H2030, H2086, H2122, and H2172 were obtained from either the American Type Culture Collection (Manassas, VA) or from Frederick Kaye (National Cancer Institute or NCI). These cell lines were started from NSCLC tumors at the NCI-Navy Medical Oncology Branch as previously described (32). The medium for H441, H522, H661, H1155, H2009, H2030 H2122, and H2172 was RPMI (Invitrogen/Life Technologies, San Diego, CA), containing 10% calf serum (Biofluids, Rockville, MD). H838 was grown in RPMI supplemented with 10% calf serum, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate. H2086 was grown in ACI-L with 10% calf serum. When necessary, cells were serum starved in the appropriate medium lacking 10% calf serum for 72 hours. Normal human bronchial epithelial (NHBE) cells from patients without cancer (Cambrex Bio Science, Hopkinton, MA) were grown in BEGM (BEBM supplemented with SingleQuots, Cambrex Bio Science) following manufacturer’s instructions. All cells were maintained at 37°C in 5% CO2 NSCLC tumors and paired normal tissues were from patients with stage I or II NSCLC resected at the Minneapolis VA Medical Center and enrolled in the Institutional Review Board–approved tumor banking program.

**Cell lysate preparation.** Adherent cells were trypsinized, collected, centrifuged, washed with cold PBS, centrifuged, and resuspended in five times the pellet volume of freeze-thaw lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 50 mmol/L NaF, 1 mmol/L EDTA, 10 mmol/L tetrasodium pyrophosphate, 1 mmol/L phenylmethylsulfonyl fluoride, 5 mmol/L leupeptin, 2 μg/mL aprotinin, 1 μg/mL pepstatin A, 100 mmol/L Na3VO4, 20 mmol/L β-glycerophosphate]. This cell mixture was next subjected to three consecutive freeze (15 minutes at –80°C)/thaw (2 minutes at 37°C) cycles followed by centrifugation (14,000 rpm, 4°C) for 10 minutes. The cell lysates were removed, and the protein concentration was determined by Bradford assay and stored at –80°C.

**Cell transfection.** NSCLC cell lines H522, H838, H2009, and H2030 were transfected with plasmid pACTAG2, encoding the neomycin resistance gene, pACTAG neo/HA-4E-BP1, or pACTAG neo/HA-TTAA using Lipofectin reagent (Invitrogen/Life Technologies) according to manufacturer’s protocol. The HA–TTAA expressing construct has substituted alanine for threonine at residues 37 and 46, the first two sequentially phosphorylated sites in 4E-BP1, resulting in a dominantly active form of 4E-BP1 incapable of being phosphorylated (5). Cells were plated at ~50% to 70% confluence, and 5 μg of the plasmid DNA were used for transfection. After 72 hours, the medium was replaced with medium containing G-418 (Cellgro; 300 μg/mL). Resistant colonies were collected and propagated.

**Immunoblot analysis.** Protein samples were separated by SDS-PAGE on either straight 15% or an 8% to 15% gradient (cap-affinity assay samples) and then transferred to a polyvinylidene difluoride membrane (Hybond-P, Amersham Biosciences, Piscataway, NJ). The membranes were blocked in 5% nonfat dry milk for 1 hour at room temperature in TBST-Tween 20 (TBST: 0.15 mol/L NaCl, 0.01 mol/L Tris-HCl [pH 7.6], 0.05% Tween 20), rinsed, and incubated for 1 hour at room temperature with the appropriate primary antibody. Whole and portions of blots were probed separately with either rabbit α-PHAS-I (4E-BP1) antibody (Zymed Laboratories, South San Francisco, CA) or a 1:166 dilution to detect 4E-BP1, mouse α-eIF4E antibody (BD Biosciences, San Jose, CA) at 1:500 dilution to detect eIF4E, mouse α-actin (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:1000 dilution, or rat α-HA antibody (Roche, Indianapolis, IN) at a 1:20,000 dilution to detect the tagged HA–4E-BP1 and HA–TTAA proteins, respectively. The blots were washed thrice for 5 minutes in TBST before incubation with the appropriate horseradish peroxidase–labeled secondary antibodies followed by incubation for 1 hour at room temperature and three more washes in TBST. Detection was carried out using Enhanced Chemiluminescence Plus Western Blotting System (Amersham Biosciences) to visualize the bands of interest. The density of protein bands was determined using ImageJ a public domain Java image processing program.

**In vitro cap-affinity assay.** Cap-affinity assay was done as before with minor changes (14): 300 μL (1 μg/μL) of cell lysate were incubated while mixing for 2 hours at 4°C, with 50 μL of suspended (50% mixture) 7-methyl GTP-Sepharose 4B (Amersham Biosciences), to capture eIF4E and its binding partners eIF4G and 4E-BP1. The captured proteins were eluted with 35 μL of elution buffer [25 mmol/L Tris-HCl [pH 7.5], 150 mmol/L KCl] containing 100 μmol/L 7-methylguanosine 5'-triphosphate (Sigma-Aldrich, St. Louis, MO) and prepared for immunoblotting.

**Cloning efficiency assay.** NSCLC cells were transfected with the identical procedure as above except when the resistant colonies were of appropriate size; the plates were fixed in 10% methanol and 5% acetic acid for 10 minutes, stained in 0.1% crystal violet/20% ethanol for 5 minutes, and scored for G418-resistant colonies as previously described (33). The transfections were carried out in triplicate, and ANOVA analysis was carried out to test for significance.

**Enhanced cytotoxicity assays.** Cells were seeded (2 × 104 per well) in six-well plates in the appropriate growth medium. After overnight incubation, the medium was replaced by fresh medium containing gemcitabine HCl (Eli Lilly, Indianapolis, IN: 2′,2′-difluoro-2′-deoxycytidine) at various concentrations. Seventy-two hours later, the cells were washed twice with PBS, trypsinized, collected, and resuspended, and the cell number was determined by counting viable cells after treatment with trypan blue. Each experiment was done in triplicate, and the effect of gemcitabine was determined using a paired t test. Only the total number of cells was reported.

**Xenograft experiment.** H2009 cells (5.66 × 106) stably transfected with either pACTAGneo or pACTAG neo/HA-TTAA were suspended in 1.0 mL of PBS and s.c. injected into the left (pACTAGneo) or right (pACTAG neo/HA-TTAA) flank of 10 nude mice (nu/nu; Harlan, Iowa, IA) as previously described (34). Tumor formation was monitored, and the mice were sacrificed when control tumors were ~1 cm2 in size or 28 days had passed. Tumors were excised and weighed. Identical experiments were carried out with H2030 cells. Xenograft size was analyzed with a t test. Immunoblot analysis confirmed the presence of HA–TTAA protein in the right flank tumors.

**Results**

Cancers employ a variety of strategies to activate cap-dependent translation, including increasing the steady-state levels of eIF4E and eIF4G and hyperphosphorylating 4E-BP1. In NSCLC, we examined the expression level of eIF4E in six NSCLC tumors and matching normal adjacent tissue as controls (Fig. 1A). Expression levels for eIF4E were substantially increased in NSCLC tissues compared with adjacent normal tissues (1.0 ± 0.27 versus 1.67 ± 0.28 relative levels). The increase for these six samples seems similar to that previously shown for lung adenocarcinomas (21). The steady-state eIF4E expression levels in the panel of NSCLC cell lines was determined and compared with the level in NBHE cells (Fig. 1B). Relative eIF4E levels in the NSCLC cell lines were elevated in each cell line, and the composite level was 2-fold enhanced compared with NBHE cells. These results for both NSCLC tissue and cell lines predict an increase in eIF4F assembly.

**Conclusion.** Immunoblot analysis was also done to evaluate eIF4G expression in the panel of NSCLC cell lines (Fig. 1C). In contrast to previous reports in other cancers (10, 35), comparison of the NBHE level of eIF4G was identical to the average of the panel of NSCLC cell lines. It is unlikely that alterations in eIF4G levels significantly affect deregulation of cap-dependent translation in NSCLC.

To further understand the mechanism of aberrant translation, we examined the level of 4E-BP1 isoforms in NSCLC. The steady-state 4E-BP1 levels were found to be substantially greater in NSCLC cells than in NBHE cells (Fig. 2B and C). Only one of the 10 cell lines
exhibited a normal 4E-BP1 level, whereas the remaining nine had >2-fold elevated 4E-BP1 compared with NHBE cells. The average increase for the NSCLC cell lines was 4-fold. For cancer cells that require enhanced protein synthesis, it might be predicted that expression of cap-dependent translation suppressors would remain the same or decrease. However, because inactive 4E-BP1 is hyperphosphorylated whereas the active form is not, it was important to assess the relative levels of the phosphorylated isoforms. Using immunoblot analysis, the band intensities for the α (active, hypophosphorylated), β (inactive, phosphorylated), and γ (inactive, hyperphosphorylated) 4E-BP1 isoforms were measured, and the percentage of the total 4E-BP1 for each isoform was calculated in each cell line (Fig. 2A). The combined results for all 10 NSCLC cell lines were also calculated (Fig. 2D). In NHBE cells, the majority (77.6%) of 4E-BP1 was active and capable of binding to eIF4E and suppressing cap-dependent translation, whereas a minority (22.4%) was inactive. For the panel of NSCLC cell lines, active hypophosphorylated 4E-BP1 decreased an average of 31.9%. These results indicate a significant decrease in the α (active) form of 4E-BP1 in NSCLC compared with normal cells.

Inactivation of 4E-BP1 by hyperphosphorylation could be driven by either constitutive action of kinase pathways or exogenous growth factor stimulation of these same pathways. In normal cells, serum and a host of extracellular stimuli (hormones, growth factors, mitogens, cytokines, or G-protein–coupled receptor agonists) promote 4E-BP1 phosphorylation inducing eIF4F assembly (36). Conversely, nutrient deprivation leads to dephosphorylation of 4E-BP1 and a consequent decrease in cap-dependent translation. To identify whether activation of cap-dependent translation is driven by endogenous activation of signal transduction pathways or exogenous stimulation of receptor tyrosine kinases, we examined if withdrawal of exogenous growth factors was associated with repression of cap-mediated translation. To examine the phosphorylation state of 4E-BP1 in NSCLC in the presence and absence of FCS, proliferating cells were grown in the appropriate medium with or without serum (Fig. 3). 4E-BP1 phosphorylation in H838 and H2030 shifted from phosphorylated to hypophosphorylated isoforms in response to serum withdrawal. In contrast, H522 and H2009 did not change their patterns of 4E-BP1 phosphorylation, apparently insensitive to serum stimulation, suggesting a constitutive mode of 4E-BP1 phosphorylation. These results indicate deregulation of 4E-BP1 phosphorylation in NSCLC may be driven by divergent mechanisms. NSCLC cells, such as H522 and H2009, seem to regulate 4E-BP1 phosphorylation independent of exogenous growth factors, whereas H838 and H2030 are partially dependent on added growth factors. Levels of eIF4E and eIF4G were also examined during serum withdrawal. The amount of each cap-dependent initiation factor did not change significantly in the presence or absence of serum (Fig. 3). As indicated in Fig. 3, the expression of eIF4E in NSCLC may be constitutively up-regulated and have lost sensitivity to serum stimulation. Proteolytic cleavage of eIF4G occurs in cells treated with apoptosis inducers or serum deprivation, which is consistent with the finding that cap-dependent translation also decreases (36). In our panel of NSCLC cells, eIF4G did not decrease in level in response to serum withdrawal consistent with a loss of sensitivity to normal cell signaling that allows cells to continue to translate proteins in an aberrant manner.

Deregulation of protein synthesis is a major component in tumorigenesis, and enhanced expression levels of eIF4E are implicated in NSCLC. To identify if NSCLC cells forced to ectopically express 4E-BP1 activity suppress the transformed phenotype HA–4E-BP1wild or the phosphorylation-defective HA–TTAA protein were expressed at levels sufficient to inhibit eIF4E

Fig. 1. Assessment of eIF4G and eIF4E levels in NSCLC. A, immunoblot analysis showing levels of eIF4E in matched normal (N) and NSCLC tumor tissue (T). B, immunoblot detecting steady-state levels of eIF4E and eIF4G in cultures of NHBE and NSCLC cell lines. Actin represents a loading control. C, relative levels of eIF4E in normal tissue (open columns) compared with NSCLC tissue (filled columns) and relative levels of eIF4E and eIF4G in NHBE cells (open columns) compared with NSCLC cell lines (filled columns).
function. To assess the ability of either HA-4E-BP1wt or HA-TTAA to interfere with assembly of eIF4F initiation complex, a cap analogue capture of eIF4E and its binding partners in cell lysates was employed followed by immunoblot analysis. NSCLC cell lines were stably transfected with an empty vector or the same vector containing either HA-4E-BP1wt or HA-TTAA. The level of exogenous 4E-BP1 was determined in cell lysates for all the cell lines (Fig. 4A, top). Ectopically expressed HA-tagged 4E-BP1 and TTAA migrate more slowly on SDS-PAGE because of the hemagglutinin (HA) tag. The level of the binding partners of eIF4E, eIF4G, and 4E-BP1 eluted from the cap analogue was also determined (Fig. 4A, bottom). The amount of eIF4G normalized to the level of eIF4E bound to the cap analogue is shown for the four NSCLC cell lines stably transfected with empty vector or the same vector containing either HA-4E-BP1wt or HA-TTAA (Fig. 4B). The expression of HA-TTAA in cell lysates was always less than the expression of HA-4E-BP1wt except in cell line H522. This presumably reflects differential tolerance for suppression of cap-dependent translation in the different lung cancer cell lines.

In the cell-free system, the relative level of cap-bound eIF4G in cell lysates signifies the functional potency of eIF4F, whereas the level of cap-captured 4E-BP1 estimates the negative effect on eIF4F assembly. For each NSCLC cell line transfected with empty vector, eIF4G avidly bound to eIF4E consistent with the translationally active state. The level of eIF4E bound to eIF4G for cells transfected with HA-4E-BP1wt and HA-TTAA was normalized to the level of cells transfected with empty vector for each cell line. Expression of ectopic 4E-BP1wt decreased binding of eIF4E to eIF4G in each NSCLC cell line (Fig. 4A). For each cell line, the repression of the association of eIF4E to eIF4G was greater when cells expressed HA-TTAA than when cells expressed the wild-type form of 4E-BP1 (Fig. 4B). The level of 4E-BP1 bound to eIF4E for the cap-affinity assay inversely correlates with the level of eIF4G bound to eIF4E (Fig. 4A). The level of both the mutually exclusive cap-bound proteins eIF4G and 4E-BP1 was assessed and ectopically expressed HA-TTAA diminishes cap-dependent translation.

The malignant potential of NSCLC overexpressing 4E-BP1 activity was assessed using cloning assays. Each of the four NSCLC cell lines was transfected with a neomycin resistance vector bearing HA-4E-BP1wt, the HA-TTAA gene, or empty vector. In all NSCLC ectopically expressing the phosphorylation-defective HA-TTAA, there was an average cell survival of 51.1%. Cell survival was greater for cells expressing the wild-type form of 4E-BP1 with the average cell survival at 81.2% (Fig. 5). Although the suppressive effect was most pronounced in the cells receiving the HA-TTAA gene, an effect was also seen in cells receiving the HA-4E-BP1wt. The difference between all three transfectants was significant ($P < 0.0001$).
It has been reported that repression of either mTOR and/or cap-mediated translation results in enhancement of cell killing by cytotoxic agents (30, 37). Gemcitabine is a deoxycytidine analogue that incorporates into DNA, resulting in cell death. Enhanced cell death for cells expressing HA-TTAA was found with gemcitabine compared with parental cells (Fig. 6). In the absence of gemcitabine treatment, there was little significant difference in proliferation rates of NSCLC cells following the introduction of the HA-TTAA activity. Surprisingly, in the case of the H2030 cell line, there was a statistically significant difference favoring the proliferation of clones containing the HA-TTAA gene ($P = 0.0187$). None of the other three cell lines showed any difference in proliferation at 72 hours in the absence of gemcitabine. Collectively, gemcitabine-induced cell death was enhanced by 30.2% (10 nmol/L), 30.0% (25 nmol/L), and 20.3% (50 nmol/L) when comparing cells expressing HA-TTAA with those with empty vector alone (Fig. 6). H2030 was the most sensitive NSCLC cell line to gemcitabine with an average increased cell death of 45.7% for all three concentrations. For each cell line, the cytotoxicity of gemcitabine at 10 nmol/L was nearly equivalent or greater for cells producing HA-TTAA than the cell killing at 50 nmol/L in the cells producing no ectopic HA-TTAA (Fig. 6). Considering an ANOVA model on the two factors (drug concentration and presence of HA-TTAA), both are significant with $P < 0.0001$. Because the coefficient of absence of HA-TTAA is positive, the presence of HA-TTAA enhances cell killing.

To study the relationship between cap-mediated translation and in vivo tumor formation, NSCLC cells expressing the HA-TTAA or the vector alone were injected into nude mice. Expression of activated 4E-BP1 caused a marked suppression of xenograft growth. H2009 and H2030 cells expressing HA-TTAA produced tumors that were 5.2% and 17.9% the size of parental controls, respectively (Fig. 7A). The lysates from cells not producing (vector alone) or producing HA-TTAA were subjected to immunoblot analysis both before and after implantation into the mice (Fig. 7B). One of the cell lines, H2009, the level of expression of HA-TTAA protein in the xenograft diminished to nearly undetectable levels, suggesting a strong selective pressure in NSCLC to reverse translational repression. Overall, these results show that when aberrant cap-dependent translation in NSCLC cells is abrogated by HA-TTAA expression, tumor growth is drastically inhibited.

**Discussion**

Aberrant translational control has been implicated in the development of many human cancers, including lung cancer (38). The presence of enhanced cap-mediated translation is thought to lead to the translation of a limited subset of critical proteins that establish the transformed phenotype and affect malignant and metastatic potential (39). As such, unregulated cap-mediated translation is an attractive target for cancer therapeutics. One example of this strategy, rapamycin, specifically inhibits mTOR and subsequently prevents phosphorylation of 4E-BP1.

In contrast, we were interested in exploring specific inhibition of cap-dependent translation as mediated by 4E-BP1 phosphorylation, a downstream target of mTOR. To validate cap-mediated translation as a target in NSCLC, as well as gather further information on mechanisms of its activation, we carried out a series of studies identifying 4E-BP1 hyperphosphorylation as a common phenotype in NSCLC. Steady-state levels of 4E-BP1 are increased in NSCLC, and subsequently prevents phosphorylation of 4E-BP1. In contrast, we were interested in exploring specific inhibition of cap-dependent translation as mediated by 4E-BP1 phosphorylation, a downstream target of mTOR. To validate cap-mediated translation as a target in NSCLC, as well as gather further information on mechanisms of its activation, we carried out a series of studies identifying 4E-BP1 hyperphosphorylation as a common phenotype in NSCLC. Steady-state levels of 4E-BP1 were increased in NSCLC cell lines expressing HA-TTAA than the cell killing at 50 nmol/L in the cells producing no ectopic HA-TTAA (Fig. 6). Considering an ANOVA model on the two factors (drug concentration and presence of HA-TTAA), both are significant with $P < 0.0001$. Because the coefficient of absence of HA-TTAA is positive, the presence of HA-TTAA enhances cell killing.

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in some but not all NSCLC cell lines. Activation of eIF4F complex by increased levels of eIF4E and continuous inactivation of 4E-BP1 seems to be a common phenotype for NSCLC. Whether this is the result of disruptions of signaling pathways, tyrosine kinase activation by mutation, or autocrine stimulation is an area for future studies. Alterations in upstream factors that influence mTOR activity control phosphorylation of 4E-BP1 and may intimately link eIF4F assembly and NSCLC. The same should be true of genetic alterations that increase eIF4E activity.

There is likely a direct relationship between oncogenic activation and enhanced translational activity that may be independent from cell proliferation. For example, in ras-transformed fibroblasts, exogenous expression of 4E-BP1 was shown to be proapoptotic, and enhanced cytotoxic induced cell killing (13, 14). Similarly, c-myc- and eIF4E-induced transformation was blocked, and G1 progression was inhibited by a constitutively active form of 4E-BP1 without inhibiting cell growth (40). It is likely that the pathways mediating enhanced translational activity in NSCLC are also

**Fig. 6.** Enhanced susceptibility of NSCLC cells expressing 4E-BP1 to cytotoxic drugs. NSCLC cell lines (H2009, H522, H2030, and H838) either expressing activated HA-TTAA (filled columns) or not (open columns) were treated with the indicated concentration of gemcitabine for 72 hours, and viable cells were counted. Columns, mean of three independent determinations of cell number; bars, SD.

**Fig. 7.** Functionally active 4E-BP1 potently inhibits xenograft tumor growth. A, immunoblot of lysates from cells with (no vector) or without HA-TTAA before and after xenograft growth. Level of ectopic HA-TTAA. B, tumor size for 10 animals from cell lines H2009 and H2030 either expressing or not expressing HA-TTAA. Columns, means; bars, SD.
activated by acquired mutations in signaling pathways. Our studies were carried out in malignant NSCLC cell lines and tumors and cannot adequately address this hypothesis. It is interesting to note, however, that two of the four cell lines studied (H2009 and H838) possess activating mutations in Kras, but only one of these (H2009) seemed to have inactivation of 4E-BP1 independent of exogenous growth factors. An attractive hypothesis to test would be if direct alterations in levels of components of elF-4 complex leads to lung cancer in murine models similar to enforced expression of lung-specific mutant Kras (41).

Overexpression of 4E-BP1 does not lead to apoptosis in nontransformed fibroblasts, making it an attractive target for treatment specifically aimed at malignant cells (14). In light of our results, development of anticancer therapy that effects correction of the aberrant cap-dependent translation in NSCLC is warranted. For example, therapies that mimic phosphorylation-defective elF-4 may contribute to the development of non-small-cell lung cancer and other neoplasms.

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