Research Article

Reduced Eukaryotic Initiation Factor 2Bε-Subunit Expression Suppresses the Transformed Phenotype of Cells Overexpressing the Protein

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Abstract

Eukaryotic initiation factor 2B (eIF2B), a five-subunit guanine nucleotide exchange factor, plays a key role in the regulation of mRNA translation. Expression of its ε-subunit is specifically up-regulated in certain conditions associated with increased cell growth. Therefore, the purpose of the present study was to examine the effect of repressing eIF2Bε expression on growth rate, protein synthesis, and other characteristics of two tumorigenic cell lines that display up-regulated expression of the ε-subunit. Experiments were designed to compare spontaneously transformed fibroblasts to transformed mouse embryonic fibroblasts infected with a lentivirus containing a short hairpin RNA directed against eIF2Bε. Cells expressing the short hairpin RNA displayed a reduction in eIF2Bε abundance to 30% of the value observed in uninfected transformed mouse embryonic fibroblasts, with no change in the expression of any of the other four subunits. The repression of eIF2Bε expression was accompanied by reductions in guanine nucleotide exchange factor activity and global rates of protein synthesis. Moreover, repressed eIF2Bε expression led to marked reductions in cell growth rate in culture, colony formation in soft agar, and tumor progression in nude mice. Similar results were obtained in MCF-7 human breast cancer cells in which eIF2Bε expression was repressed through transient transfection with a small interfering RNA directed against the ε-subunit. Overall, the results support a role for eIF2Bε in the regulation of cell growth and suggest that it might represent a therapeutic target for the treatment of human cancer. [Cancer Res 2008;68(21):8752–60]

Introduction

Translation of mRNA occurs through a series of events that can be grouped into three distinct stages: initiation, elongation, and termination. The majority of the regulatory mechanisms of protein synthesis identified thus far mediate control of initiation of mRNA translation. The reactions that mediate the initiation of mRNA translation can be briefly summarized as follows: an initiator methionyl-tRNA (met-$tRNA_i$) binds to the 40S ribosomal subunit forming the preinitiation complex that binds to the mRNA and localizes to the AUG start codon. The initiation factors associated with the 40S ribosomal complex are then released allowing the 60S ribosomal subunit to join to form the 80S ribosomal complex which proceeds to the elongation phase of translation. Binding of met-$tRNA_i$ to the 40S ribosomal subunit is mediated through eukaryotic initiation factor 2 (eIF2), which is complexed to GTP. The GTP bound to eIF2 is hydrolyzed to GDP when eIF2 is released from the 40S subunit, leaving behind the met-$tRNA_i$ and allowing translation to progress. However, eIF2 must be bound to GTP to proceed to another round of initiation. Under physiologic conditions, eIF2 has a higher affinity for GDP than GTP (1). Consequently, a catalyst is needed to promote the exchange of GDP for GTP. Regeneration of the eIF2•GTP is mediated by the guanine nucleotide exchange factor (GEF), eIF2B. Therefore, the activity of eIF2B is critical for allowing mRNA translation to proceed.

eIF2B is a five-subunit complex consisting of five subunits: eIF2Bα, eIF2Bβ, eIF2Bγ, eIF2Bδ, and eIF2Bε, which are encoded by the genes *EIF2B1, EIF2B2, EIF2B3, EIF2B4*, and *EIF2B5*, respectively. The best characterized mechanism through which eIF2B is regulated involves phosphorylation of the α-subunit of eIF2 on Ser^{51}, an event that converts eIF2 from a substrate into a competitive inhibitor of eIF2B (2). Moreover, the GEF activity of eIF2B is also altered by direct phosphorylation of the ε-subunit of the protein by at least four different protein kinases including casein kinase-I, casein kinase-II, glycogen synthase kinase-3 (3, 4), and the dual-specificity tyrosine-phosphorylation kinase (5).

The ε-subunit of eIF2B is believed to be responsible for the catalytic activity of the complex because it is the only subunit that individually shows activity *in vitro* (6, 7). Although eIF2Bε exhibits some GEF activity when expressed alone, this activity is ~10% of that observed when it is expressed together with at least the β-subunit, γ-subunit, and δ-subunit of eIF2B (7, 8). The α-subunit, β-subunit, and δ-subunit of eIF2B exhibit protein sequence homology, and are believed to sense the phosphorylation state of eIF2α through a poorly defined mechanism (6, 9). The γ-subunit and ε-subunit of eIF2B bind directly to the substrate, eIF2 (10). Furthermore, deletion of the α-subunit of eIF2B prevents eIF2α phosphorylation–mediated inhibition of eIF2B (7, 11).

The role of eIF2B in human cancer is not well understood. However, recent evidence suggests that it may serve as an oncogene. Phosphorylation of eIF2α on Ser^{51} is elevated in mammary carcinoma cell lines compared with nontransformed mammary epithelial cell lines (12). One would expect that phosphorylation of eIF2α would decrease protein synthetic rates via competitive inhibition of eIF2B; however, no such repression has been observed. This suggests that higher activity or expression of eIF2B compensates for the expected repression due to eIF2α Ser^{51} phosphorylation, or that eIF2B has become unresponsive to this inhibitory mechanism. More recently, it has been shown that spontaneously transformed mouse embryonic fibroblasts (MEF) display elevated eIF2B activity relative to genetically matched

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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parental control cells (13). The increase in eIF2B activity leads to an approximate doubling in protein synthesis and cell growth. Unexpectedly, expression of the catalytic ε-subunit of eIF2B, but not the other four subunits, is up-regulated in transformed MEFs (TMEF), a finding that may account for the relative insensitivity of eIF2B activity and protein synthesis to increases in eIF2α phosphorylation. Further evidence linking up-regulated eIF2Bε expression to oncogenesis is provided by studies showing that eIF2Bε mRNA is up-regulated in a variety of tumors compared with normal surrounding tissue, suggesting that part of the transformation process involves loss of regulation of this gene (13). These results suggest that the catalytic subunit of eIF2B, i.e., eIF2Bε, might be a target for cancer therapy.

The present study was designed to examine the contribution of eIF2Bε overexpression to the transformed phenotype of two cell lines, TMEFs and MCF-7 cells. Expression of eIF2Bε was repressed using either lentiviral delivery of a short hairpin RNA (shRNA) or transfection of a small interfering RNA (siRNA) duplex, both specifically targeting eIF2Bε. Knockdown of eIF2Bε expression in transformed cells resulted in a reduction in eIF2B activity, global rates of protein synthesis, and cell growth rates, as well as an impairment in growth in soft agar and nude mice.

Materials and Methods

Reagents and antibodies. The monoclonal antibodies against mouse eIF2Bε, eIF2Bγ, eIF2Bδ, and eIF2Bα were developed in our laboratory as described (7, 14, 15). Antibody against human eIF2Bε was acquired from Santa Cruz Biotechnology. The antiactin antibody and all other reagents were from Sigma, except as listed below.

Cell culture. Spontaneously transformed MEFs, wild-type MEFs (both generous gifts from Dr. Glen Barber, University of Miami, Miami, FL), and MCF7 cells were maintained in high-glucose DMEM (Life Technologies/Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Atlas Biologicals) and 1% (v/v) penicillin/streptomycin (Life Technologies/Invitrogen). Unless otherwise noted, 4×10^5 cells were seeded onto 60 mm dishes and incubated in culture medium overnight before use.

For experiments involving siRNA transfection, 4×10^5 cells were seeded onto 60 mm dishes and grown in culture medium without penicillin/streptomycin overnight. The following day, cells were transfected using DeliverX Plus siRNA transfection kit (Panomics) according to the manufacturer's protocol using 30 nmol/L of siRNA.

Plasmids and virus production. HEK-293T cells (1.0×10^6) were seeded onto 100 mm tissue culture plates and incubated overnight at 37° in 5% CO_2 in the absence of antibiotics. The following day, cells were transfected with 1.5 μg of pCMV-VSVG packaging plasmid, 1.5 μg of pCMV-dR8.2 δvpr, and 3 μg of either the eIF2Bε-shRNA (targeted against sequence 5′-AAG-UGGUGCCAUCCUACGUCC-3′) in the vector pLKO.1 or the empty pLKO.1 vector as a control using Fugene 6 (Roche) as described (16). The following day, the medium was changed in transfected cells. Forty-eight hours post-seeding of cells into plates, target TMEFs were plated in 100 mm dishes and transfected cells were allowed to incubate for another 24 h. The next day, the medium was harvested from transfected HEK-293T cells and was filtered through a 45-μm filter to remove cellular debris. TMEFs were infected by the addition of filtered virus-containing medium (2 mL) followed by incubation for 8 h. Infection medium was then removed and replaced with growth medium and cells were permitted to recover overnight. Stably infected cells were selected with puromycin, and clonal (2Bε-1, 2Bε-2) and a pooled collection of transfected cells (2Bε-3) were selected for expansion.

The mouse eIF2Bε, in the expression plasmid pCMV-Sport6 (M-2Be), was acquired from the I.M.A.G.E. Consortium and was purchased through Invitrogen [MGC:103029 (IMAGE:5342410)].

Plasmid transfection. For experiments involving HEK293 cells, cells (2.5×10^4) were plated into individual wells of six-well dishes. Twenty-four

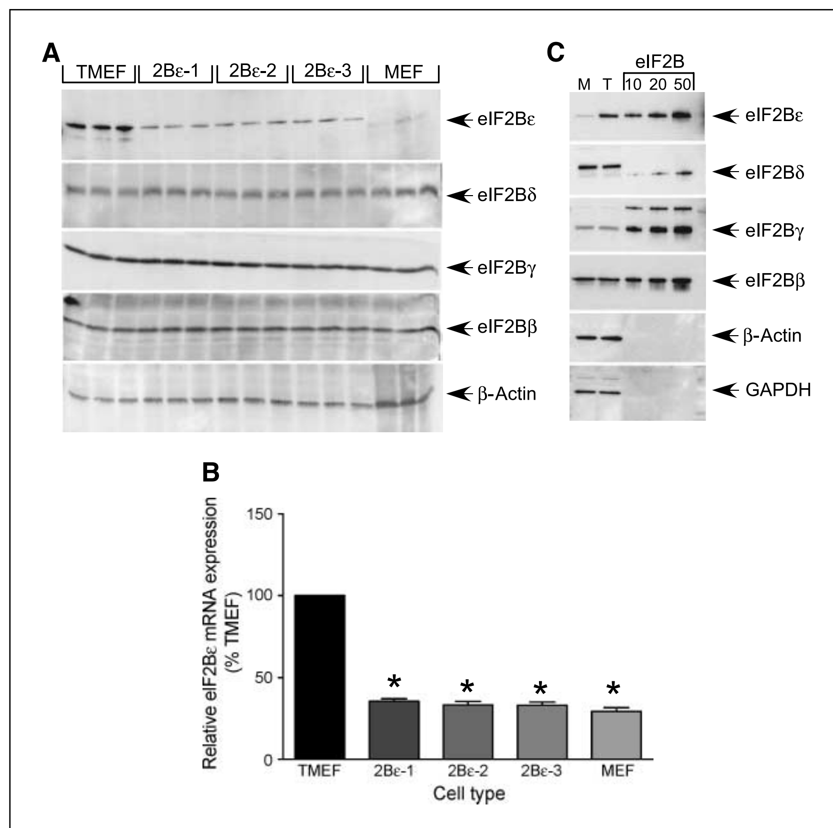

Figure 1. shRNA-induced knockdown of eIF2Bε protein and mRNA expression in TMEFs. TMEFs were infected with either a lentivirus encoding an shRNA against eIF2Bε or a virus not expressing an shRNA (pLKO.1) as a control. Cell lysates were prepared from equal numbers of TMEFs, two clonal isolates of TMEFs infected with virus expressing eIF2Bε shRNA (*2Bε-1* and *2Bε-2*), a pool of several groups of TMEFs expressing eIF2Bε shRNA (*2Bε-3*), and nontransformed MEFs. *A,* protein from the cell lysates was separated by SDS-PAGE and then electrophoretically transferred onto a PVDF membrane and blotted using antibodies against eIF2Bε, eIF2Bδ, eIF2Bγ, eIF2Bβ, or β-actin. Results of a typical experiment; within each experiment, three individual dishes of cells were analyzed. *B,* mRNA was isolated from the lysates using TRIZOL, reverse-transcribed, and analyzed for eIF2Bε and GAPDH mRNA expression using SYBR Green quantitative real-time PCR as described under Materials and Methods. Values for eIF2Bε mRNA were normalized to GAPDH mRNA. *Columns,* mean of three experiments; *bars,* SE (*, $P < 0.01$). *C,* 50 μg of protein from MEF (*M*) and TMEF (*T*) cell lysates were subjected to SDS-PAGE and Western blot analysis using antibodies against eIF2Bε, eIF2Bδ, eIF2Bγ, eIF2Bβ, β-actin, and GAPDH. A series of dilutions of eIF2B purified from rat liver (15) was analyzed in parallel lanes.

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hours later, cells were transfected with 2 μg of either pcDNA3.1 or a plasmid (M-2Be)–expressing mouse eIF2Bε per well via calcium phosphate as described previously (17). Twenty-four hours post-transfection, cells were counted or protein synthesis was measured as described below. For experiments involving MEF cells, cells (2×10^6 of either wild-type MEF or 2Bε-1 shRNA–expressing cells) were transfected with 10 μg of either pcDNA3.1 or M-2Be by electroporation using a protocol optimized for MEF cells by Amaxa. Equal numbers of transfected cells were then seeded into three wells of a six-well dish and maintained in growth medium for 24 h.

Western blotting. Cells were washed twice in ice-cold PBS and then scraped either directly into 1× SDS sample buffer or 4E lysis buffer (20 mmol/L HEPES, 2 mmol/L EGTA, 50 nmol/L NaF, 100 mmol/L KCl, 0.2 mmol/L EDTA, 50 mmol/L β-glycerophosphate, 2.5% Triton X-100, and 0.25% deoxycholate). After scraping into 4E lysis buffer, an aliquot of the lysate was centrifuged at $1{,}000 \times g$ for 3 min at 4°C and a volume of the supernatant was combined with an equal volume of 2× SDS sample buffer. The samples were boiled for 5 min at 95°C and resolved by SDS-PAGE. Proteins were then electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane, incubated with the appropriate primary and secondary antibodies, and visualized using Pierce ECL Western blotting substrate (Pierce) or ECL Plus Western blotting detection system (Amersham Biosciences).

RNA isolation. Total cellular RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol and resuspended in RNA storage solution (Ambion). RNA samples were analyzed for quality using the Agilent 2100 bioanalyzer microfluidics platform (Agilent Biotechnologies) and standard spectrophotometric techniques.

Quantitative real-time PCR. Quantitative real-time PCR was conducted on the RNA samples derived from intact cells. RNA from each sample was converted to cDNA using the SuperScript First-Strand synthesis system for real-time PCR (Invitrogen). The resulting cDNA was assayed to quantify the relative abundance of various mRNA species using the QuantiTect SYBR Green real-time PCR kit (Qiagen) according to the manufacturer's protocol. For assessment of individual mRNA abundance from intact cells, relative expression values were normalized to relative glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression. For analysis of individual mRNA, the data were expressed as the percentage of the total relative mRNA expression compared with controls. The primer sets used were eIF2Bε sense (5′-TCCCCCATCTCCAAGGACC-3′) and antisense (5′-TCGATCAGCGCGA-CATTG-3′), and GAPDH sense (5′-GGGCTGCCTTCTCTTGTGA-3′) and antisense (5′-TGAACTTGCCGTGGGTAGA-3′).

Protein synthesis. Cells were seeded into 60 mm dishes such that they would be 50% confluent the following day. Treated cells were then metabolically labeled for 30 min using 100 μCi/mL [^{35}S]methionine/cysteine followed by preparation of cell lysates in 4E lysis buffer. The cell lysate was applied to absorbent filters and protein was precipitated in the filter with 10% trichloroacetic acid followed by washing thrice with 5% trichloroacetic acid. Filters were then dried and protein was solubilized followed by scintillation counting. The amount of radioactivity incorporated into protein was normalized to overall protein levels in the lysate and is reported as a percentage of the control value. Protein from the cell lysate was also subjected to Western blot analysis using anti-eIF2Bε antibodies as described above.

Soft agar assay. Nontransformed MEFs or TMEFs infected with either a lentivirus encoding an shRNA against eIF2Bε or a control virus not expressing an shRNA were mixed into 0.5 mL of 0.35% agar containing growth medium and layered over a base of 0.5% agar to prevent anchorage-dependent cell growth. Once this layer was solidified, it was overlaid with 1 mL of normal growth medium, which was replaced every 2 days for 14 days. A colony is defined as a cell aggregate larger than 100 μm. Pictures were taken and visible colonies were counted after 14 and 28 days.

eIF2B activity assay. GEF activity of eIF2B in lysates from TMEFs infected with control and eIF2Bε shRNA were measured as previously described (18, 19). Briefly, eIF2 was complexed to [^{3}H]GDP for 10 min. The

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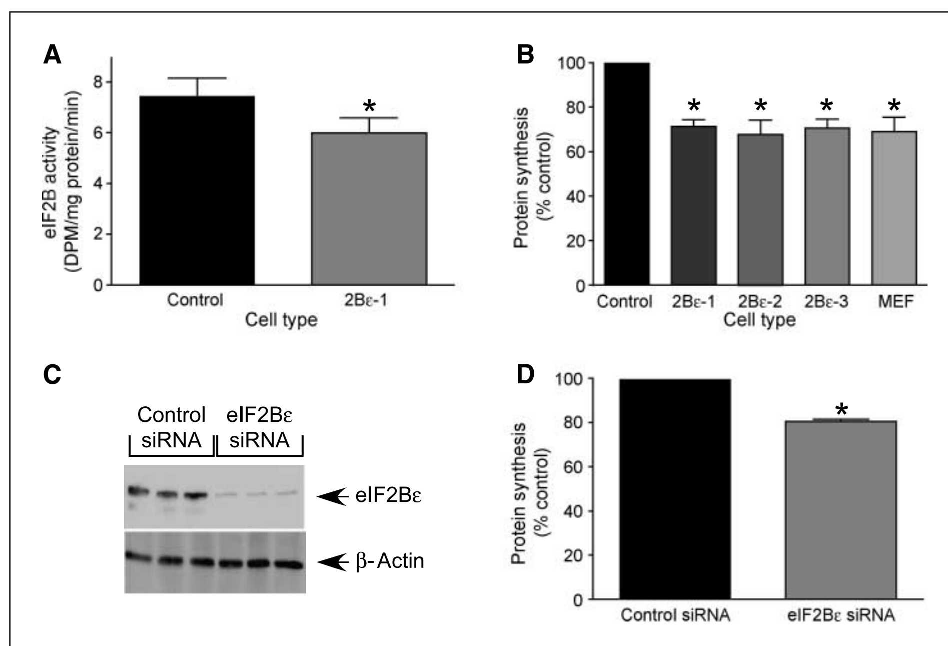

Figure 2. shRNA-mediated and siRNA-mediated knockdown of eIF2Bε expression in TMEFs. Cell lysates were prepared as described in the legend to Fig. 1. *A,* eIF2B GEF activity was measured as described under Materials and Methods. The data is expressed as the rate of exchange of [^{3}H]GDP bound to eIF2 for nonradioactive GDP. *Columns,* mean of three experiments; *bars,* SE (*, $P < 0.05$, compared with TMEFs). *B,* cells were seeded onto 60 mm dishes and the global rates of protein synthesis were measured as described under Materials and Methods. *Columns,* mean values relative to the rate observed in TMEFs; *bars,* SE (*, $P < 0.05$, compared with TMEFs). *C* and *D,* TMEF cells were transfected with either scrambled (*Control*) or eIF2Bε siRNA targeted against a different region of eIF2Bε than that of the shRNA used above. Twenty-four hours later, 100 μCi of [^{35}S]methionine/cysteine was added to the cell culture medium and the cells were returned to the incubator for 30 min. *C,* protein was separated by SDS-PAGE and electrophoretically transferred onto a PVDF membrane and blotted using antibodies against eIF2Bε or β-actin. *D,* global rates of protein synthesis were measured as described under Materials and Methods. *Columns,* mean percentage of control values from siRNA-treated cells of three experiments; *bars,* SE (*, $P < 0.05$, compared with control siRNA).

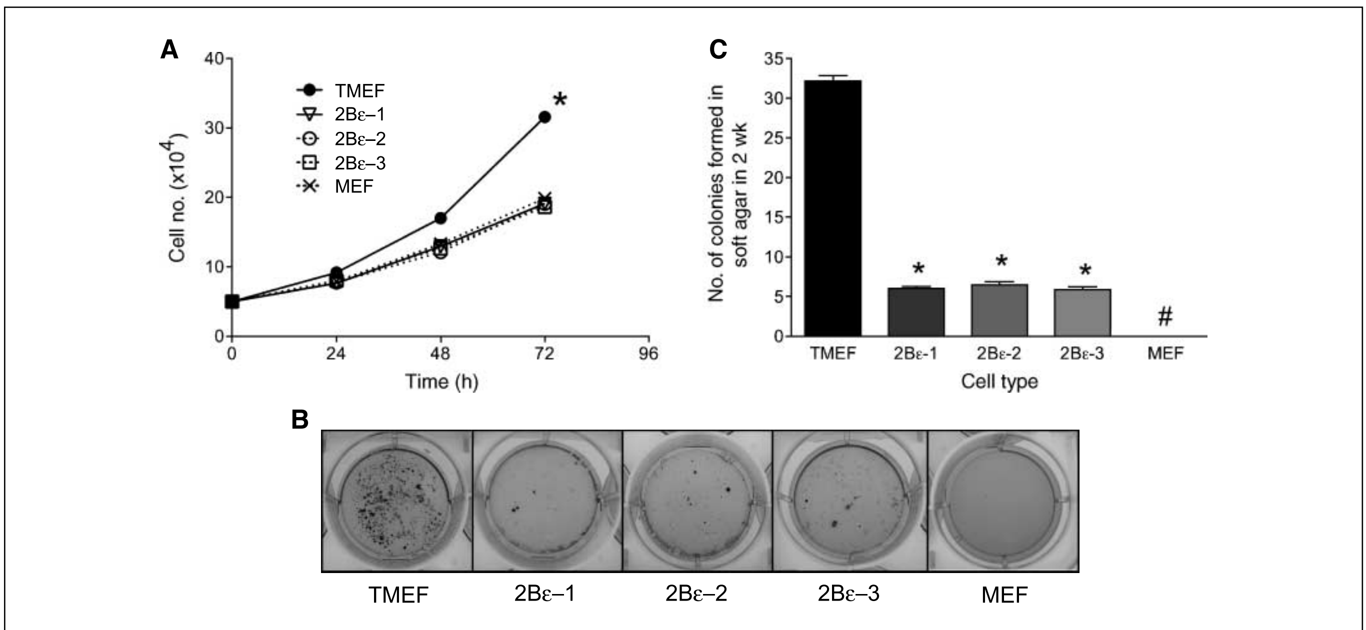

Figure 3. Growth rate and colony formation in soft agar of TMEFs after shRNA-induced knockdown of eIF2Bε expression. *A,* the cells described in the legend to Fig. 1 were seeded onto 100 mm dishes, harvested at the times indicated in the figure, and counted. *Points,* mean of three experiments; *bars,* SE (*, $P < 0.01$, growth rate using ANOVA between cell types). *B,* cells were seeded in soft agar as described under Materials and Methods. Representative results after 2 wk of growth are shown for TMEFs infected with a control virus (*TMEF*), 2Bε-1, 2Bε-2, 2Bε-3, and MEFs. *C,* colonies were counted 2 wk after seeding; *columns,* mean of three experiments; *bars,* SE (*, $P < 0.01$, compared with TMEF; #, no detectable colonies observed).

assay was started with the addition of cell lysate to eIF2-[^{3}H]GDP. Aliquots were taken at 0, 2, 4, and 6 min, and the remaining eIF2-[^{3}H]GDP complex was captured on nitrocellulose filters, and β-radiation was quantified using liquid scintillation counting, with appropriate correction for quench due to the dissolved filters.

Tumor formation in nude mice. The animal protocol used for the studies described herein was reviewed and approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University College of Medicine. Nontransformed MEFs and TMEFs infected with either a virus encoding an shRNA against eIF2Bε or a control virus not expressing an shRNA were dispersed in 200 μL of serum-free DMEM and injected s.c. into nude mice. Tumor progression and size were measured daily using calipers until animal sacrifice when tumors reached 20 mm in width. Tumors were removed from mice by dissection, protein lysates were prepared and subjected to SDS-PAGE, and Western blots for actin and eIF2Bε were performed as described above.

Statistical calculations. One-way ANOVA analysis was carried out in Prism 4 (Graphpad Software) to assess statistical differences among groups. When significant differences were detected by ANOVA, test conditions were compared with controls using a standard *t* test. $P < 0.05$ was considered significant for all comparisons and data are presented as means ± SD.

Results

The TMEF and MCF7 cells used in the present study, as well as many other transformed cell lines and human cancers, display a relative overexpression of eIF2Bε compared with nontransformed cells (13, 20). Therefore, we sought to investigate the contribution of the overexpression of eIF2Bε to the up-regulated growth phenotype of these cells. To accomplish this, a shRNA developed against eIF2Bε was delivered to the cells via a lentiviral delivery system. Clones 2Bε-1 and 2Bε-2, as well as a pooled sample of infected cells (2Bε-3), were then verified for repressed eIF2Bε protein expression via Western blot analysis (Fig. 1*A*) and mRNA expression via real-time PCR (Fig. 1*B*). In agreement with previous studies (13), the expression of eIF2Bε in TMEFs was >10-fold above the level in control MEFs, although the expression of three other subunits of the protein (β, γ, and δ) was similar in TMEFs and control MEFs. Stable expression of shRNA against eIF2Bε in TMEFs reduced expression of the protein to ∼35% of the level in TMEFs infected with a virus not expressing a shRNA. Interestingly, although eIF2Bε mRNA expression was returned to the value observed in control MEFs in response to expression of shRNA (Fig. 1*B*), the protein remained consistently elevated. This finding suggests that both transcriptional and posttranscriptional regulation of eIF2Bε protein expression is involved in the elevation of eIF2Bε expression in TMEFs. The reduction in expression was specific, as there were no observed effects on eIF2Bβ, eIF2Bγ, or eIF2Bδ (Fig. 1*A*). Although eIF2Bε exhibits GEF activity when expressed in the absence of the other four subunits of the protein, its activity is significantly greater when present in the five-subunit holocomplex (7). To assess whether the other eIF2B subunits are present in TMEFs in quantities sufficient to allow the formation of the holocomplex, the amounts of the β, γ, δ, and ε subunits were measured by Western blot analysis. As shown in Fig. 1*C*, the intensity of signal observed for eIF2Bγ and eIF2Bε was similar in both MEF and TMEF cells and equivalent to ∼16 μg of purified eIF2B analyzed on the same blot. Surprisingly, the eIF2Bγ content (∼5 μg) was less than that of either the β- or δ-subunits, but was similar in both cell lines. In MEF and TMEF cells, the eIF2Bε content was equivalent to ∼3 and 14 μg of purified eIF2B, respectively. The results suggest that in MEFs, both the γ- and ε-subunits are present in limiting amounts, and that in TMEFs, the γ-subunit is limiting for the formation of the eIF2B holoprotein.

To assess the effectiveness of reducing eIF2Bε expression on eIF2B function, eIF2B GEF activity was measured in cell lysates. As shown in Fig. 2*A*, knockdown of eIF2Bε expression significantly repressed eIF2B GEF activity to ∼80% of that observed in the

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control cells ($P < 0.05$). Inhibition of eIF2B GEF activity, e.g., by increased phosphorylation of eIF2α, typically results in a decrease in global rates of protein synthesis (21). To determine whether the magnitude of the shRNA-induced reduction in eIF2B activity was sufficient to alter protein synthesis, the cells were subjected to metabolic labeling with [^{35}S]methionine/cysteine, and the rate of incorporation of radiolabel into protein was measured. As shown in Fig. 2*B*, protein synthesis in TMEFs expressing eIF2Bε shRNA was ~70% of the control value ($P < 0.05$), a result consistent with the central role of eIF2B in regulating translation initiation. To confirm that the results obtained using shRNA were specific for eIF2Bε, we also used a siRNA knockdown approach that targeted a different portion of the eIF2Bε mRNA. TMEFs subjected to siRNA knockdown exhibited a reduction in eIF2Bε protein expression to ~30% of the control value ($P < 0.05$; Fig. 2*C*) and a corresponding 25% decrease in protein synthesis ($P < 0.05$; Fig. 2*D*), strongly suggesting that shRNA knockdown of eIF2Bε was specifically responsible for the reduction of global rates of protein synthesis. To further verify that the results observed were not due to off-target effects, we sought to restore the parental phenotype of the TMEFs expressing the eIF2Bε shRNA through exogenous expression of the targeted protein, i.e., eIF2Bε. As shown in Supplemental Fig. S1*A*, transfection of a plasmid-expressing mouse eIF2Bε in the TMEFs already expressing eIF2Bε shRNA resulted in a 4-fold increase in expression of the protein, a value intermediate between the parental cells (TMEF) and the cells expressing the shRNA, but transfected with an empty plasmid (2Bε-1). Similarly, exogenous expression of eIF2Bε in TMEFs expressing the shRNA resulted in a significant increase in protein synthetic rate that was intermediate between that observed in TMEF and 2Bε-1 cells.

Because protein synthesis is an essential component to cell growth and replication, the reduced rate observed in cells expressing eIF2Bε shRNA would be expected to affect cell growth. To determine whether the reduced rate of protein synthesis was sufficient to repress cell growth, equal numbers of TMEFs expressing eIF2Bε shRNA or not expressing an shRNA were seeded into culture dishes and, at 24-hour intervals, the number of cells on each plate was measured. Nontransformed MEFs were used as a control. A shown in Fig. 3*A*, the growth rate of control TMEFs was significantly greater than that of nontransformed MEFs. Moreover, as observed for protein synthesis, knockdown of eIF2Bε resulted in a decrease in growth rate to a value that was indistinguishable from nontransformed MEFs.

The ability to grow in soft agar is a characteristic shared by many transformed cells (22–24). Indeed, as previously reported (13), the TMEFs used in the present study exhibited a large number of colonies 2 weeks after being plated in soft agar (Fig. 3*B*). In contrast, wild-type MEFs failed to form colonies under identical conditions. Expression of eIF2Bε shRNA in the TMEFs was greatly reduced, such that the number of colonies formed in cells expressing eIF2Bε shRNA was only 20% of the value in the control TMEFs (Fig. 3*C*). A possible explanation for the limited colony formation in TMEFs expressing eIF2Bε shRNA was that the reduced rate of cell growth prolonged the time needed for colonies to reach a size that could be visualized. Therefore, incubation of the cultures was extended for another 2 weeks. Although the existing colonies became larger, no new colonies were observed in TMEFs expressing eIF2Bε shRNA (data not shown), demonstrating that the reduction in colony formation was not simply due to decreased cell growth but likely due to at least a partial reversal in transformation.

To obtain further evidence that knockdown of eIF2Bε expression attenuated cell transformation, nude mice were inoculated with either TMEFs infected with a control virus, TMEFs infected with virus expressing eIF2Bε shRNA, or wild-type MEFs, and the mice were examined every 24 hours thereafter for signs of tumor development. Once visible, tumor size was recorded daily. Within 8 days of inoculation, all of the control TMEF-inoculated animals exhibited tumors, and the tumors grew aggressively until animal sacrifice at day 13 (Fig. 4*A*). In contrast, the onset of tumor appearance in the animals inoculated with eIF2Bε-shRNA expressing TMEFs was delayed compared with animals inoculated with control TMEFs, with the first animals having visible tumor formation on day 17. The tumors also grew slower, such that animals did not have to be sacrificed until approximately day 28. Animals inoculated with wild-type MEFs showed no tumor formation during the 28-day experiment. Because tumor formation was not completely ablated by expression of eIF2Bε shRNA, we hypothesized that perhaps a small subset of cells had escaped the shRNA-mediated knockdown of eIF2Bε followed by clonal expansion, thereby allowing the protein to return to the levels present in the control TMEFs. Therefore, at the time of sacrifice, tumors were excised and homogenized, and eIF2Bε protein expression was quantitated by Western blot analysis. As shown in Fig. 4*B*, even after 28 days of growth, the tumors excised from the animals

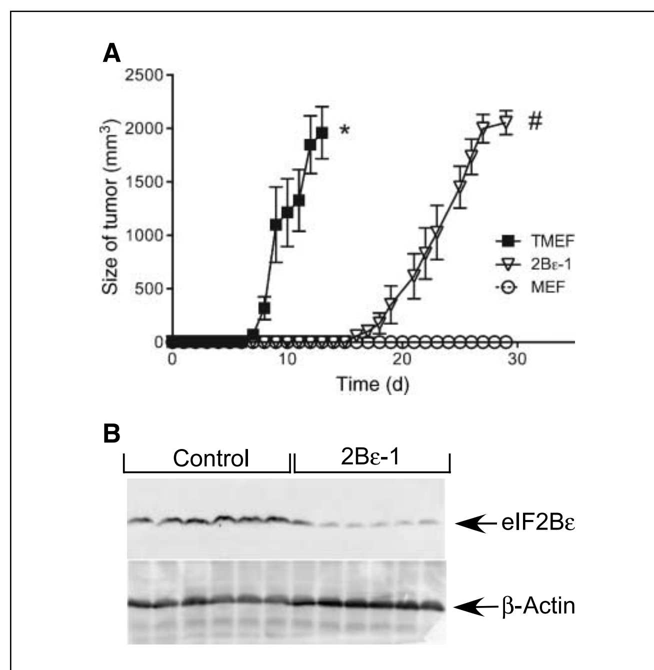

Figure 4. Time of onset and rate of tumor progression in nude mice after shRNA-induced knockdown of eIF2Bε expression in TMEFs. *A,* 4- to 6-week-old nude mice were injected s.c. with 5×10^6 cells of TMEFs infected with either a control virus (*TMEF*), a virus expressing eIF2Bε-shRNA (*2Bε-1*), or noninfected, nontransformed MEFs cells. Tumor size was measured daily from the time of appearance until the animals were sacrificed. *B,* tumors were isolated from mice injected with TMEFs infected with a control virus (*Control*) or with TMEFs infected with a virus expressing eIF2Bε-shRNA (*2Bε-1*) and then homogenized. The homogenates were subjected to Western blot analysis using antibodies against eIF2Bε or β-actin as described under Materials and Methods. The blot shows results for six tumors randomly selected from mice injected with control TMEFs and TMEFs expressing eIF2Bε shRNA. *Points,* mean of two experiments; *bars,* SE; within each experiment, 6 or 10 mice were analyzed per condition, respectively (*, $P < 0.01$, compared with 2Bε-1 or MEFs; #, $P < 0.01$, compared with MEFs).

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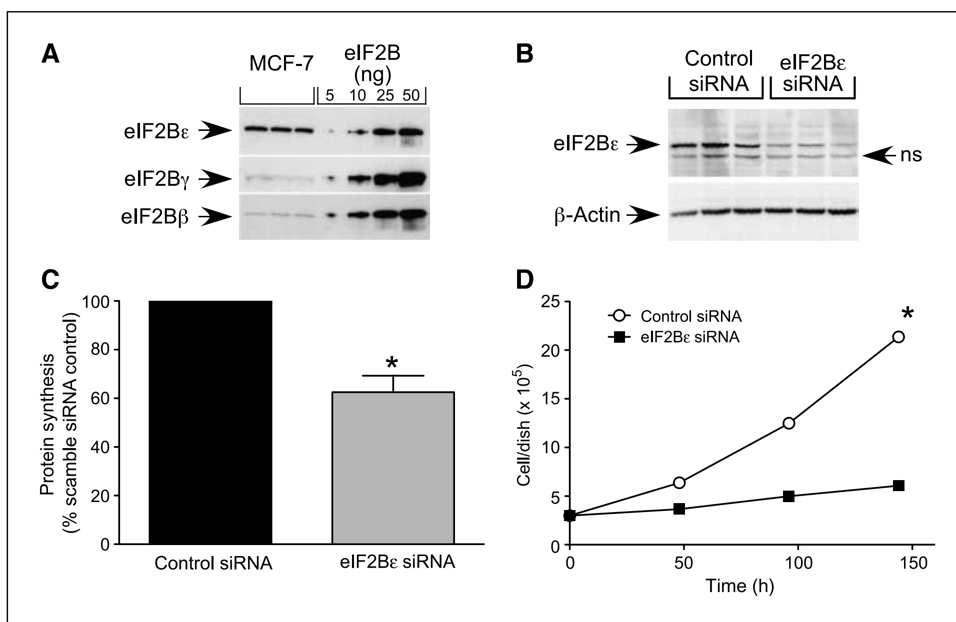

Figure 5. Protein synthesis and cell growth rate after siRNA-induced knockdown of eIF2Bε expression in MCF-7 cells. *A,* MCF-7 homogenates from three dishes of cells and eIF2B purified from rat liver were subjected to Western blot analysis using antibodies against eIF2Bε, eIF2Bγ, and eIF2Bβδ. A series of dilutions of eIF2B purified from rat liver (15) was electrophoresed in parallel lanes. *B* to *D,* MCF-7 cells were transfected with either scrambled (*Control*) or eIF2Bε siRNA. Twenty-four hours later, cells were incubated with 100 μCi of [^{35}S]methionine/cysteine in normal growth medium for 30 min. Cell lysates were prepared and (*B*) protein was separated by SDS-PAGE and subjected to Western blot analysis using antibodies against eIF2Bε or β-actin (*ns*, nonspecific) and (*C*) the amount of radioactivity incorporated into protein was measured by scintillation counting. *Columns,* mean percentage of the control value for three experiments; *bars,* SE (*, $P < 0.01$, compared with control). *D,* cells were transfected with the siRNAs described above, and 3×10^5 cells were seeded onto 60 mm dishes. Cells were harvested at the times indicated in the figure and counted. *Points,* mean of three experiments; *bars,* SE (*, $P < 0.01$, compared with cells transfected with the scrambled siRNA).

inoculated with eIF2Bε-shRNA–expressing cells still had reduced levels of eIF2Bε protein compared with levels observed in tumors from mice inoculated with control TMEFs.

To confirm the findings shown above using TMEFs, the effect of repressing eIF2Bε expression on cell growth was examined in another cell line that specifically overexpresses eIF2Bε. As shown in Fig. 5*A*, MCF7 cells exhibit a specific elevation of eIF2Bε protein compared with eIF2Bβ and eIF2Bγ. To repress eIF2Bε expression, MCF7 cells were transiently transfected with the same siRNA targeting the protein as was used in the studies shown in Fig. 2*C* and *D*. Similar to the results obtained in TMEFs, eIF2Bε expression was reduced in cells transfected with eIF2Bε siRNA to <40% of the level observed in cells transfected with a control siRNA (Fig. 5*B*). Moreover, global rates of protein synthesis in cells transfected with eIF2Bε siRNA were reduced to ~60% of the value observed in cells transfected with the control siRNA ($P < 0.01$; Fig. 5*C*). Similar to that observed for TMEFs, the decrease in eIF2Bε expression and protein synthesis engendered by eIF2Bε siRNA was sufficient to significantly reduce the rate of cell growth in MCF7 cells such that 150 hours after transfection, cell number was reduced to <30% of the control value ($P < 0.01$; Fig. 5*D*).

The studies described above suggest that overexpression of eIF2Bε in TMEFs is largely responsible for the increased rates of protein synthesis and cell growth. This idea was further explored by exogenous expression of the protein in HEK293 cells. As shown in Fig. 6*A*, HEK293 cells transfected with a plasmid expressing mouse eIF2Bε exhibited an ~5-fold increase in expression of the protein. Moreover, cells exogenously expressing eIF2Bε displayed both increased rates of protein synthesis (Fig. 6*B*) and growth (Fig. 6*D*). Overall, the results suggest that up-regulated expression of eIF2Bε is sufficient to increase cell growth rates and transformation.

Discussion

There are at least two steps in translation initiation that can be rate controlling, the binding of met-tRNA$_i$ and the binding of mRNA to the 40S ribosomal subunit. Increased expression of translation initiation factors that participate in either step has been linked to tumorigenesis. For example, the mRNA cap binding protein, eIF4E, is a potent oncogene whose expression is up-regulated in a variety of tumors including breast, head and neck, colon, cervix, and lung (reviewed in refs. 25, 26). Moreover, exogenous overexpression of eIF4E in cells in culture leads to cellular transformation (27–30). The importance of the mRNA binding step in the transformation process is highlighted by the observation that expression of eIF4A and eIF4G, proteins that function in complex with eIF4E to mediate the binding of mRNA to the 40S ribosomal subunit, is also increased in certain types of cancer (31–33), and exogenous expression of eIF4G leads to malignant transformation (34). The finding that exogenous expression of the eIF4E binding protein 1 (4E-BP1), a protein that binds to eIF4E and prevents it from assembling into the eIF4F complex with eIF4A and eIF4G, reverses the transformed phenotype of cells overexpressing eIF4E (35, 36), suggesting that pharmaceutical interventions that target the mRNA binding step in initiation may be beneficial in treating tumors that exhibit up-regulation of proteins that mediate this step in initiation. This idea is supported by the results of a more recent study showing that antisense oligonucleotide-mediated knockdown of eIF4E is also effective in repressing tumor growth (37).

Modulation of the met-tRNA$_i$ binding step in translation initiation has also been linked to tumorigenesis. In this regard, up-regulated expression of eIF2α has been identified in mammary tumors (38) and non–Hodgkin's lymphoma (39) as well as in cells

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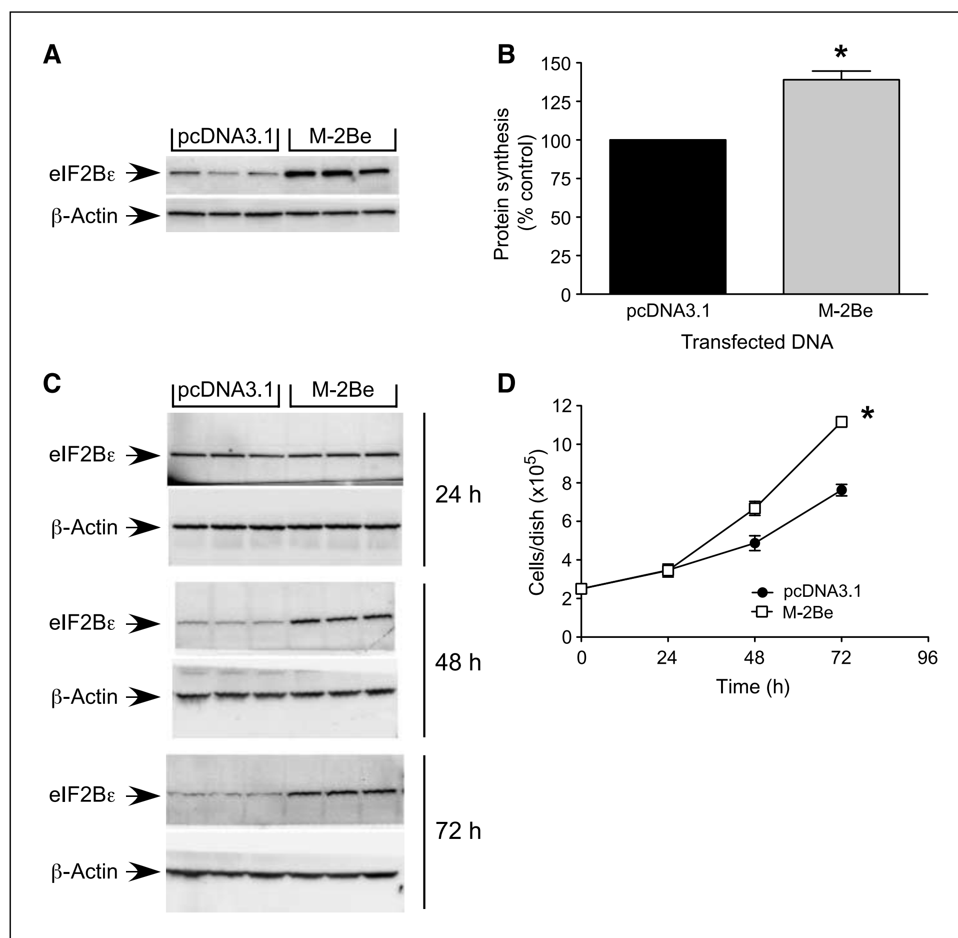

Figure 6. Protein synthesis and cell growth in HEK293 cell overexpression of eIF2Bε. HEK293 cells were transfected with either pcDNA3.1 (control) or a plasmid expressing mouse eIF2Bε (*M-2Be*). Twenty-four hours later, cells were incubated with 100 μCi of [^{35}S]methionine/cysteine in normal growth medium for 30 min. Cell lysates were prepared and (*A*) protein was separated by SDS-PAGE and subjected to Western blot analysis using antibodies against eIF2Bε or β-actin and (*B*) the amount of radioactivity incorporated into protein was measured by scintillation counting. *Columns,* mean percentage of the control value for three experiments; *bars,* SE (*, $P < 0.01$, compared with control using standard *t* test). *C* and *D,* HEK293 cells (2×10^5) were seeded onto six-well dishes and subsequently transfected with either control or mouse eIF2Bε–containing plasmid M-2Be. Cells were harvested at the times indicated and counted. Cell lysates were prepared and protein was separated by SDS-PAGE followed by Western blot analysis using antibodies against eIF2Bε or β-actin. *Points,* mean of three experiments; *bars,* SE (*, $P < 0.01$, growth rate compared with cells transfected with pcDNA3.1).

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transformed by myc, src, or abl (40, 41). Other studies have shown that the IFN-regulated eIF2α kinase, protein kinase R, acts as a tumor suppressor (42–44) and that exogenous expression of an eIF2α variant that cannot be phosphorylated on Ser^{51} results in transformation (42). In addition, exogenous expression of p58, a protein that acts to repress protein kinase R function, leads to transformation (45). Collectively, the available evidence suggests that increases in the availability of the met-tRNA$_i$ binding protein, eIF2, enhances the transformation process, whereas inhibition of eIF2 function, e.g., by up-regulating eIF2α phosphorylation on Ser^{51}, attenuates the process.

A more recent study (13) suggests that increased expression of eIF2Bε also leads to transformation and tumorigenesis. Thus, eIF2Bε expression is specifically up-regulated in a variety of transformed cell lines compared with nontransformed controls. eIF2B GEF activity is also higher in TMEFs compared with control MEFs, and, in comparison with control MEFs, the activity is relatively insensitive to inhibition by eIF2α phosphorylation. Furthermore, expression of eIF2Bε mRNA is up-regulated in a variety of human tumors, including a high percentage of those isolated from ovary, cervix, stomach, lung, and testis (13).

In the present study, the importance of eIF2Bε overexpression in the transformation process was examined using shRNA and siRNA approaches targeted against eIF2Bε to knock down its expression in two different cell lines that overexpress the protein, TMEFs and MCF7 cells. The results show that in TMEFs, knockdown of eIF2Bε expression leads to a reduction in eIF2B activity and overall protein synthesis. Moreover, knockdown of eIF2Bε expression reduces cell growth and proliferation to levels similar to those observed for the nontransformed parental MEF cell line. More importantly, eIF2Bε knockdown seems to impede the ability of transformed cells to form tumors, as shown by the studies examining growth in soft agar and tumor formation in nude mice, and the tumors that do form grow more slowly. The findings are not unique to TMEFs. Indeed, siRNA-mediated reduction of eIF2Bε expression in MCF7 cells similarly resulted in reductions in eIF2B GEF activity, protein synthesis, and cell growth rate.

The mechanism by which eIF2Bε overexpression promotes tumorigenesis is unknown, but because the only known function of eIF2B is to promote guanine nucleotide exchange on eIF2, it probably involves changes in mRNA translation. Thus, the increase in global rates of protein synthesis observed in cells that overexpress eIF2Bε is almost certainly involved in the enhanced growth rate. It is also possible that increased eIF2B activity up-regulates the translation of mRNAs encoding certain proteins that mediate the transformation process and/or that overexpression of eIF2Bε allows for continued translation of mRNAs encoding specific proteins that would normally be repressed under conditions that promote eIF2α phosphorylation. For example, in nontransformed cells, phosphorylation of eIF2α leads to repressed global rates of mRNA translation, but up-regulated translation of mRNAs encoding proteins such as ATF4 (46) and ATF5 (47) which function to adapt the cell to the stress that induced the phosphorylation. If the stress is not relieved, apoptosis ensues. In

cells in which eIF2Bε expression is specifically up-regulated, global rates of protein synthesis would not be repressed to the same extent as in control cells, and induction of translation of mRNAs such as that encoding ATF4 and ATF5 would also be blunted. It should also be noted that, in contrast to most GEFs that are typically single subunit proteins (48–50), eIF2B is relatively large and is comprised of five dissimilar subunits. Thus, a possible role for eIF2Bε distinct from mRNA translation cannot be discounted.

Overall, the data presented herein strongly suggest that down-regulation of eIF2Bε expression reduces cell growth rate. Whether lowering the expression of the protein leads to a complete reversal of the transformation process is equivocal. For example, the finding that reducing eIF2Bε expression in TMEFs dramatically decreases the number of colonies formed in soft agar suggests that the transformed phenotype is at least partially reversed in such cells. Although unproven, it is tempting to speculate that the incomplete reversal of the transformed phenotype is due to a failure to completely repress eIF2Bε expression in TMEFs to the level of control MEFs. A similar explanation could be proposed for the finding that, although the rate of appearance and growth of tumors in nude mice was slowed, tumors eventually formed in mice injected with TMEFs expressing eIF2Bε shRNA. Regardless of whether decreasing eIF2Bε by itself is sufficient to completely reverse the transformed phenotype, the results of the present study suggest that, at least in tumors that specifically overexpress eIF2Bε, the protein represents an unexplored target for therapeutic intervention to control tumor growth.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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