Control of eIF4E cellular localization by eIF4E-binding proteins, 4E-BPs

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ABSTRACT

Eukaryotic initiation factor (eIF) 4E, the mRNA 5′-cap-binding protein, mediates the association of eIF4F with the mRNA 5′-cap structure to stimulate cap-dependent translation initiation in the cytoplasm. The assembly of eIF4E into the eIF4F complex is negatively regulated through a family of repressor proteins, called the eIF4E-binding proteins (4E-BPs). eIF4E is also present in the nucleus, where it is thought to stimulate nuclear-cytoplasmic transport of certain mRNAs. eIF4E is transported to the nucleus via its interaction with 4E-T (4E-transporter), but it is unclear how it is retained in the nucleus. Here we show that a sizable fraction (~30%) of 4E-BP1 is localized to the nucleus, where it binds eIF4E. In mouse embryo fibroblasts (MEFs) subjected to serum starvation and/or rapamycin treatment, nuclear 4E-BPs sequester eIF4E in the nucleus. A dramatic loss of nuclear 4E-BP1 occurs in c-Ha-Ras–expressing MEFs, which fail to show starvation-induced nuclear accumulation of eIF4E. Therefore, 4E-BP1 is a regulator of eIF4E cellular localization.

Keywords: extracellular stimuli; intracellular localization; mRNA translation control; mTor signaling

INTRODUCTION

Cap-dependent translation initiation entails the recruitment of the 40S small ribosomal subunits (and associated factors) to the 5′ end of the mRNAs (Hershey and Merrick 2000; Pestova et al. 2007). In this process, the mRNA 5′-cap structure, m^7GpppN (where N is any nucleotide), is recognized by eukaryotic initiation factor (eIF) 4E—one of the subunits of the eIF4F complex. The eIF4F complex also contains eIF4A, an ATP-dependent RNA helicase, which is thought to unwind secondary structure present at the 5′ end of the mRNA, and eIF4G (Rozen et al. 1990), a large scaffolding protein that binds to eIF4E, eIF4A, PABP, and eIF3, and consequently bridges the ribosome and the mRNA (Gingras et al. 1999b). eIF4E is the limiting factor in translation initiation under most circumstances and is an important effector of cellular proliferation, survival, and malignant transformation (Gingras et al. 1999a; Mamane et al. 2004; Graff et al. 2007).

The activity of eIF4E is regulated by a family of translational suppressors called the 4E-binding proteins (4E-BPs), which in mammals consists of three members: 4E-BP1, 4E-BP2, and 4E-BP3. 4E-BP1 and 4E-BP2 are expressed in most tissues, whereas 4E-BP3 exhibits a more restricted expression pattern (Poulin et al. 1998; Tsukiyama-Kohara et al. 2001). Binding of the 4E-BPs to eIF4E is controlled by the phosphorylation status of 4E-BPs (Pause et al. 1994; Gingras et al. 1998). The hypophosphorylated forms of 4E-BP bind to eIF4E and prevent interaction of eIF4E with eIF4G, thus impairing cap-dependent translation (Haghighat et al. 1995). Conversely, in nutrient- or serum-stimulated cells, 4E-BPs become hyperphosphorylated, releasing eIF4E for interaction with eIF4G and assembly into the eIF4F complex, resulting in enhanced translation.

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Abbreviations: 4E-BPs, eIF4E binding proteins; eIF, eukaryotic [translation] initiation factor; c-Ha-Ras, cellular Harvey rat sarcoma viral oncogene; DKO, [4E-BP1/4E-BP2] double knock-out; HA, hemagglutinin epitope; MEF, mouse embryonic fibroblast; mTOR, mammalian target of rapamycin.

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The best-characterized 4E-BP is 4E-BP1, which contains six known proline-directed Ser/Thr phosphorylation sites, among which at least two sites are phosphorylated directly by mTOR (mammalian target of rapamycin) (Mothe-Satney et al. 2000; Gingras et al. 2001; Wang et al. 2003). mTOR is a phylogenetically conserved Ser/Thr kinase that regulates cell growth and metabolism in response to diverse extracellular and intracellular cues. Growth factors and hormones (insulin/IGF), nutrients (amino acids/glucose), and high ATP/AMP ratio activate mTOR, resulting in hyperphosphorylation of 4E-BP1 (Fingar and Blenis 2004; Tokunaga et al. 2004; Wullschleger et al. 2006). Rapamycin, an inhibitor of mTOR, impairs the phosphorylation of 4E-BP1 (Lin et al. 1995; Beretta et al. 1996).

While eIF4E is predominantly cytoplasmic, in mammalian cells and in yeast, a significant fraction (12%–33% in mammalian) is localized to the nucleus at steady-state levels as determined by biochemical fractionation studies and immunofluorescence analysis using several antibodies (Lejbkowicz et al. 1992; Lang et al. 1994; Dostie et al. 2000a). In the nucleus, eIF4E colocalizes with splicing factors in speckles (Lejbkowicz et al. 1992; Dostie et al. 2000a, b). The nuclear import of eIF4E is mediated by 4E-T (eIF4E-transporter), which binds to eIF4E through a conserved binding motif shared with 4E-BPs and eIF4G, and simultaneously interacts with nuclear import receptors, importin α/β (Dostie et al. 2000a). While the role of eIF4E in the nucleus has not been as extensively studied as its cytoplasmic role, it is known to promote the nuclear export of a subset of mRNAs (Rousseau et al. 1996; Topisirovic et al. 2003). How the steady-state pool of nuclear eIF4E is maintained and regulated is not clear.

Biochemical fractionation experiments have previously led to the conclusion that 4E-BP1 localization is restricted to the cytoplasm (Kim and Chen 2000; Kleijn et al. 2002; Zhang et al. 2002). However, in these studies the possibility that 4E-BP1 leaked from the nucleus during the fractionation procedures due to its small molecular mass was not addressed. Studies were done on the localization of 4E-BP by immunofluorescence and immunohistocytochemistry, but they did not include the use of 4E-BP knock-out or knocked-down cells (Castellvi et al. 2006). Therefore, the question of whether 4E-BP1 localizes to the nucleus under physiological conditions still remains unresolved. Here, using a monoclonal antibody to 4E-BP1, we show that a sizeable fraction (~30%) of endogenous 4E-BP1 is localized to the nucleus by immunofluorescence. Importantly, we demonstrate that 4E-BP1 regulates the subcellular localization of eIF4E under stress conditions. The 4E-BP1–dependent regulation of eIF4E localization becomes conspicuous upon serum starvation or rapamycin treatment and correlates well with the dephosphorylation of 4E-BP1 and its increased binding to eIF4E. We also show that 4E-BP1, and consequently eIF4E, can no longer localize to the nucleus in MEFs that express the c-Ha-Ras protein.

RESULTS

4E-BP1 is present in the nuclei of cultured cells and adult mouse tissues

To study the localization of 4E-BP1 in cells, a rabbit monoclonal 4E-BP1 antibody (Cell Signaling Technology; clone 53H11) was used in immunofluorescence studies. We first assessed the specificity of this antibody by Western blot analysis of total protein lysates from wild-type, 4E-BP1$^{-/-}$, 4E-BP2$^{-/-}$, and DKO (4E-BP1$^{-/-}$/4E-BP2$^{-/-}$) MEFs. The antibody recognized specifically 4E-BP1, as it did not interact with proteins from 4E-BP1$^{-/-}$ MEFs (Fig. 1A, cf. lanes 1 and 2). A comparison of extracts from wild-type and 4E-BP2$^{-/-}$ MEFs demonstrates that this anti-4E-BP1 antibody does not cross-react even with the closest related protein, 4E-BP2 (Fig. 1A, cf. lanes 1,3 and lane 2). Both wild-type and DKO MEFs were stained with this antibody and subjected to analysis by flow cytometry to measure relative fluorescence intensity (Fig. 1B). When the primary antibody was omitted, both wild-type and DKO MEFs exhibited very similar low levels of fluorescence intensity; however, when the 4E-BP1 antibody was included, wild-type and DKO MEFs exhibit more than a 10-fold difference in mean fluorescence intensity, representing a 4E-BP1–dependent retention of the Alexa 488-conjugated secondary antibody in MEFs (Fig. 1B). These data clearly demonstrate that the anti-4E-BP1 rabbit monoclonal antibody specifically recognizes 4E-BP1 under conditions used in the subsequent immunostaining experiments. Indeed, when immunostained cells were scanned by confocal microscopy, a very strong signal was detected in wild-type but not DKO cells (Fig. 1C). Strikingly, a prominent nuclear staining of 4E-BP1 was observed. The nuclear localization of 4E-BP1 is not restricted to MEFs, as immunostaining with this antibody yielded a nuclear signal in a variety of cell types, including NIH/3T3, Rat1A, COS-7, and HeLa S3 (data not shown).

To determine the relative concentration of 4E-BP1 in the nucleus versus the cytoplasm, the signal intensity was quantified in MEFs using confocal microscopy. Twenty immunostained cells were randomly chosen for scanning, and no significant difference from cell to cell was observed. Each cell was scanned using a Z section with a disk thickness of 0.65 μm. This manipulation produced 12–16 continuous image slices from the dorsal to the basal facade of every scanned cell. Using ImageJ (National Institutes of Health), the pixel intensity from each slice was measured and summed to represent the immunofluorescence of a cell. The intensity of 4E-BP1 nuclear fluorescence is ~5.5-fold greater than that in the cytoplasm. Because the volume of the cytoplasm is ~10 times larger than that of the nucleus in MEFs, the amount of 4E-BP1 in the nucleus is estimated to be ~30% of the total protein.

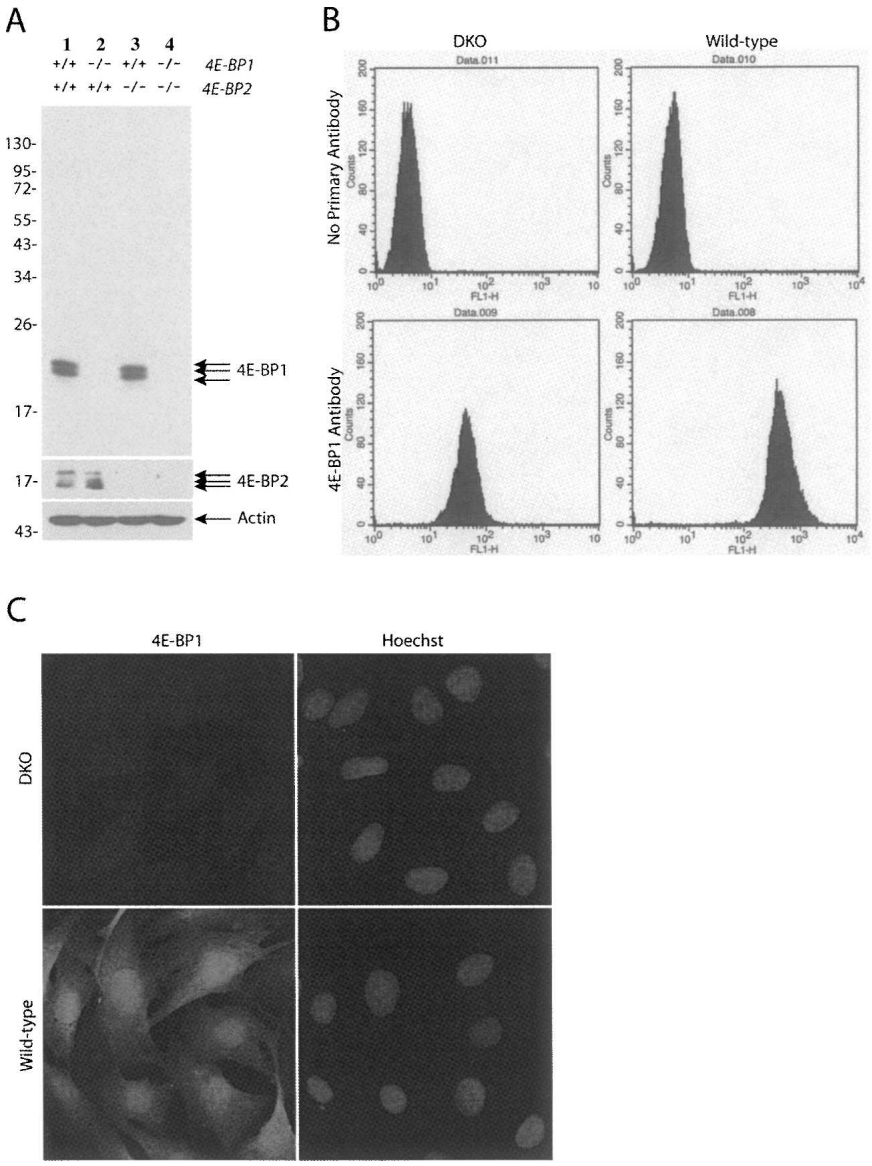

FIGURE 1. Characterization of anti-4E-BP1 rabbit monoclonal antibody (clone 53H11). (*A*) Western blot analysis of lysates from wild-type, 4E-BP1$^{-/-}$, 4E-BP2$^{-/-}$, and DKO (4E-BP1$^{-/-}$/4E-BP2$^{-/-}$) MEFs, using anti-4E-BP1 and anti-4E-BP2 antibodies. (*B*) Flow cytometric analysis of wild-type and DKO MEFs subjected to immunostaining with anti-4E-BP1 antibody. (*C*) Immunofluorescent detection of 4E-BP1 in wild-type and DKO MEFs. 4E-BP1 was stained by anti-4E-BP1 and AlexaFluor488 conjugated secondary antibody.

To rule out the possibility that the presence of 4E-BP1 in the nucleus is caused by fixation or other manipulations of the cells, living cells transiently expressing 4E-BP1-CFP fusion protein were subjected to photobleaching assays (Fig. 2A, a representative experiment). Quantification of the average nuclear fluorescent intensity following photobleaching is shown in Figure 2B. After bleaching of the nucleus for 5 sec, total recovery of the nuclear fluorescence was observed within 300 sec (Fig. 2B). These results demonstrate that 4E-BP1 localizes to the nucleus in vivo and that the nuclear import of 4E-BP1 is a rapid process.

To verify that the observed nuclear localization of 4E-BP1 occurs in tissues, immunohistochemical analysis of multiple murine tissues was performed. 4E-BP1$^{-/-}$ mouse tissues were used as a negative control. 4E-BP1 staining was observed in kidney (Fig. 3), adipose tissue, colon, liver, heart, lung, muscle, ovary, pancreas, and spleen (data not shown). Higher magnification images reveal that 4E-BP1 is present in the nuclei and cytoplasm of adult mouse kidney (Fig. 3B).

Localization of 4E-BP1 to the nucleus is independent of phosphorylation or ability to bind eIF4E

Phosphorylation of 4E-BP1 occurs on several sites (Thr37/46, Ser65, Thr70) in response to various intrinsic and environmental cues (Gingras et al. 1999a). To examine whether phosphorylation of 4E-BP1 affects its localization, we employed various conditions to alter the phosphorylation status of the protein. The conditions that induced 4E-BP1 dephosphorylation included serum starvation or rapamycin treatment for 24 h, or serum starvation for 24 h followed by rapamycin treatment for 1 h. None of these conditions resulted in a noticeable redistribution of 4E-BP1 in MEFs (Fig. 4A), NIH/3T3, Rat1A, COS-7, or HeLa S3 cells (data not shown). Serum-deprivation and rapamycin treatment each decreased the phosphorylation of 4E-BP1, while the combination of serum starvation followed by rapamycin treatment most dramatically reduced 4E-BP1 phosphorylation (Fig. 4B). Consistent with these data, inhibition of 4E-BP1 phosphorylation by PI3K inhibitors such as Wortmannin and LY294002 (Sigma) or by knockdown of mTOR using a commercially available siRNA (Cell Signaling Technology, no. 6381) failed to alter 4E-BP1 subcellular localization (data not shown).

To study whether the association of 4E-BP1 with eIF4E affects 4E-BP1 localization, DKO MEFs were infected with retroviruses expressing HA-4E-BP1 or the mutant proteins, 4E-BP1(4Ala) or 4E-BP1(Δ4EBS). 4E-BP1(4Ala) constitutively binds to eIF4E (data not shown) because four of its Ser/Thr-Pro phosphorylation sites have been mutated to

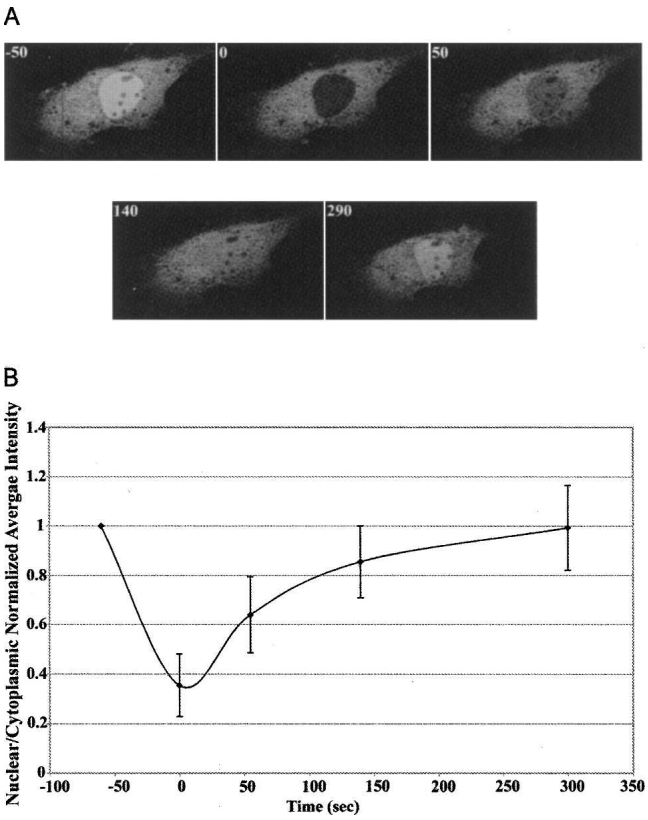

FIGURE 2. Nuclear localization of 4E-BP1 in living cells. MEFs transiently transfected with pcDNA3-4E-BP1-CFP were subjected to selective photobleaching of the nucleus and fluorescence recovery was monitored. (*A*) The nuclear area was photobleached for 5 sec, recovery of fluorescence was monitored continuously, and pictures were taken at the indicated time intervals over a 300- to 600-sec period. (*B*) The average ratio of nuclear to cytoplasmic pixel intensity during the process of recovering from photobleaching. The results are the average of readings taken from seven different cells. Error bars represent standard deviation.

alanines. 4E-BP1(Δ4EBS) is a mutant in which the eIF4E binding site was deleted and, consequently, can no longer bind to eIF4E (Gingras et al. 1999a). Stable cell lines expressing wild-type 4E-BP1 or mutants, in which exogenous 4E-BP was expressed at levels that were comparable to endogenous levels, were established. Staining of the stably expressing cell lines with an anti-HA antibody shows a similar distribution pattern to that of endogenous protein (Fig. 5). Therefore, the presence of 4E-BP1 in the nucleus is neither affected by constitutive binding to eIF4E nor by a deficiency in binding to eIF4E. Because a 4E-BP2 antibody suitable for immunostaining experiments was not identified, HA-4E-BP2 expression was monitored using an anti-HA antibody for immunofluorescence in DKO MEFs. HA-tagged 4E-BP2 exhibited similar localization to that of HA-4E-BP1 in these cells, and the nuclear-to-cytoplasmic ratio of HA-4E-BP2 did not change following serum starvation and rapamycin treatment (Fig. 5).

eIF4E is excluded from the nucleus in 4E-BP1/4E-BP2 DKO MEFs

Because 4E-BPs bind to eIF4E, it was pertinent to study eIF4E localization in MEFs lacking 4E-BPs. We used a monoclonal antibody (BD Transduction Labs) that recognizes almost exclusively cytoplasmic eIF4E under physiological conditions (see manufacturer's technical data sheet; catalog no. 610270). In serum-deprived or rapamycin-treated cells, the fraction of eIF4E in the nucleus significantly increased from ~1% to ~20%, while serum starvation in conjunction with rapamycin had the most dramatic effect on eIF4E nuclear accumulation (~30% in the nucleus) (Fig. 6). In sharp contrast, eIF4E failed to accumulate in the nucleus of DKO cells in response to serum starvation and rapamycin treatments (Fig. 6A), demonstrating that 4E-BPs are required for eIF4E nuclear accumulation. A similar redistribution of eIF4E was observed in other cells, including HeLa S3, NIH/3T3, and

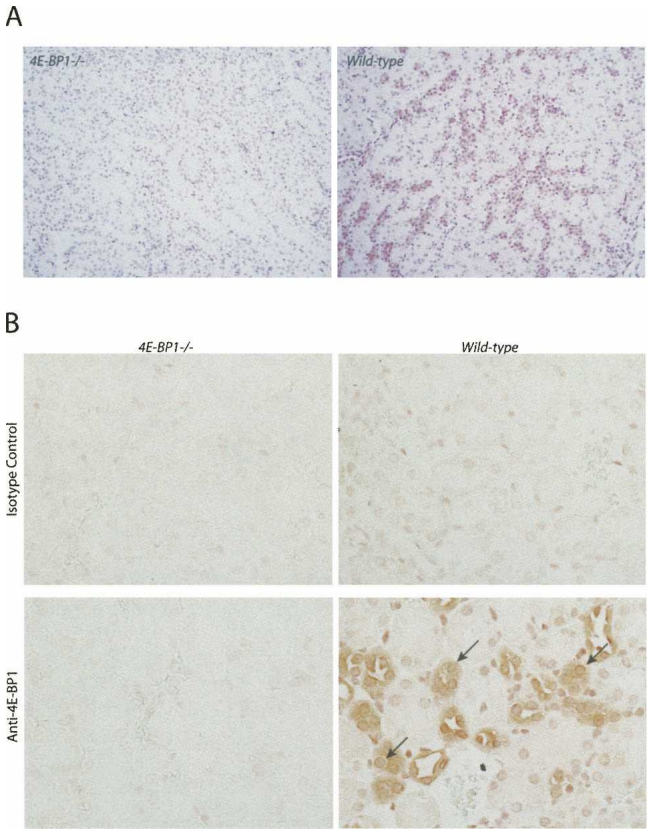

FIGURE 3. Immunohistochemical analysis of 4E-BP1 localization in adult mouse kidney demonstrates the presence of nuclear 4E-BP1 in vivo. (*A*) Low magnification comparison of wild-type and 4E-BP1$^{-/-}$ mouse kidney sections subjected to immunohistochemical staining with 4E-BP1 monoclonal antibody and hematoxylin counter-stain of select cells within the medulla. (*B*) Higher magnification and omission of hematoxylin allows visualization of nuclear 4E-BP1 (indicated by the position of the arrowheads) in adult mouse kidney.

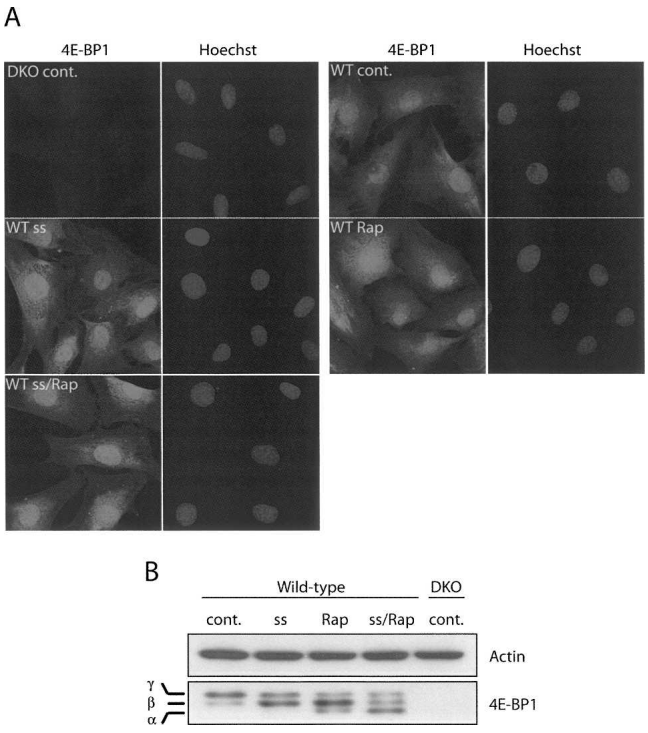

FIGURE 4. Localization of endogenous 4E-BP1 to the nucleus is unaffected by conditions that reduce its phosphorylation status. (*A*) Wild-type MEFs were subjected to immunocytochemical analysis under control (WT cont.) conditions, after 24-h serum starvation (ss), following rapamycin (Rap) treatment (10 nM, 24 h), or after serum starvation/rapamycin dual treatment (ss/Rap). DKO MEFs were stained in parallel as a negative control. (*B*) Western blotting showing phosphorylation status of 4E-BP1 in wild-type MEFs under the different conditions. The positions of hypophosphorylated (α), hyperphosphorylated (γ), and intermediately phosphorylated (β) 4E-BP1 are indicated.

Rat 1A (data not shown), and with other eIF4E antibodies, including a rabbit polyclonal antibody 5853 (Lejbkowicz et al. 1992), and another monoclonal that recognizes cytoplasmic eIF4E (Kimball et al. 2003; Ferraiuolo et al. 2005; data not shown).

To determine whether the differential staining observed in wild-type versus DKO cells under serum starvation and rapamycin treatment is a consequence of the lack of 4E-BPs, rather than other unintended effects of cell manipulations, we examined the localization of eIF4E in DKO cells that stably expressed HA-4E-BP1 or HA-4E-BP2. Exogenous expression of 4E-BP1 or 4E-BP2 restored the wild-type phenotype in DKO MEFs (Fig. 6B). This demonstrates that the defective regulation of eIF4E localization in DKO MEFs is due directly to the absence of 4E-BPs and that both 4E-BP1 and 4E-BP2 control eIF4E localization. Consistent with this conclusion, eIF4E was present in the nucleus of single knockout 4E-BP1$^{-/-}$ or 4E-BP2$^{-/-}$ MEFs in response to starvation and rapamycin treatment (data not shown).

Constitutive binding of 4E-BP to eIF4E results in nuclear accumulation of eIF4E

Next, we wished to determine whether the interaction of 4E-BP1 with eIF4E is necessary to retain eIF4E in the nucleus. To this end, we examined the cellular distribution of eIF4E in DKO MEFs expressing the 4E-BP1 mutant HA-4E-BP1(4Ala) or HA-4E-BP1(Δ4EBS). eIF4E was present in the nucleus of HA-4E-BP1(4Ala)–expressing cells, even under normal growth conditions, while it was cytoplasmic in cells expressing the 4E-BP1(Δ4EBS) (Fig. 6C). Strikingly, HA-4E-BP1(Δ4EBS) failed to confer eIF4E nuclear localization under starved/rapamycin conditions (Fig. 6C). These results clearly demonstrate that the interaction of 4E-BP with eIF4E is absolutely required for serum starvation-induced eIF4E relocalization to the nucleus.

eIF4E localization in the nucleus is dependent on nuclear 4E-BPs

To further demonstrate that eIF4E localization to the nucleus is dependent on the presence of 4E-BP in the

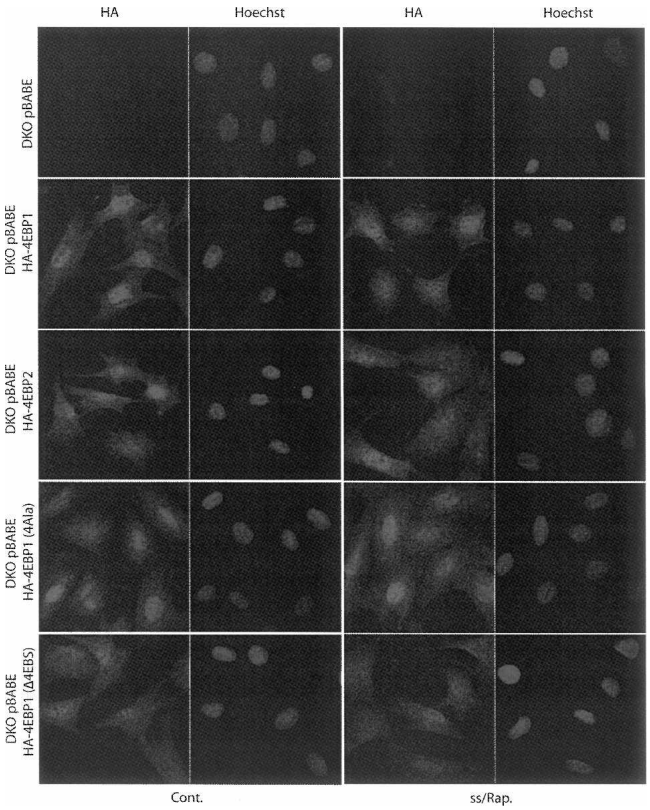

FIGURE 5. Localization of HA-4E-BPs is unaffected by mutations that alter eIF4E binding. DKO MEFs with stable expression of exogenous HA tagged wild-type 4E-BP1, 4E-BP1(4Ala), 4E-BP1(Δ4EBS), or wild-type 4E-BP2 were subjected to immunocytochemical analysis using anti-HA mouse monoclonal antibody and AlexaFluor594 conjugated secondary antibody. The stable cell lines were established by infection of DKO MEFs with retroviruses expressing various 4E-BP constructs.

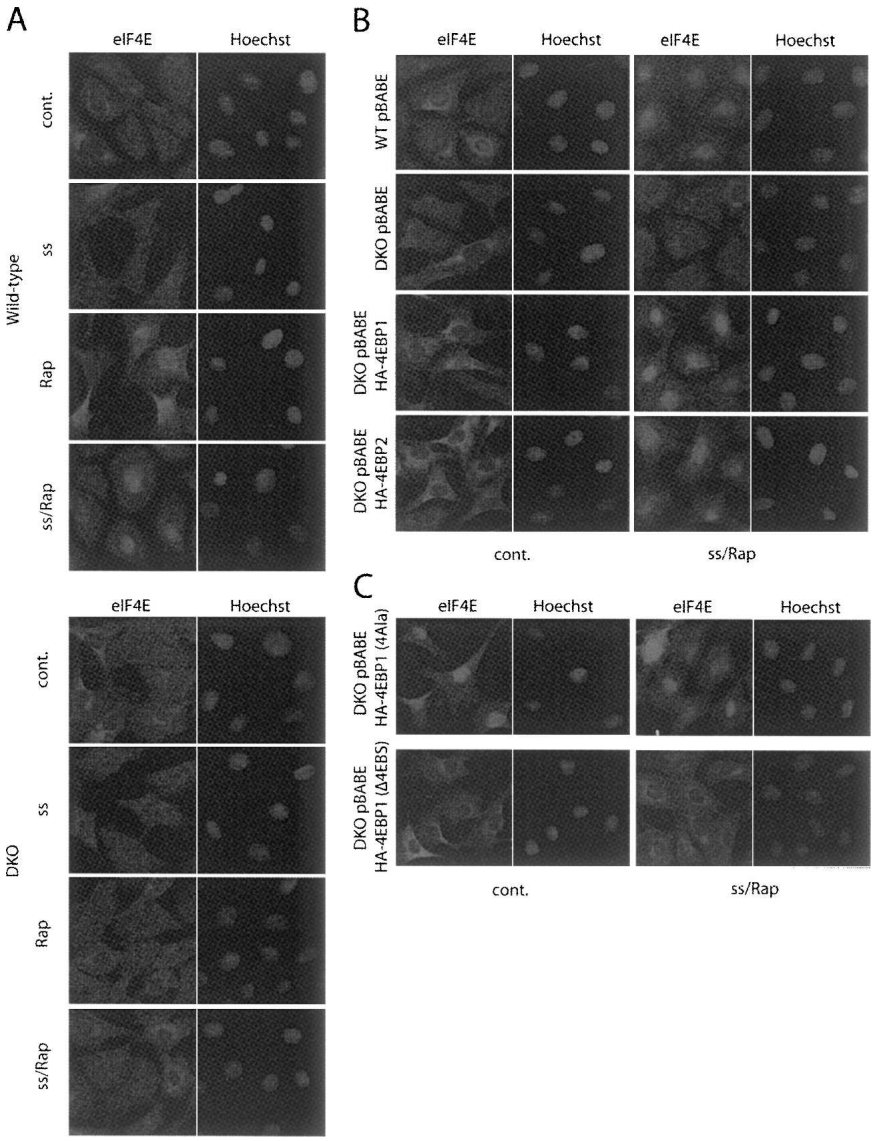

FIGURE 6. Serum starvation and/or rapamycin induce nuclear accumulation of eIF4E. (*A*) Wild-type and DKO MEFs were subjected to immunocytochemical analysis following serum starvation (ss), rapamycin treatment (Rap), and dual treatment (ss/Rap). (*B*) Localization of eIF4E in DKO cells that express HA-4E-BP1 or HA-4E-BP2. (*C*) Interaction of 4E-BP1 with eIF4E is necessary for the localization of eIF4E in the nucleus. 4E-BP1(4Ala) and 4E-BP1(Δ4EBS) mutants were used. eIF4E was stained with a mouse monoclonal antibody and AlexaFluor594 conjugated secondary antibody. The cells lines that express exogenous 4E-BP proteins were established by infection of DKO MEFs with retroviral-based constructs.

nucleus, we searched for conditions that would exclude 4E-BP1, and consequently eIF4E, from the nucleus. We found that 4E-BP1 was exclusively cytoplasmic in c-Ha-Ras–expressing MEFs (Fig. 7). This result demonstrates that the cellular localization of 4E-BP1 is subject to regulation related to expression of Ras. Consistent with our previous findings, those cells exhibiting exclusively cytoplasmic 4E-BP1 failed to display the serum starvation and/or rapamycin-induced nuclear accumulation of eIF4E phenotype (Fig. 7).

DISCUSSION

Herein we provide evidence that a sizable fraction (~30%) of 4E-BP1 is present in the nucleus, contrary to earlier reports (Kim and Chen 2000; Kleijn et al. 2002). The use of biochemical fractionation techniques suggested that 4E-BPs reside exclusively in the cytoplasm, but these techniques are subject to limitations due to leakage of small proteins from the nucleus. We provide compelling evidence for the nuclear localization of 4E-BP1 as follows: (1) a null cell line that does not express 4E-BP1 was not stained with a monoclonal antibody, providing strong evidence that the observed fluorescent signal is due to 4E-BP1 (Fig. 1); (2) in vivo localization of CFP-4E-BP, which precludes artifacts arising from fixing and/or labeling cells for indirect immunofluorescence analysis, yielded similar results; (3) Western blot analysis with an antibody generated against 4E-BP1 detects bands specific to 4E-BP1 that are not present in 4E-BP$^{-/-}$ cells; and (4) the nuclear localization of 4E-BP1 is confirmed in multiple cell lines and tissues. The mechanism by which 4E-BP enters the nucleus, however, remains to be elucidated.

We provide evidence that 4E-BP regulates the localization of its binding partner, eIF4E. We reported that 4E-T mediates the nuclear import of eIF4E by the importin α/β pathway (Dostie et al. 2000a). In the nucleus, eIF4E colocalizes with splicing factors in speckles (Dostie et al. 2000b). We propose that 4E-BP controls the amount of nuclear eIF4E by regulating its release from the nucleus. The regulation of nuclear eIF4E by 4E-BP is supported by data demonstrating that HA-4E-BP1(4Ala), which is constitutively bound to eIF4E, causes nuclear accumulation of eIF4E even under standard conditions (Fig. 6C). A 4E-BP1 mutant lacking the eIF4E binding site failed to cause the nuclear accumulation phenotype observed under stress conditions (Fig. 6C). Furthermore, a loss of nuclear 4E-BP in Ras-expressing MEFs is accompanied by a loss of nuclear eIF4E under stress conditions. Therefore, eIF4E is retained and released from the nucleus in a 4E-BP–dependent manner. The presence of 4E-BP in the nucleus provides a means to regulate the release of eIF4E from the

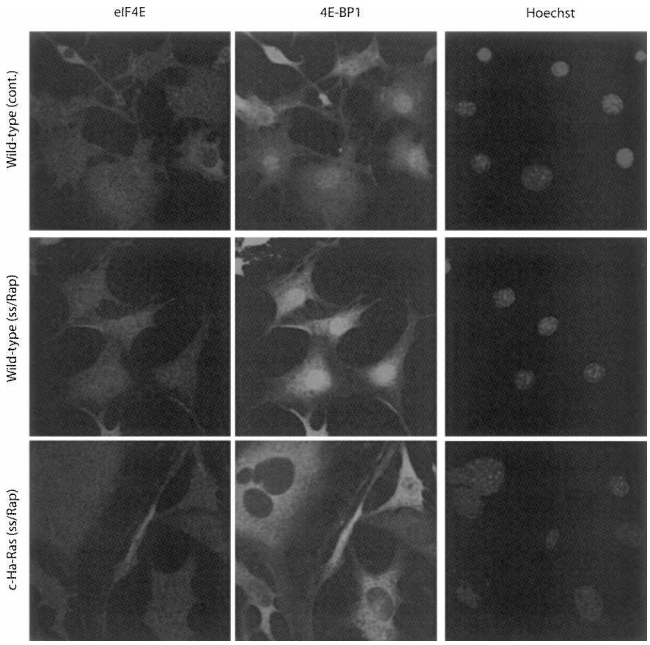

FIGURE 7. 4E-BP1 is cytoplasmic in c-Ha-Ras–expressing MEFs, precluding nuclear accumulation of eIF4E in response to serum starvation plus rapamycin (ss/Rap) dual treatment. Cells were subjected to immunofluorescence analysis using eIF4E mouse monoclonal (BD Transduction Labs) and 4E-BP1 (53H11) antibodies.

nucleus and may prevent the untimely export of eIF4E-bound mRNAs. What could be the advantage of sequestering eIF4E in the nucleus under stress conditions known to inhibit cap-dependent translation? One possibility is that sequestering eIF4E in the nucleus is more efficient than in the cytoplasm, because 4E-BPs do not need to compete with eIF4G and 4E-T, which are mainly cytoplasmic. Another possible advantage is that nuclear recruitment by 4E-BP eliminates the necessity for incorporation of eIF4E into cellular structures, such as insoluble granules formed by eIF4G and heat shock proteins (Cuesta et al. 2000). Thus, retention of eIF4E in the nucleus under stress conditions could be an effective means to inhibit cap-dependent translation. In this regard, it is of interest that mTOR was reported to be a nucleocytoplasmic shuttling protein whose nuclear localization is required for 4E-BP1 phosphorylation (Kim and Chen 2000; Zhang et al. 2002). Thus, activation of the mTOR pathway is expected to cause the phosphorylation of 4E-BP in the nucleus and the release of eIF4E to the cytoplasm to stimulate translation.

The mechanism by which 4E-BP enters and exits the nucleus is unclear; however, previous work has shown that small proteins (<70 kDa) traverse the nuclear pore complex by simple diffusion (Hicks and Raikhel 1995; Wei et al. 2003). 4E-BP is present in the nucleus at steady-state and under stress conditions and is a small protein and does not possess the canonical nuclear localization signals or nuclear export signals (Gorlich and Kutay 1999). However, given that c-Ha-Ras expression induces exclusively cytoplasmic 4E-BP1 localization, the nucleocytoplasmic trafficking of 4E-BP appears to be a regulated process and provides a model system to investigate the regulation of 4E-BP translocation. The mechanism by which c-Ha-Ras prevents nuclear 4E-BP1 localization is not immediately clear, although cytoplasmic retention of nuclear proteins has been reported in Ha-Ras overexpressing embryonic fibroblasts after focus formation (Knippschild et al. 1996). Overexpression of the c-Ha-Ras oncogene is known to induce mTOR phosphorylation through activation of the PI3K/AKT pathway (Marte and Downward 1997). Activated AKT in turn phosphorylates and suppresses TSC2, which results in mTOR activation (Inoki et al. 2002; Reiling and Sabatini 2006; Wullschleger et al. 2006). Erk1/2, another target of the Ras signal transduction route, can phosphorylate and inactivate TSC2 on Ser664 (Ma et al. 2005). It is of interest that increased phosphorylation and cytoplasmic staining of 4E-BP1 correlated with higher histological grade and poor outcome in a clinical study of human ovarian epithelial tumors (Castellvi et al. 2006).

Taken together, we have provided evidence that 4E-BP1 localizes partly to the nucleus and regulates eIF4E nuclear levels by retaining it under stress conditions. This would suggest that nuclear 4E-BP is a critical modulator of eIF4E activity and release from the nucleus. 4E-BP may regulate the export of eIF4E bound to certain mRNAs and/or release of free eIF4E to the cytoplasm for translation. Furthermore, the biological relevance of this regulation is apparent in Ras-expressing cells whereby eIF4E fails to accumulate in the nucleus in a manner that is dependent on 4E-BP (or coincident with a loss of 4E-BP in the nucleus). In this manner, 4E-BP localization may prove to be an important hallmark of oncogenic transformation (Armengol et al. 2007), and our study provides the molecular framework behind this premise.

MATERIALS AND METHODS

Plasmid constructs

pcDNA3-3HA-4E-BP1, pcDNA3-3HA-4E-BP2, and pACTAG2-3HA-4E-BP1(Δ4EBS) have been described previously (Rousseau et al. 1996; Gingras et al. 1999a). pcDNA3-3HA-4E-BP1 (4Ala) carrying alanine substitutions at four phosphorylation sites (Thr37, Thr46, Ser65, and Thr70) was produced using the method previously described to generate the Thr37Ala, Thr46Ala double mutant protein (Gingras et al. 1999a), which had been shown to constitutively bind eIF4E. 4E-BP1, 4E-BP2, and mutant cDNA were amplified by PCR and inserted into BamHI/SalI sites of the retroviral vector pBABE. 4E-BP1-CFP was generated by fusing the open reading frame of 4E-BP1 in-frame with CFP from pcDNA3-CFP (a kind gift from Dr. R.Y. Tsien, La Jolla, CA), using a PCR-based strategy. The 4E-BP1-CFP fusion is under control of the CMV promoter in pcDNA3. Primers and templates for PCRs are

listed in Table 1. The 6.6-kb fragment from pEJ6.6 (Newbold and Overell 1983) encoding the *c-Ha-ras* gene was cleaved with BamHI, blunted, and inserted into pcDNA3.1-Hygro to generate a Ras-expression construct.

Antibodies

Anti-4E-BP1 rabbit monoclonal antibody (clone 53H11) and anti-4E-BP2 rabbit polyclonal antibody were from Cell Signaling Technology; anti-eIF4E mouse monoclonal antibody was from BD Transduction Labs; anti-eIF4E rabbit polyclonal antibody 5853 was described previously (Frederickson et al. 1992; Lejbkowicz et al. 1992); another anti-eIF4E monoclonal antibody was a gift from Dr. S. Kimball (Kimball et al. 2003; Ferraiuolo et al. 2005); anti-HA and anti-actin mouse monoclonal antibodies were from Sigma-Aldrich; and AlexaFluor 488 (or 594) conjugated secondary antibody was from Molecular Probes.

Cell lines and cell culture

Primary wild-type, 4E-BP1$^{-/-}$, 4E-BP2$^{-/-}$, and DKO (4E-BP1$^{-/-}$/4E-BP2$^{-/-}$) MEFs were previously described (Le Bacquer et al. 2007). Immortalized cell lines were established by passing cells for more than 16 generations, and experiments were performed using cells between passage 20 and 30. Cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin, except during serum starvation, when DMEM containing 1% penicillin/streptomycin was applied. All plasmid and siRNA transfections were performed according to the manufacturer's instructions, using Lipofectamine Plus Reagent or Lipofectamine2000 (Invitrogen). To generate cell lines that stably express various 4E-BP mutants, pBABE constructs were transiently transfected into the Ecotropic Phoenix-293T packaging cell line. After 48 h, virus-containing medium was filtered (0.45 μm), collected, and used to infect MEFs in the presence of 5 μg/mL polybrene (Sigma-Aldrich). Infection was repeated the next day. Twenty-four hours after the second infection, medium supplemented with puromycin (1 μg/mL, Sigma-Aldrich) was added, and cells were subjected to selection for 1 wk after which time colonies were pooled. pcDNA3.1-Hygro-c-Ha-Ras was transfected into MEFs. After 48 h, medium supplemented with Hygromycin B (250 μg/mL, Bioshop) was applied for selection (1 wk).

Immunofluorescence, flow cytometry, photobleaching, and immunohistochemistry

Cells for immunofluorescence were seeded in Lab-Tek chamber slides (Nunc) at 60% confluency 24 h before treatment. After serum starvation and/or rapamycin (LC Labs) treatment, cells were fixed in 4% formaldehyde/PBS, permeabilized in 4% formaldehyde/PBS/0.1% Triton X-100, and blocked with 50% FBS, 6% skim milk, 3% BSA, 0.1% Triton X-100, and 0.05% NaN_3 in PBS (Lejbkowicz et al. 1992; Dostie et al. 2000a). Fixed cells were incubated with anti-4E-BP1 rabbit monoclonal antibody clone 53H11 (1:200), anti-eIF4E mouse monoclonal antibody (1:200), or anti-HA mouse monoclonal antibody (1:250) for 2 h at room temperature or overnight at 4°C. After washing with PBS, cells were incubated with AlexaFluor 488 (or 594) conjugated secondary antibodies (1:1000) (Molecular Probes) for 30 min. Nuclei were stained with Hoechst 33258 (Sigma-Aldrich). Cells were washed extensively eight times with PBS/0.1% Triton X-100 for 10 min each time. Images were obtained using a 63× objective of a Zeiss LSM 510 confocal microscope. For c-Ha-Ras–overexpressing MEFs, continuously growing cells were either seeded in chamber slides at 60% confluency 24 h before treatment or mixed with parental cells at a ratio of 1:9, then seeded in culture for about 3 d until foci appeared. In the latter case, immunofluorescence was performed according to the same protocol described above.

TABLE 1. The primers and templates used to generate 4E-BP constructs

Construct	Primer	Template
pcDNA3-4E-BP1-CFP	HindIII-4E-BP1-fwd: CCCAAGCTTATGTCCGGGGGCAGCAGC HindIII-4E-BP1-rev: CCCAAGCTTGGTGGCGACCGGTGGATC	pcDNA3-3HA-4E-BP1
pBABE-HA-4E-BP1	HA-Bam-fwd: GCTAGGATCCATGATCTATCCATATGAC BP1-Sal-rev: CGATGTCGACTTAAATGTCCATCTC	pcDNA3-3HA-4E-BP1
pBABE-HA-4E-BP2	HA-Bam-fwd: GCTAGGATCCATGATCTATCCATATGAC BP2-Sal-rev: CGATGTCGACTCAGATGTCCATCTC	pcDNA3-3HA-4E-BP2
pBABE-HA-4E-BP1(4Ala)	HA-Bam-fwd: GCTAGGATCCATGATCTATCCATATGAC BP1-Sal-rev: CGATGTCGACTTAAATGTCCATCTC	pcDNA3-3HA-4E-BP1(4Ala)
pBABE-HA-4E-BP1(Δ4EBS)	HA-Bam-fwd: GCTAGGATCCATGATCTATCCATATGAC BP1-Sal-rev: CGATGTCGACTTAAATGTCCATCTC	pACTAG2-3HA-4E-BP1-(Δ4EBS)

To determine the relative fluorescent intensities of wild-type and DKO MEFs following immunostaining, cells were subjected to flow cytometric analysis using the primary antibody (53H11) according to the manufacturer's recommended protocol (Cell Signaling Technology). Briefly, cells were typsinized, fixed with 4% formaldehyde for 10 min at 37°C, and then permeabilized with methanol for 30 min at −20°C. After 1 h blocking with 2% BSA (in PBS), cells were incubated with the 4E-BP1 antibody (clone 53H11, 1:200) overnight at 4°C, and AlexaFluor 488 conjugated goat anti-rabbit IgG (1:1000) for 1 h. After washing with PBS, cells were analyzed using a Beckton Dickinson FACScan.

The pcDNA3-4E-BP1-CFP mammalian expression construct was transiently transfected into wild-type MEFs plated in 3.5-cm Petri dishes with a 25-mm circular microscope cover glass (Fisher). A photobleaching assay was performed 24 h later in a 37°C chamber with a supply of 5% CO_2, using a Zeiss LSM510 Meta microscope. A 25-mW diode laser generating a 405-nm line was used in combination with a band pass filter reading of 470–500 nm. Images were acquired at 0.5-mW power prior to photobleaching. The nucleus was bleached for 5 sec using the 405-nm laser line at 100% intensity. Single images were then collected for 300–600 sec at the indicated time points using 0.5-mW power. Using the Metamorph software, fluorescence intensities of all the pixels representing the nuclear and cytoplasmic area were measured, and the average intensity of the photobleached nucleus was normalized against the average intensity of the cytoplasm. This experiment was repeated for seven different cells.

Immunohistochemistry was performed according to the primary antibody manufacturer's protocol (Cell Signaling Technology). Briefly, mouse tissues were fixed overnight at room temperature in 10% neutral buffered formalin. Four-millimeter sections were deparaffinized in xylene for 5 min three times, and rehydrated through two washes of 100% ethanol for 10 min each and two washes of 95% ethanol for 10 min each. Antigen unmasking was performed using 10 mM sodium citrate (pH 6.0) at a sub-boiling temperature for 10 min. After quenching in 3% H_2O_2 for 10 min, slides were blocked with 5% horse serum in PBS supplemented with 0.1% Tween-20 for 1 h at room temperature. Tissues were stained with primary antibody overnight at 4°C and secondary antibody for 30 min at room temperature. Detection was performed with Vector ABC Elite kit and NovaRed substrate (Vector Labs). Slides were dehydrated by incubating with 95% ethanol, 100% ethanol, and xylene for 10 sec each and mounted with coverslips.

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Control of eIF4E cellular localization by eIF4E-binding proteins, 4E-BPs

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