CPEB1 Regulates the Expression of MTDH/AEG-1 and Glioblastoma Cell Migration

Dawn M. Kochanek and David G. Wells

Abstract

Cytoplasmic polyadenylation element-binding protein 1 (CPEB1) is an mRNA-binding protein present in both neurons and glia. CPEB1 is capable of both repressing mRNA translation and activating it depending upon its phosphorylation state. CPEB1-bound mRNAs are held in translational dormancy until CPEB1 is phosphorylated, leading to the cytoplasmic polyadenylation of the bound mRNA that triggers translation. Here, we show that CPEB1 can bind to and regulate translation of the mRNA-encoding metadherin (MTDH, also known as AEG-1 and Lyric) in the rat glioblastoma cell line CNS1. MTDH/AEG-1 is being revealed as a critical signaling molecule in tumor progression, playing roles in invasion, metastasis, and chemoresistance. By using a mutant of CPEB1 that cannot be phosphorylated (thereby holding target mRNAs in translational arrest), we show that inhibiting CPEB1-mediated translation blocks MTDH/AEG-1 expression in vitro and inhibits glioblastomas tumor growth in vivo. CPEB1-mediated translation is likely to impact several signaling pathways that may promote tumor progression, but we present evidence suggesting a role in directed cell migration in glioblastoma cells. In addition, reporter mRNA containing CPEB1-binding sites is transported to the leading edge of migrating cells and translated, whereas the same mRNA with point mutations in the binding sites is synthesized perinuclearly. Our findings show that CPEB1 is hyperactive in rat glioblastoma cells and is regulating an important cohort of mRNAs whose increased translation is fueling the progression of tumor proliferation and dispersal in the brain. Thus, targeting CPEB1-mediated mRNA translation might be a sound therapeutic approach. Mol Cancer Res; 11(2); 149–60. ©2012 AACR.

Introduction

Altered protein synthesis is a hallmark of cancer with many genes having documented aberrant transcription rates. However, we were interested in what was happening to the posttranscriptional mechanisms controlling mRNA translation in transformed cells, specifically glioblastomas. The mRNA-binding protein cytoplasmic polyadenylation element-binding protein 1 (CPEB1) is present in both neurons and astrocytes, where it regulates the transport and translation of a distinct set of mRNAs (1–3). CPEB1 binds to mRNAs that contain a cis-element in their 3′-untranslated region (UTR) called a cytoplasmic polyadenylation element (CPE; ref. 4). Once bound to the CPE, CPEB1 initially silences mRNA translation but can activate translation following phosphorylation (4, 5). In astrocytes, CPEB1 is phosphorylated by Aurora kinase A and confirmed CPEB1 targets include β-catenin and cyclin B1 mRNA (3, 6), both proteins and the kinase have been implicated in cancer. To determine a functional role for CPEB1 in glioblastoma, we used a mutant version of CPEB1 that maintains the ability to bind specific mRNA, but does not contain the phosphorylation site necessary for activation, thus inhibiting translation of bound mRNA.

The mutant CPEB1 protein we termed DN-CPEB1 (dominant negative-CPEB1) because it binds specifically to CPEB1 targets, blocking the endogenous CPEB1 from binding, and reduces the translation of bound mRNA (1, 3, 7). Our rationale for using this approach was predicated on our goal of turning off CPEB1-mediated mRNA translation. By using siRNA, or other means to knockdown the expression of CPEB1 protein, the result would be to liberate bound mRNA from repression, thus in essence activating translation of CPEB1 targets (6). If we overexpress CPEB1 protein, we cannot be sure what fraction of the expressed CPEB1 is phosphorylated, again complicating the interpretation as the translation of some bound mRNA would be dormant and some activated. However, by expressing the phospho-mutant, we can attain the greatest certainty that we are actually inhibiting CPEB1 function.

In astrocytes, CPEB1 was first described as a regulator of β-catenin mRNA and DN-CPEB1 expression blocked cells from migrating in a traditional scratch assay in vitro (3). Left unanswered in that study was whether DN-CPEB1 expression blocked all movement or rather altered the cells ability
to move in a directed or progressive manner. Directional cell migration requires cell polarity allowing for protrusion at the leading edge and retraction at the rear. Indeed, movement is largely driven by actin polymerization and acto-myosin contraction at the leading edge of a cell (8). Much of the asymmetric localization of organelles and proteins required for cell migration are dependent upon microtubules (9). Perhaps surprisingly, one component that is asymmetrically localized and required for directional migration is mRNA (9, 10). In fact, local protein synthesis may be an important feature in establishing cellular asymmetry during cellular development and migration (10, 11).

By examining the 3'-UTR of mRNAs encoding proteins misregulated in glioblastoma, we identified a number that could potentially be targets for CPEB1 regulation (see Table 1). Among that group, we selected MTDH/AEG-1 for further analysis based on 2 properties. The first was its ability to interact and activate the β-catenin pathway (12); and second, the emerging evidence that MTDH/AEG-1 may coordinate the activity of several signaling pathways all leading to the progression of tumor growth and spread in a number of different tumor types (including glioblastoma; ref. 13–17). If CPEB1 is regulating the synthesis of MTDH/AEG-1, it would suggest that CPEB1 is coordinating the synthesis of proteins that are functionally related (e.g., β-catenin and MTDH/AEG-1), and that targeting CPEB1-mediated protein synthesis might be useful in many types of cancer not just glioblastoma.

Materials and Methods

Cell culture preparation

Astrocytes were isolated as previously described (3). Briefly, astrocytes were isolated from postnatal day 1 mice pups of either sex, treated with trypsin, physically dissociated, and plated at approximately 100,000 cells/mL on 60-mm tissue culture dishes, glass bottom tissue culture dishes, 6-well plates or 18-mm glass coverslips (MatTek, Carolina Biologicals). Astrocytes were maintained in growth medium containing minimum essential medium (MEM) with glutamine (Invitrogen), 10% horse serum (Atlanta Biologicals), glucose (1 mg/mL), pyruvate (1 mmol/L), and penicillin/streptomycin (100 mg/mL). For neuron-free cultures, cells were plated in 75-mm flasks and grown for 1 to 2 weeks before trypsinization and replating on tissue culture dishes. CNS-1 cells, a gift from Rick Matthews (State University of New York (SUNY), Upstate Medical University, Syracuse, NY), were cultured as previously described (3). Wounds were carried out by scratching the confluent monolayers with a 0.1 to 10 μL plastic pipette tip. Human U87 astrocytoma cells were cultured in growth medium containing Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% FBS (Invitrogen) and penicillin/streptomycin (100 mg/mL).

DN-CPEB1-GFP expression

CNS-1 cells were transfected using Lipofectamine 2000 (Invitrogen). Briefly, 0.5 μg of plasmid DNA was mixed with 3 μL of transfection reagent and mixed in 200 μL of Opti-Mem (Invitrogen). DNA and reagent were incubated for up to 6 hours at room temperature before addition to the CNS-1 cells. Cells were transfected in antibiotic-free MEM (Invitrogen) for 4 hours before the transfection mixture was replaced with fresh growth medium. Constructs used for transfection encoded either enhanced GFP (EGFP) or an EGFP-tagged truncated CPEB1 protein that does not contain the phosphorylation truncated CPEB1 protein that does not contain the phosphorylation site needed for activation but does not

Table 1. CPE-containing mRNAs mis-regulated in various stages of glioblastoma

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Accession #</th>
<th>Mis-regulated in</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine deaminase</td>
<td>NM_015840.3</td>
<td>Grade III</td>
<td>Chumbalkar et al. 2005</td>
</tr>
<tr>
<td>Cachexin related tumor suppressor</td>
<td>NM_052453.5</td>
<td>GBMs (secondary)</td>
<td>Schwartz et al. 2005</td>
</tr>
<tr>
<td>Calpain 1 light chain</td>
<td>NM_174650.1</td>
<td>GBM</td>
<td>Hobbs et al. 2003</td>
</tr>
<tr>
<td>Centrosome associated protein 350</td>
<td>NM_018410.2</td>
<td>GBMs (primary)</td>
<td>Schwartz et al. 2005</td>
</tr>
<tr>
<td>Copine1</td>
<td>NM_001915.5</td>
<td>Gliomas</td>
<td>Chakravarti et al. 2001</td>
</tr>
<tr>
<td>Cyclin B1</td>
<td>NM_012382.2</td>
<td>Gliomas</td>
<td>Holtkamp et al. 2005, Kim et al. 2011</td>
</tr>
<tr>
<td>Dihydropteridine reductase</td>
<td>NM_003202.2</td>
<td>Grade II</td>
<td>Schwartz et al. 2004</td>
</tr>
<tr>
<td>FABP5</td>
<td>NM_001444.2</td>
<td>Grade III</td>
<td>Hobbs et al. 2003</td>
</tr>
<tr>
<td>FABP7</td>
<td>NM_001446.3</td>
<td>Gliomas</td>
<td>Chakravarti et al. 2001, Gerstner et al. 2012</td>
</tr>
<tr>
<td>Glutamate dehydrogenase 1</td>
<td>NM_005271.3</td>
<td>Grades I&amp;II</td>
<td>Hiratsuka et al. 2003</td>
</tr>
<tr>
<td>Metadherin (AEG-1)</td>
<td>NM_17812.3</td>
<td>GBM</td>
<td>Emdad et al. 2006</td>
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<tr>
<td>Nucleolar GTP binding protein 1</td>
<td>NM_012341.2</td>
<td>GBM &amp; Grade III</td>
<td>Hiratsuka et al. 2003</td>
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<tr>
<td>PTEN†</td>
<td>NM_003134.4</td>
<td>GBM</td>
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<tr>
<td>Tropomodulin 2</td>
<td>NM_014548.3</td>
<td>Grade III</td>
<td>Chumbalkar et al. 2005</td>
</tr>
<tr>
<td>Tubulin specific chaperone A</td>
<td>NM_004607.2</td>
<td>GBM</td>
<td>Hobbs et al. 2003</td>
</tr>
<tr>
<td>Tumor protein p53</td>
<td>NM_000456.5</td>
<td>GBM</td>
<td>Odreman et al. 2005, Burns et al. 2011</td>
</tr>
</tbody>
</table>

†Validated CPEB1 targets.
**Validated in this manuscript.
contain the mRNA-binding domain (DN-CPEB1-GFP). Cloning of the DN-CPEB1-GFP construct into pEGFP-C1 (BD Biosciences) has been previously described (3).

Immunoprecipitation and DN-CPEB1-GFP pull-down

Total protein lysate was isolated from CNS1 cells, or CNS1 cells that had been transfected with DN-CPEB1-GFP, using the miRVana PARIS Kit (Life Technologies). This lysate was then mixed with rabbit anti-CPEB1 (1:1,000) or anti-GFP (1:100; Invitrogen) for 30 minutes before the addition of 50 µL Protein A MAG beads (GE Healthcare) for 30 minutes. Beads were pelleted, washed 3 times, and RNA was isolated by ethanol precipitation using miRVana PARIS kit. DNA was resuspended in water and treated with DNase I (Ambion) for 15 minutes at 37°C followed by reverse transcription (RT) and PCR analysis to detect mRNA-encoding metadherin (MTDH) and glial fibrillary acidic protein (GFAP) mRNAs. Primer sequences were as follows: MTDH/AEG-1: (forward) 5'-gagcagagacacagcttac-3' and (reverse) 5'-cgcttcctctctctcga-3'; GFAP: (forward) 5'-agcaggggcagggcgatgc-3' and (reverse) 5'-ggaacgcccgtcagggcacctca-3'.
Quantification of directed cell migration
CNS-1 cells were transfected as described earlier with either the DN-CPEB1-GFP or control GFP constructs, 12 to 16 hours after transfection a wound was introduced. Using live cell confocal microscopy, transfected cells along the wound edge were located and imaged every 30 seconds for 45 minutes. The individual trajectories of the cells were then normalized to a starting point and tracked using MetaMorph software (Molecular Devices).

Reporter construct generation
Constructs pSL-MS2-12x and pMS2-GFP were gifts from Robert Singer (Albert Einstein Medical College, Bronx, NY). The CPE sequences used for the reporter were based on the β-catenin 3’-UTR, a well-characterized target of CPEB1 (3, 7). Approximately, 250 nucleotides of the 900-nucleotide β-catenin, 3’-UTR was amplified from mouse cDNA as previously described (7). Constructs used for transfection encoded either the wild-type or mutated CPE sequences digested out of pEGFP-C1 at the BglII and BamHI sites (3) and subcloned in frame into the BamHI site of pBluescriptII KS+ (Clontech). The 3’-UTR’s were then digested out of pBluescriptII KS+ at the NotI and XhoI sites and cloned in frame into pcDNA3 (Invitrogen). The 12 MS2-binding domains and multiple cloning site (MCS) were digested out of pSL-MS2-12x and cloned in frame into the 3’-UTR containing pcDNA3 at the BamHI and XhoI sites. The MS2 protein, GFP, and nuclear localization signal (NLS) were digested out of pMS2-GFP and cloned into pcDNA3 (Invitrogen). The destabilized EGFP (dEGFP) was PCR-amplified out of pZSGreen1-DR (Clontech) using a forward primer designed to add in a Kozak sequence and the KpnI restriction site: 5’-AAAAAGTGACCATGGCCCAGTCCAAG-3’. To the reverse primer, we added a farnesylation sequence and the BamHI restriction site: 5’-AAAAAGGATCCCTAGAGGACACACTTGCAGCTTGACGCGGCCAACTCTCATCAGGAGGTTCAGCTTAGATCTGAGTCCGGACACATATTGATCCTAGC-3’. The PCR product was purified then ligated in frame into pcDNA3 at the KpnI and BamHI sites. The CPE-containing and CPE-mutated 3’-UTR’s were digested out of pcDNA3 (mentioned earlier) and cloned in frame into the NotI and XhoI sites.

Visualizing mRNA localization
CNS-1 cells were cultured on 18-mm glass coverslips (Carolina Biologicals) and then cotransfected as described earlier with the MS2-GFP-NLS construct and either the CPE-containing or CPE-mutated MS2 constructs. Twelve to 16 hours after transfection, the cells were fixed with 4% paraformaldehyde in PBS, mounted onto slides using ProLong Gold with DAPI (Invitrogen), and imaged under confocal microscopy. Quantification of mRNA expression was determined by counting the number of transfected cells in 3 fields per coverslip. Fields were visualized and selected under ×63 microscopy. Blinded to condition, each transfected cell in the field was visualized under brightfield and fluorescence to determine the location of the mRNA. mRNA expression was classified as either perinuclear or expressed throughout the cell including in the periphery.

Quantification of fluorescence
Primary astrocytes were cultured as described earlier and transfected with the farnesylated-dEGFP construct containing either the CPE-containing or CPE-mutated reporter, and 12 to 16 hours after transfection a wound was introduced. One to 2 hours after the wound using live confocal microscopy under a ×63 objective, transfected cells along the wound edge were located and imaged, photobleached, and subsequently imaged every 30 seconds after the bleaching for 15 minutes. To quantify fluorescence in the perinuclear region and at the peripheral edge of the cell, a region of interest in both locations was defined in the phase image. The areas were then overlaid on the fluorescent image and the mean pixel intensity was calculated using the T-stacks, intensity versus time monitor function plug-in on MacBiophotonics ImageJ (U.S. NIH). To correct for background fluorescence, pixel intensity of the same size area was measured immediately adjacent to the transfected cell and this value was subtracted.

Results
CPEB1 regulates MTDH/AEG-1 expression
Table 1 lists proteins that have been implicated in glioblastoma progression that we determined to contain a CPE sequence in their 3’-UTR by sequence analysis. Some of these have previously been verified targets of CPEB1; here, we selected to examine MTDH/AEG-1 for the reasons outlined in the introduction. The mRNA encoding MTDH/AEG-1 contains a conserved CPE sequence in the 3’-UTR that is located within 100 nucleotides of the hexanucleotide sequence, which is the cleavage and polyadenylation signal present just 5’ to the poly(A) tail in nearly all mRNA (Fig. 1A). To determine if CPEB1 binds to MTDH/AEG-1 mRNA in CNS1 glioblastoma cells, we immunoprecipitated CPEB1 protein under conditions that allowed us to isolate the bound mRNA. The RNA was isolated and underwent RT-PCR using primers specific for MTDH/AEG-1 or the non-CPE-containing mRNA encoding GFAP. MTDH/AEG-1 mRNA is only detected in the CPEB1 immunoprecipitation, not when a nonspecific antibody is used for immunoprecipitation. The interaction of MTDH/AEG-1 mRNA is specific in that the non-CPE-containing mRNA GFAP is not bound to CPEB1 protein (Fig. 1B). To confirm this interaction, we expressed a mutated version of CPEB1 that contains the mRNA-binding domain but not the activation (phosphorylation) domain tagged with GFP (DN-CPEB1-GFP) in CNS1 cells and immunoprecipitated using an antibody specific for GFP (Fig. 1C). Again, the MTDH/AEG-1 mRNA specifically interacts with the DN-CPEB1-GFP protein. This result also indicates that the DN-CPEB1-GFP protein is capable of competing with the endogenous CPEB1 for target mRNA.

MTDH/AEG-1 immunoreactivity was detected at low levels in primary rat astrocytes in culture, but seems greatly
Because CPEB1 stimulates the translation of bound mRNA, we compared the pCPEB1 state of both astrocytes and CNS1 cells indicating active translation of bound mRNA. We compared the pCPEB1 state of both astrocytes and CNS1 cells using a phospho-specific antibody we raised against this site (Supplementary Fig. S1A). Although the total level of CPEB1 protein in astrocytes and CNS1 cells is about equal, pCPEB1 levels are significantly higher in CNS1 cells (Supplementary Fig. S1B). Thus, following disruption of CPEB1 function there is a significant inhibition of tumor volume in glioblastoma that may be due in part to a lack of cell migration away from the injection site.

CPE-dependent local protein synthesis in astrocytes

The apparent lack of migration in vivo in cells expressing DN-CPEB1-GFP was similar to the effect we observed in cultured cells (3). Because directed cell migration requires asymmetric localization of proteins, we examined if CPEB1-mediated protein synthesis could contribute to the localization of proteins to the leading edge of migrating cells. We asked if the presence of a CPE sequence in the 3'-UTR can direct protein synthesis into the periphery of the cell by expressing a GFP reporter construct in astrocytes. Astrocytes are much broader and flatter cells in vitro than CNS1 cells and the levels of MTDH/AEG-1 protein is drastically and significantly diminished, whereas the level of mRNA encoding MTDG/AEG-1 does not change (Fig. 2B). Finally, this decrease in MTDH/AEG-1 is detected specifically in cells expressing the DN-CPEB1 protein (Fig. 2C).

Disruption of CPEB1 inhibits glioblastoma growth in vivo

To determine if CPEB1 inhibition could affect tumor growth in vivo, we expressed either the DN-CPEB1-GFP or GFP alone in CNS-1 cells before implanting them into the central striatum of adult Lewis rats. A total of 150,000 CNS1 cells were injected into the right hemisphere and animals were perfused 10 days after implantation. Brains were processed and serial sectioned. To assay for cell migration, we used fluorescence microscopy to image transfected cells in serial sections and determined spread away from the injection site (Fig. 3A). We counted the number of GFP-expressing cells in 10-μm wide bins (medial-laterally from injection site), normalizing each bin to the number of cells in the 10-μm bin centered on the injection site. This method normalizes the counts and allows for comparison between groups. The cell density was significantly increased in regions distal to the injection site in the GFP-expressing controls compared with the DN-CPEB1-GFP–expressing cells (Fig. 3B). Brains were also processed, stained (Fig. 3C and D), and subjected to 3D volume reconstruction. In agreement with the cell spread data, tumor reconstructions revealed that expression of DN-CPEB1-GFP resulted in a significant reduction in total tumor volume compared with the control GFP-expressing tumors (Fig. 3E and F). The decrease in tumor size observed by inhibiting CPEB1 could also be visualized in rostral-caudal spread of the DN-CPEB1-GFP–transfected tumors (Fig. 3G). When we quantified the total tumor volume as a group, we found the DN-CPEB1-GFP–transfected tumors displayed a significant 45% decrease in total volume compared with control GFP-expressing tumors (Fig. 3H; GFP, n = 10; DN-CPEB1, n = 10; P < 0.05; Student t test, two-tailed). Brains were also processed and immunostained for pCPEB expression. Tumors transfected with the GFP alone showed increased pCPEB expression as compared with adjacent healthy tissue (Supplementary Fig. S1B). Thus, following disruption of CPEB1 function there is a significant inhibition of tumor volume in glioblastoma that may be due in part to a lack of cell migration away from the injection site.
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and allow for greater spatial resolution when imaging. The reporter encoded a farneylation sequence fused to the C-terminus of a dEGFP to anchor the GFP into the membrane near the site of synthesis (19). This reporter contained either CPE sequences in the 3'-UTR or the same 3'-UTR but with the CPE sequences mutated (Fig. 4A). Astrocytes were grown to near confluence and cells were transfected with either the CPE-containing or mutated construct. Sixteen hours after transfection, a wound was introduced. Using live cell confocal imaging, transfected cells on the leading edge of the wound were located, imaged, photobleached, and subsequently imaged every 30 seconds for the reappearance of fluorescence (Fig. 4B). We quantified GFP expression by selecting 2 regions of interest: one at the peripheral edge of the cell and the other adjacent to the nucleus (Fig. 4B; black boxes). Cells expressing the CPE-containing 3'-UTR showed expression of GFP both around the nucleus and at the distal edge of the cell within minutes (Fig. 4B and C). However, cells expressing the mutated CPE construct had a clear delay in fluorescence recovery at the peripheral edge of the cell (Fig. 4B and C). Thus, the presence of CPE sequences in the 3'-UTR can localize protein synthesis to the leading edge of migrating astrocytes.

CPE-dependent mRNA localization

The lack of localized protein synthesis in the absence of the CPE in Fig. 4 could either be misregulation of mRNA translation or mRNA localization. To assess if mRNA-containing CPE sequences are trafficked differently from non-CPE–containing mRNA, we used the MS2 RNA visualization assay pioneered in the Singer laboratory (20). Here, the single-stranded mRNA-binding phage coat protein MS2 is fused to a fluorescent reporter and expressed along with mRNA engineered to contain MS2-binding sites (21). We generated 2 reporter constructs, one containing 12 MS2-binding sites fused to the CPE-containing 3'-UTR of the MTDH/AEG-1 mRNA and the other containing 12 MS2-binding sites fused to the mutated CPE 3'-UTR containing AEG-1 mRNA levels. A, MTDH is upregulated in glioblastoma cells. Astrocyte and CNS1 cultures were probed with anti-MTDH/AEG-1 antibody showing increase immunoreactivity in the CNS1 cells. Astrocyte, CNS1, and U87 cultures were analyzed by Western blot analysis for MTDH/AEG-1 expression and levels quantified. Loading was normalized to β-actin and the relative amount of MTDH expressed in the glioblastomas line was compared with astrocytes (n = 3; *, P < 0.05 by Student t test, two-tailed). B and C, CNS1 cultures were transfected with either GFP or DN-CPEB1-GFP and processed for MTDH/AEG-1 immunoreactivity. Cells expressing GFP alone showed robust MTDH/AEG-1 expression (arrows in left), whereas cells expressing DN-CPEB1-GFP show decreased expression (arrows in right) even though neighboring nontransfected cells show robust expression. CNS1 and U87 cultures expressing GFP or DN-CPEB1-GFP analyzed by Western blot analysis for MTDH/AEG-1 expression show a significant decrease in MTDH/AEG-1 expression in DN-CPEB-GFP–expressing cultures. Loading was normalized to β-actin (n = 3; *, P < 0.05; **, P < 0.005 by Student t test, two-tailed). However, RT-PCR showed no change in MTDH/AEG-1 mRNA levels.
to mRNA (Fig. 5A). To take advantage of a higher transfection efficiency and rapid migration rate, the constructs were cotransfected into CNS-1 cells (18). Transfected monolayers were subjected to a scratch assay and assessed for mRNA localization 1 to 2 hours after the wound. Cells that were transfected with the CPE-containing construct displayed mRNA expression throughout the cell including the periphery. However, cells transfected with the mutated CPE construct expressed the tagged mRNA in a perinuclear location only (Fig. 5B). 70% of cells transfected with the CPE-3’-UTR construct expressed the MS2-tagged mRNA at the peripheral edge. While, only 28% of cells transfected with the mutated CPE-3’-UTR visualized MS2-tagged mRNA outside of the perinuclear region (Fig. 5C). This dramatic nuclear localization of the CPE-lacking reporter suggests that CPEB1 may play a role in not only targeting the mRNA to the periphery but shuttling the mRNA between the nucleus and the cytoplasm. To confirm in astrocytes that CPEB1 is shuttled in and out of the nucleus, we transfected cells with a construct containing the full length CPEB1 fused to mCherry. The cells were then left untreated or treated with the nuclear export inhibitor LMB. In the untreated cells, mCherry-tagged CPEB1 was expressed throughout the cytoplasm including the peripheral edge of the cell (Fig 5D). This localization of mCherry-CPEB1 is identical to that of endogenous CPEB1 (3). However, after treatment with LMB for 6 hours, the expression of mCherry-tagged CPEB1 was completely restricted to the nucleus in both astrocytes and CNS1 cells (Fig. 5D). Interestingly, CNS1 cells exhibit a faster response to LMB than astrocytes, localizing the majority of CPEB1 to the nucleus after just 1 hour of treatment (Fig. 5D).
These data suggest that CPEB1 plays a role in the localization of CPE-containing mRNA in astrocytes and glioblastoma cells and that the nuclear shuttling of CPEB1 in CNS1 cells is enhanced. Further evidence, along with the increase in pCPEB1, that the activity of this pathway is enhanced in glioblastoma cells.

CPEB1 regulates directed cell migration

In our initial characterization of CPEB1 in astrocytes and CNS1 cells, the expression of DN-CPEB1-GFP resulted in a failure of expressing cells to migrate in a wound healing assay in vitro (3). While it was determined that cells transfected with the dominant negative CPEB1 were less likely to migrate into the wound, we were not able to determine if the cells had lost their ability to migrate or if they lacked directional movement. To address this distinction, CNS1 cells were transfected with either DN-CPEB1-GFP or GFP alone and visualized 1 to 2 hours after inducing a wound in the cultures. Transfected cells along the edge of the wound were located and imaged every 30 seconds for 45 minutes. When individual trajectories were normalized to a starting point, 72% of the GFP-transfected control cells migrated toward the wound. In contrast, only 16% of the DN-CPEB1-GFP cells migrated toward the wound (Fig. 6A). When compared as a group, control cells displayed a clear trajectory, steadily migrating into the wound. However, cells transfected with the DN-CPEB1-GFP displayed movement but this movement was random (Fig. 6B). We next quantified the amount of time the cells spent migrating toward the wound. Over a 45-minute time course, cells transfected with the control GFP spent 85% of the time moving with directionality toward the wound. In contrast, cells transfected with the DN-CPEB1-GFP spent 25% moving with a preference toward the wound (Fig. 6C). These results suggest that disruption of CPEB1 function results in a loss of directed cell migration.
Metadherin/AEG-1 is upregulated in many types of cancer including glioma, breast, prostate, and liver, and has been linked to increased proliferation, migration, cell survival, chemoresistance, and a poor clinical outcome (14, 22). Emerging evidence is suggesting it acts to coordinate many signaling pathways to impart these diverse effects. For example, MTDH/AEG-1 expression is enhanced by the activation PI3K/Akt pathway via phosphorylation of GSK3β (23). GSK3β phosphorylation also stabilizes β-catenin protein and MTDH/AEG-1 increases LEF-1/TCF1, the transcription factor that mediates Wnt/β-catenin signaling (12).

The findings presented in our current work show that the synthesis of MTDH/AEG-1 can be regulated by the mRNA-binding protein CPEB1 in astrocytes and glioblastoma cells. Our figure 5. mRNA trafficking is regulated by the presence of a CPE. A, schematic describing the constructs used in this approach. The mRNA reporter constructs contained 12 binding sites for the coat protein of the bacterial phage MS2 and either CPE-containing 3′-UTR or one in which the CPE sequences had been mutated. A third construct contained the GFP-tagged MS2 protein along with a NLS. B, CNS-1 cells cotransfected with the GFP-MS2 construct and either the CPE-containing (top) or CPE-mutated (bottom) reporter constructs. Cells expressing the CPE-containing construct displayed labeled mRNA throughout the nucleus and the periphery of the cell (arrows) in contrast to cells expressing the mutated CPE construct, which expressed the tagged mRNA only in and around the nucleus. C, quantification of the location of the mRNA (wild-type, n = 87; mutated, n = 50; *, P < 0.01 by one-way ANOVA; post-Tukey). D, astrocyte and CNS1 cultures were transfected with mCherry-tagged CPEB1 and expression was observed throughout the cytoplasm. Six hours after LMB treatment (20 nmol/L), expression of mCherry-tagged CPEB1 was restricted to the nucleus. However, CNS1 cells show a faster response to LMB, localizing the majority of mCherry-tagged CPEB1 to the nucleus after 1 hour (middle).
work suggests that CPEB1 may be regulating a cohort of mRNAs that work together to fuel the progression of tumors.

Alteration in protein expression has been subject to intense investigation in glioblastoma. While many screens have been applied to identify protein expression patterns in glioblastoma, mechanisms that involve translational regulation of a group of mRNAs are still being elucidated. One example of such regulation is the RNA-binding protein Musashi1 (MSI1). MSI1 induces glioma growth by regulating several proteins that promote cell proliferation and survival (24). Similarly, in the glioblastoma cell line LN827, the RNA-binding protein ZFP36 binds to and regulates the expression of PIM-1, PIM3, and XIAP leading to a reduction in the cell lines viability and migration (25). We now add CPEB1 to the list of mRNA-binding proteins that may play a role in tumor progression. Our results suggest that targeting the CPEB1 pathway may be an effective way to limit glioblastoma cell migration and tumor progression in vivo.

By inhibiting CPEB1 function, we observe a statistically significant reduction in glioblastoma tumor spread and growth in vivo. This inhibition in tumor size is similar to that which we described in vitro by blocking CPEB1 function (3). Likely, contributing to this decrease in tumor progression by DN-CPEB1-GFP is the inhibition of MTDH/AEG-1 expression shown here as well as the inhibition of β-catenin synthesis showed in a previous study (3).

Undoubtedly, expressing DN-CPEB1 will inhibit the synthesis of many CPE-containing mRNAs and neither the lack of migration nor the reduced tumor size in vivo can be attributed to merely inhibiting the synthesis of these 2 proteins. Rather multiple proteins necessary for both migration and growth are likely contributing to this effect. For example, CPEB1 regulates the expression of cyclin B1 in astrocytes, and blocking CPEB1 function prevents entrance into mitosis, leading to an inhibition of proliferation (6). Furthermore, we have shown in hippocampal neurons that tissue plasminogen activator (tPA), a serine protease that regulates the physiologic processes that necessitate tissue remodeling and cell migration, is regulated by CPEB1 (26). Along with β-catenin, MTDH, and cyclin B1, FABP7 is regulated by CPEB1 in astrocytes (27). Not surprisingly, FABP7 is one of multiple CPE-containing mRNAs that are misregulated in various stages of glioblastoma (Table 1).

In agreement with a role for CPEB in tumor growth, the CPEB1 paralog CPEB4 regulates the translation of a group of proteins necessary for pancreatic ductal adenocarcinoma growth, invasion, and vascularization (28). Originally thought to differ in their mRNA targets (29), recent studies have shown that CPEB4 does recognize and bind to some of the same CPE targets as CPEB1, though with a lower affinity (30, 31). For example, like CPEB1 in hippocampal neurons, CPEB4 has been shown to bind and regulate tPA in both normal and cancerous pancreatic tissue (28). Interestingly, several reports implicate a downregulation of CPEB1 in cancer (32–34), whereas the results presented here suggest that an increase in CPEB1-mediated translation may support the progression of cancer. Because CPEB1 can both inhibit and activate translation, reducing the levels of CPEB1 protein would free mRNA from repression and have the same result as an increase in phosphorylation of CPEB1—namely an increase in target protein expression. For example, the increase in NF-kB signaling and the
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decrease in p53 levels detected in CPEB1 knockout mouse embryo fibroblasts (MEF; refs. 32, 35) could be in part accounted for by an increase in MTDH/AEG-1 expression (12) following the release of its mRNA from translational repression by CPEB1. In either case, inhibiting CPEB1-mediated mRNA translation, with the use of DN-CPEB1-GFP, clearly inhibits tumor growth.

Increasingly, roles for localized mRNA translation in specific cellular compartments are being established and the molecular mechanisms regulating mRNA trafficking and translation are now being elucidated (36, 37). One well-studied example of an element in the 3′-UTR being responsible for cellular trafficking is the β-actin zipcode. In 1994, Kislauskis and colleagues showed that a 54-nucleotide sequence in the 3′-UTR of β-actin was responsible for its intracellular trafficking in chicken embryo fibroblasts and they termed this sequence “zipcode” (38). It was later reported that the zipcode was being bound by an mRNA-binding protein named zipcode-binding protein1 (ZBP1) and the interaction between the 2 was necessary for the localization and translation of the β-actin mRNA (39, 40).

Here, we show that trafficking of a CPEB1-regulated mRNA is dependent upon the presence of CPE sequences in the 3′-UTR. The role of CPE sequences in targeting mRNA has been disputed in neurons, where it has been examined in the context of regulating CaMKII mRNA. In hippocampal neurons, a reporter RNA containing the wild-type CPE sequence from αCaMKII mRNA is more efficiently transported than one containing a mutated CPE (1). However, in this study only the distal 170 nucleotides of the approximately 3,000-nucleotide long αCaMKII 3′-UTR (~5%) was used, and this report was in stark contrast to a previous study, which showed that the most proximal 94 nucleotides of the αCaMKII 3′-UTR were essential for the mRNAs localization (41). Indeed, another group reported a different, 1,200-nucleotide stretch of the αCaMKII 3′-UTR is essential for mediating its dendritic localization (42). It may in fact be that many regions of the 3′-UTR contribute to mRNA localization; thus, for our reporter constructs, we used a larger region (30%) of the native 3′-UTR. We found the presence of the CPEs necessary to mediate mRNA localization and protein synthesis at the periphery of the neurons. Given that MTDH/AEG-1 has distinct functions if expressed in the nucleus or cell surface (14), these data suggest the possibility that local synthesis of MTDH/AEG-1 could play a role in protein localization, and thus function inside the cell. Nuclear shuttling of CPEB1 has been reported in both Xenopus oocytes and HeLa cells in which nuclear binding of CPEB1 to mRNA is thought to regulate the degree to which the mRNAs are expressed in the cytoplasm (43, 44). Several findings support our conclusion that the CPE is necessary for CPEB1-mediated localization and local synthesis in astrocytes and glioblastoma cells. First, constructs in which the CPE sequences have been mutated failed to localize the mRNA to the periphery (Fig. 5). Second, constructs in which the CPE had been mutated were no longer synthesized at the leading edge of migrating astrocytes (Fig. 4). Third, CPEB1 moves between nucleus and cytoplasm (Fig. 5). Fourth, expression of a dominant negative CPEB1 causes the loss of directed cell migration (Fig. 6); likely due to the loss of local synthesis of CPE containing mRNAs. Our data suggest a model in which CPE-containing mRNAs are bound by CPEB1 in the nucleus and trafficked to the periphery of the cell and subsequently translated. In support of this, studies in hippocampal neurons have shown expression of the RNA-binding domain of CPEB1 (akin to our DN-CPEB1-GFP) is primarily localized to the cell body (1, 7) and that the CPEB1-RBD construct is no longer able to bind to dynemin and kinesin (1), the motor proteins thought to be responsible for CPEB1 transport.

Our findings describe a novel role for CPEB1-mediated mRNA translation in tumor progression and suggest disrupting CPEB1-mediated protein synthesis may be a viable therapeutic approach in many types of cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: D.M. Kochanek, D.G. Wells
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.M. Kochanek
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.M. Kochanek
Writing, review, and/or revision of the manuscript: D.M. Kochanek, D.G. Wells
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