Cancer Cells Exploit eIF4E2-Directed Synthesis of Hypoxia Response Proteins to Drive Tumor Progression

James Uniacke, J. Kishan Perera, Gabriel Lachance, Camille B. Francisco, and Stephen Lee

Abstract

Human tumors display considerable diversity in their genetic makeup but share common physiologic attributes such as a hypoxic microenvironment that contribute to the malignant phenotype. Hypoxic cells switch from eukaryotic initiation factor 4E (eIF4E) to eIF4E2 cap-dependent translation to synthesize a portion of their proteins. Here, we show that genetically distinct human cancer cells exploit eIF4E2-directed protein synthesis to form cellular masses larger than approximately 0.15 mm, the diffusion limit of oxygen. Cancer cells depleted of eIF4E2 are indistinguishable from control cells under normoxic conditions, but are unable to survive and proliferate in low oxygen conditions. Activation of eIF4E2-directed translation is essential for cancer cells to form a hypoxic tumor core in in vitro spheroids and to form detectable tumors in in vivo xenograft assays. In contrast, the eIF4E-directed protein synthesis pathway alone cannot sustain cellular adaptation to hypoxia in vitro or confer tumorigenic potential in xenograft assays. These data demonstrate that the phenotypic expression of the cancer genome requires translation by the eIF4E2-directed hypoxic protein synthesis machinery. Cancer Res; 74(5): 1–11. ©2014 AACR.

Introduction

A salient feature of expanding tumors is their ability to outgrow their vasculatures, causing regions in which cells are exposed to low oxygen tension (hypoxia; refs. 1, 2). As oxygen diffuses through approximately 10 layers of cells (3), the majority of cancer cells that populate a tumor are thought to be exposed to hypoxic conditions. Tumor cells must, therefore, adapt to transient fluctuations in the supply of oxygen and nutrients (4). One of the primary cellular responses to low oxygen availability is the activation of the hypoxia-inducible factor (HIF) transcription program (5–8). HIFs are degraded in the presence of oxygen but stabilized by hypoxia to activate an array of genes involved in cellular adaptation to low oxygen availability, including VEGF and glucose transporter 1 (9). Outside of embryogenesis, these extreme oxygen-depleted conditions are rarely experienced by the cells of the body except during ischemic diseases such as cancer. It is believed that activation of the HIF transcription program plays a central role in the ability of cancer cells to thrive in the hypoxic core of tumors.

Once synthesized, mRNAs must undergo translation to produce their corresponding proteins. Typically, protein synthesis is initiated by the binding of the eukaryotic initiation factor 4F (eIF4F) to the 5′ cap found on the majority of mRNAs (10). eIF4F is composed of the cap-binding protein eIF4E, the RNA helicase eIF4A, and the scaffold protein eIF4G (11). The activity of this complex is primarily regulated by the mammalian target of rapamycin complex 1 (mTORC1; refs. 12, 13). During periods of active translation, mTORC1 phosphorylates members of the 4E-binding proteins (4E-BP) to prevent their ability to interact with, and inhibit, eIF4E. In contrast, inhibition of mTORC1 results in the accumulation of hypophosphorylated 4E-BPs that are capable of assembling with eIF4E and prevent cap-dependent translation (14–16). Hypoxia is a potent inhibitor of mTORC1 activity causing 4E-BP–mediated inhibition of eIF4E-dependent translation and a decrease in global translation rates (17, 18). The most commonly accepted interpretation of the hypoxic inhibition of eIF4E-mediated translation is a process whereby cells attenuate protein synthesis to preserve energy, whereas anaerobic metabolism is the sole provider of ATP. Indeed, there is a hypoxia-controlled switch in breast cancer from global eIF4E-mediated cap-dependent translation to selective cap-independent translation (19) that enables cells to synthesize at least a fraction of their proteins (20). Although cancer cells may reduce their energy consumption by decreasing the rate of global translation, they are still fully capable of engaging in intense protein synthesis during the adaptation to hypoxia (20–22). This raises a fundamental question in our understanding of tumor biology about how malignant cells can translate their genetic makeup into a hypoxic cancer cell phenotype during periods of oxygen scarcity.

We recently demonstrated that various normal cells and cancer cells use an alternative translation initiation scheme to
synthesize a portion of their proteins during hypoxia (21). This system relies on the participation of several components. First, the assembly of the oxygen-regulated HIF-2α and the RNA-binding protein RBM4 at 3’ untranslated region (UTR) RNA hypoxia response elements (HRE) found in hundreds of mRNAs that code for proteins with roles in cancer hallmarks such as survival, proliferation, invasion, angiogenesis, and the evasion of apoptosis. Second, the recruitment of the cap-binding protein elf4E2, a homolog of elf4E, to the HIF-2α/ RBM4 complex followed by the capture of the 5’ cap of HRE-containing mRNAs. elf4E2 is an inhibitor of translation during Drosophila development (23) and in normoxic human-derived cells (24). Consistent with these observations, silencing of elf4E2 has little detectable effect on the global translation capacity of cells maintained in normal oxygen tension (21). In contrast, elf4E2-depleted cells have a reduced capacity to synthesize proteins under hypoxic conditions. This is likely because 4E-BPs have a stronger affinity for elf4E than elf4E2, enabling HRE-containing mRNAs to evade mTORC1-mediated arrest of elf4E-dependent translation and contribute significantly to hypoxic protein synthesis (21, 25, 26). Therefore, we hypothesized that tumors require elf4E2-mediated protein synthesis to sustain hypoxic regions and, consequently, grow to significant sizes.

In this report, we show that in vitro and in vivo cancer cells exploit elf4E2-directed translation to express their tumorigenic potential. Depletion of elf4E2 protein prevents genetically diverse cancer cells from forming tumors and slows the growth of already-established tumors as they are unable to adapt to the hypoxic microenvironment. In addition, the elf4E2-mediated protein synthesis machinery alone is insufficient to confer adaptation to low oxygen availability. These data provide evidence that cancer cells exploit the hypoxic translation initiation machinery to express the hypoxic cancer cell phenotype.

Materials and Methods
Cell culture and cell lines
U87MG glioblastoma, 786-O renal cell carcinoma, HCT116 colorectal carcinoma, and renal proximal tubular epithelial cells were used within 6 months of being obtained from the American Type Culture Collection and maintained as suggested. These were characterized by short tandem repeat, Y-chromosome paint and Q-band assays. Cells were incubated at 37°C in ambient O2 levels and a 5% CO2 environment. Hypoxia was induced by incubating at 1% O2, for 24 hours unless otherwise indicated. Cell number assays were performed by plating 10^5 cells in 6-cm plates and measuring the cell number after 48 hours with a hemocytometer.

Polysomal analysis
Polysome analysis was performed as previously described (27).

Protein synthesis by 35S-Met incorporation
De novo protein synthesis was measured as previously described (21).

In vitro spheroids
Performed as previously described (27).

Constructs and short hairpin RNAs
Luciferase constructs were generated and used as previously described (21). GIPZ Lentiviral Human elf4E2 shRNA-mir (ThermoScientific) was used to target the elf4E2 CD5 [V2LHS_68041 short hairpin RNA (shRNA)-I sequence TGAACAGAATATCAA] or the 3’UTR (V3LHS_405000 shRNA-2 sequence CAGCCTGAGATCCTAATAA). A non-targeting shRNA in a pGIPZ vector was used as a control. Clones are identified by their shRNA sequence and clone number (i.e., 1.1 represents shRNA sequence 1 clone 1). Rescue clones were generated by transfecting U87MG elf4E2 knockdown cells (clones 2.1 and 2.3) with an elf4E2 ORF cDNA construct (GeneCopoeia) or a vehicle control containing a neomycin resistance gene.

Western blot analysis and antibodies
Membranes were incubated with primary antibodies anti-EGFR (Ab-12; LabVision), anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Genetex), anti-HIF-2α (Novus), anti-actin (Sigma), anti-L5 (Abcam), anti-S13 (Abcam), anti-elf4E (Genetex), and anti-elf4E2 (Genetex). Secondary antibodies were horseradish peroxidase–conjugated anti-mouse (Amersham Biosciences) or anti-rabbit (Jackson ImmunoResearch Inc.).

Immunohistochemistry
Four-day-old spheroids or biopsied xenografts were frozen in optimum cutting temperature compound. Of note, 10-μm sections were fixed in cold acetone for 10 minutes, and blocked with 10% goat serum in PBS for 1 hour at room temperature followed by incubation overnight at 4°C with primary antibody (1:300) in 5% goat serum. TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling) was performed using the In Situ Cell Death Detection Kit (Roche) as per the manufacturer’s protocol. All specimens counterstained with Hoechst 33242 reagent (Sigma). Blood vessels visualized with hematoxylin and eosin (H&E) as previously described (28).

Cell proliferation and activated programmed cell death assays
For measuring proliferation, cells were incubated for 24 hours in normoxia or hypoxia, then incubated in 10 μmol/L BrdUrd (bromodeoxyuridine; Jackson ImmunoResearch Inc.) for 3 hours, and fixed in 70% ethanol in 50 mmol/L glycine (pH 2.0) for 30 minutes at room temperature. An anti-BrdUrd antibody (1:40) and anti-mouse Alexa594–conjugated secondary antibody (1:200; Invitrogen) were used for detection. For measuring the activation of apoptosis, cells were incubated for

polysomes

polysome

protein synthesis

Spheroids

in vivo

in vitro

Western blot

antibodies

Immunohistochemistry

Cell proliferation

assays
48 hours in normoxia or hypoxia, and fixed in cold methanol for 10 minutes followed by cold acetone for 1 minute. Cells were blocked overnight in 10% FBS and incubated with antiactive caspase-3 primary antibody (Abcam; 1:300) followed by incubation with an anti-rabbit Alexa594–conjugated secondary antibody (1:200). For measuring proliferation in a spheroid section, acetone-fixed slides were blocked with 5% goat serum for 1 hour and washed for 5 minutes in PBS. Slides were incubated with anti-mouse Ki-67 antibody (Dako; 1:150) for 1 hour and washed for 5 minutes in PBS. Slides were incubated with anti-mouse Alexa594–conjugated secondary antibody (1:200) for 1 hour at room temperature.

**Clonogenic and sub-G1 analysis**

For clonogenic analysis, 200 cells were plated and incubated in normoxia or hypoxia for 48 hours. Plates were then incubated in normoxia for 2 weeks to allow colony formation. Colonies were visualized by fixing in 6% glutaraldehyde and staining with 0.5% crystal violet. For sub-G1 analysis, $10^7$ cells were fixed in 70% ethanol for 30 minutes on ice. Cells were pelleted and washed in phosphate–citrate buffer. Cells were treated with 50 μL RNase A (100 μg/mL) and 450 μL propidium iodide (50 μg/mL) for 15 minutes and analyzed by flow cytometry (Beckman Coulter Cyan ADP 9 Analyzer).

**Nude mice xenograft studies**

Female CD-1 nude mice (Charles River Laboratories) were injected in their flanks with $10^6$ or $10^7$ cells in 200 μL sterile PBS. Mice were euthanized 6 to 8 weeks after injection according to facility protocols (University of Ottawa, Ottawa, Canada). Lentiviral infection efficiency was monitored by GFP expression from the pGIPZ vector. Titers yielded $4 \times 10^8$ transduction units per sample. Lentiviral infections for xenografts were designed for a multiplicity of infection of at least four per cell injected. For a xenograft injection of $10^6$ cells, lentiviral injections contained $4 \times 10^8$ transduction unit diluted in 50 μL PBS. When tumors reached a volume of 50 mm$^3$, injections were performed every 48 hours for 8 days with lentivirus containing either control shRNA or shRNA-targeting eIF4E2. To determine tumor volume by external caliper, the greatest longitudinal diameter (length) and the greatest transverse diameter (width) were determined: $1/2 \times (\text{length} \times \text{width})^2$. All experiments were performed double-blinded.

**Statistical analysis**

$P$ values associated with all comparisons were based on two-tailed Student $t$ tests. Results are mean ($n \geq 3$) ± SEM.

**Results**

**Tumor cells activate the eIF4E2 translation machinery**

Normal cells and cancer cells incubated in the absence of oxygen switch from eIF4E to eIF4E2-mediated cap-dependent translation (Fig. 1A and B) in a HIF-2α–dependent manner (21). It is known that a considerable fraction of cells that form a tumor are exposed to a hypoxic microenvironment (3, 29). We thus hypothesized that cancer cells in the hypoxic core of...
tumors require eIF4E2-directed protein synthesis for tumorigenesis. We began our investigation by examining the phosphorylation status of mTORC1 targets in tumors. The kinase activity of mTORC1 is reliant on normal oxygen tension (30). Tumors are exposed to an oxygen environment that is more heterogeneous than in monolayers. Therefore, to determine whether tumors are appropriate models for studying hypoxic translation, we measured their oxygenation and mTORC1 activity. The oxygenation of a spheroid, an avascular in vitro tumor model, was revealed by incubating with hypoxyprobe.

This compound is a pimonidazole hydrochloride that forms reductively activated protein adducts at, or below, 10 mmHg (1.3% O₂), which can be recognized by specific antibodies (31). Spheroids of HCT116 colorectal carcinoma and U87MG glioblastoma cells revealed a thin nonhypoxic edge (~100 μm) and a hypoxic core that accounted for 80% to 90% of the area of the section (Fig. 1C and Supplementary Fig. S1A). Immuno-staining of mTORC1 targets revealed a biphasic staining pattern where phosphorylated inactive 4E-BP or ribosomal protein S6 were detected solely at the oxygenated edge (Fig. 1D and E and Supplementary Fig. S1B). In contrast, total 4E-BP levels were induced in the majority of cells that composed the hypoxic core of a spheroid (Fig. 1F and Supplementary Fig. S1C). Consistent with these observations, eIF4E protein was observed mostly in the monosomes fractions of spheroids, suggesting that it does not participate in active protein synthesis (Fig. 1B). This differed considerably with eIF4E2, which was observed in monosomes of oxygenated monolayers of cells, but abundantly present in polysomes of spheroids (Fig. 1B). An enrichment toward the eIF4E2-directed translation machinery was also apparent in vivo in which there is an added layer of complexity due to abnormal vascularization creating pockets of oxygen (32, 33). We confirmed that there are indeed hypoxic pockets with inactive mTORC1 by probing for phosphorylated 4E-BP in xenografts of glioblastoma cells. Sections immunolabeled for phosphorylated 4E-BP revealed mTORC1 inactivity in the core, as well as in a region between the core and the surface (Supplementary Fig. S1D). A similar region was stained with H&E to highlight the presence of blood vessels (Supplementary Fig. S1E). Furthermore, eIF4E2 was observed in polysome fractions of xenografts (Fig. 1B). These data suggest that eIF4E2 actively participates in the translation machinery of the majority of cancer cells that constitute a tumor.

**Tumor cells require eIF4E2-directed protein synthesis to proliferate and survive under hypoxia**

Activation of eIF4E2-mediated translation occurs in hypoxic but not normoxic conditions (21). We reasoned that inhibiting eIF4E2 could selectively inhibit the survival of tumor cells exposed to hypoxia. To address this, we silenced eIF4E2 in three genetically distinct cancer cell lines: U87MG glioblastoma (PTEN-null), 786-O renal cell carcinoma (VHL-null), and HCT116 colorectal carcinoma (KRAS mutation). Partial or complete reduction of eIF4E2 protein was achieved using two independent shRNAs (Fig. 2A and Supplementary Fig. S2A). The eIF4E2 knockdown cell lines were viable, displayed unaltered morphology compared with control cells, and proliferated normally under normoxic conditions. In contrast, we failed to generate stable cell lines with detectable reduction of eIF4E. This was likely due to the arrest of normoxic protein synthesis followed by cell death during the selection process. Cell lines stably expressing shRNA-targeting eIF4E2 failed to induce the expression of the EGF receptor (EGFR) under hypoxic conditions (Fig. 2B and Supplementary Fig. S2B). The hypoxic induction of EGFR has been previously shown to be dependent on HIF-2α (27, 28) and eIF4E2 (21). Renal cell carcinoma cells did not require hypoxia to induce EGFR because they constitutively express HIF-2α (Supplementary Fig. S2B). Stably silencing eIF4E2 considerably reduced the rate of global hypoxic translation highlighting its role as an activator of global hypoxic protein synthesis (Fig. 2C). Furthermore, eIF4E2 was required to activate the hypoxic expression of a luciferase reporter containing a 3′UTR rHRE (Fig. 2D). Silencing eIF4E2 had no effect on proliferation and number of glioblastoma, renal, and colorectal cancer cells maintained in normoxic conditions (Fig. 2E and Supplementary Fig. S2C and S2D). However, a significant decrease in proliferation and number of the same cells relative to control cells was observed in hypoxic conditions (Fig. 2E and Supplementary Fig. S2C and S2D). These same cells failed to adapt to low oxygen tension and subsequently entered apoptosis (Supplementary Fig. S3A–S3C) or failed to form colonies (Supplementary Fig. S3D). Importantly, normal human renal proximal tubular epithelial cells transiently expressing shRNA-targeting eIF4E2 displayed decreased survival and proliferation only under hypoxia (Supplementary Fig. S4). These data suggest that eIF4E2 dependence is not a cancer-dependent event, but a physiologic mechanism that is exploited by cancer cells attempting to survive low oxygen conditions.

**eIF4E2 depletion prevents tumorigenesis of genetically diverse human cancers**

The ability of cancer cells to adapt to a hypoxic tumor microenvironment is believed to be a key determinant in the evolution of malignancy. In principle, cancer cells would be required to engage in eIF4E2-directed hypoxic protein synthesis during the initial steps of tumorigenesis. To test this, nude mice were injected subcutaneously in their flanks with cancer cells stably expressing either control shRNA or shRNA-targeting eIF4E2. Four to 5 weeks after injection, glioblastoma cells stably expressing control shRNA produced large tumors more than 200 to 300 mm³ in volume relative to cells stably expressing shRNA-targeting eIF4E2, which formed initial minimal masses that were eventually no longer visible (Fig. 3A and Supplementary Fig. S5A). Analogous results using eIF4E2-depleted renal (Fig. 3B) and colorectal carcinomas (Fig. 3C) were obtained. To emphasize eIF4E2 as an attractive antitumor target, established tumors (50 mm³) in nude mice were injected every 2 days for 8 days with lentiviruses harboring shRNA-targeting eIF4E2 or non-targeting control shRNA. Glioblastoma and colorectal carcinoma xenografts injected with shRNA-targeting eIF4E2 decreased in size after the second injection and had an average final volume that was 5-fold and 2-fold smaller relative to the control, respectively (Fig. 3D and E). Immunohistochemistry (IHC) revealed that the hypoxic regions of these xenografts had similar levels of proliferation,
Figure 2. Cancer cells require eIF4E2-directed protein synthesis to survive and proliferate during hypoxia. A, two shRNAs were used independently. shRNA-1 targeted the coding sequence (CDS) and shRNA-2 the 3’UTR of eIF4E2 mRNA. Three clones were selected from three genetically diverse cancer cell lines (U87MG glioblastoma, 786-O renal cell carcinoma, and HCT116 colorectal carcinoma) stably expressing shRNA targeting the eIF4E2 CDS (1.1, 1.2, and 1.3) or eIF4E2 3’UTR (2.1, 2.2, and 2.3). Clones stably expressing a nontargeting control shRNA (C1.1, C1.2, and C1.3) were also selected. See also Supplementary Fig. S2A. B, the ability of each clone to induce EGFR protein when exposed to normoxia or hypoxia was determined. GAPDH was used as a loading control. C, global translation rates in normoxia or hypoxia of cells stably expressing shRNA-targeting eIF4E2 (1.1 and 2.1) or control shRNA (C1.1). GLUT mRNA used as a marker for hypoxia. D, luciferase expression was measured in eIF4E2-depleted cells (1.1 and 2.1) relative to control cells (C1.1) expressing a reporter construct containing a 3’UTR RNA hypoxia response element. E, the ability of cells stably expressing control shRNA (C1.1) or shRNA-targeting eIF4E2 (1.1, 2.1, and 2.2) to proliferate was measured following incubation in normoxia or hypoxia for 24 hours. F, active caspase-3-positive cells stably expressing control shRNA (C1.1) or shRNA-targeting eIF4E2 (1.1, 2.1, and 2.2) incubated in normoxia or hypoxia for 48 hours. E and F, data reported relative to cells stably expressing nontargeting shRNA (C1.1). Columns, mean (n = 3); error bars, SEM. Significance measured by the Student t test; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
but eIF4E2-depleted xenografts had smaller regions of hypoxia that were adjacent to large areas of cell death (Fig. 3F). The xenografts were confirmed to be infected with lentiviruses by detecting the GFP expressed by the pGIPZ backbone (Supplementary Fig. S5B and S5C). Although not every cell in the tumor was infected, a corresponding decrease in eIF4E2 protein levels was observed (Supplementary Fig. S5B and S5C). We were unable to excise and study tumors from cells stably expressing shRNA-targeting eIF4E2 because of their absence.

Formation of a hypoxic tumor core necessitates eIF4E2-directed protein synthesis

Regions of hypoxia occur early in tumor formation as oxygen diffuses through approximately 10 cellular layers (3). Therefore, we used 4-day-old spheroids, an in vitro tumor model, to observe the effects of eIF4E2 depletion at the early stages of tumor formation. Typically, cancer cells are able to produce hypoxic spheroids that enlarge over time. However, eIF4E2-depleted spheroids formed fragile, loosely packed aggregates (Fig. 4A and Supplementary Fig. S6A) that failed to increase to sizes larger than 500 mm even after several days in culture relative to control spheroids (Fig. 4B and Supplementary Fig. S6B). Interestingly, eIF4E2-depleted spheroids had no detectable expression of the hypoxia markers HIF-1α or HIF-2α, compared with spheroids expressing control shRNA (Fig. 4C and Supplementary Fig. S6C). Furthermore, staining of spheroids with hypoxyprobe revealed that they were unable to produce a hypoxic tumor microenvironment (Fig. 4D), unlike spheroids generated from control cells (Fig. 1C and Supplementary Fig. S1A), even though their radii exceeded the oxygen

Figure 3. Silencing of eIF4E2 prevents tumorigenesis in genetically diverse human cancers. A–C, mice were injected with 10^7 U87MG glioblastoma (A), 786-O renal cell carcinoma (B), and HCT116 colorectal carcinoma (C) cells stably expressing nontargeting shRNA (C1.1 and C1.2) or shRNA-targeting eIF4E2 (1.1, 1.2, 2.1, and 2.2). D and E, established tumors (~50 mm^3) of glioblastoma (D) or colorectal carcinoma (E) were injected every 2 days for 8 days with lentiviruses harboring shRNA-targeting eIF4E2 (shRNA-1 or shRNA-2) or control shRNA. Change in tumor volume reported relative to volume before first lentiviral injection. Data, mean ± SEM of at least three independent experiments. Significance measured by the Student t test; *, P < 0.05; **, P < 0.01; ***, P < 0.001. F, IHC of serial sections from D and E, staining for hypoxia (CAIX), proliferation (Ki-67, brown nuclei), and cell death (TUNEL). Dotted line, hypoxia. Scale bar, 100 μm.
diffusion limit (3). Consistent with the presence of ample oxygen in the eIF4E2-depleted in vitro tumors, mTORC1 activity was observed throughout the spheroids. IHC revealed phosphorylated 4E-BP to be evenly distributed throughout the spheroid (Fig. 4E), as opposed to being localized to the outer, more oxygenated edge in the controls (Fig. 1D and Supplementary Fig. S1B). Immunostaining for antiactive caspase-3 and TUNEL assays revealed less cell death in controls (Fig. 5A) compared with eIF4E2-depleted spheroids (Fig. 5B). Finally, the distribution of proliferative cells differed between control and eIF4E2-depleted spheroid sections. In a control spheroid, a higher concentration of proliferative cells was observed in the oxygenated edge than in the core (Fig. 5C). This compartmentalization is consistent with the spheroid oxygen gradients shown with hypoxyprobe (Fig. 1B and Supplementary Fig. S1A). Conversely, an eIF4E2-depleted spheroid did not have a distinct border between the edge and core, but a uniform distribution of proliferative cells (Fig. 5D). These data demonstrate that eIF4E2-directed translation confers the ability to cancer cells to form large tumors with hypoxic cores in vitro. These results also suggest that eIF4E2-depletion leads to more rapid cell death in the cores of early tumors.

Figure 4. Formation of a hypoxic tumor core necessitates eIF4E2-directed translation. A, light micrographs of U87MG glioblastoma and HCT116 colorectal control spheroids (C1.1) compared with eIF4E2-depleted spheroids (1.1 and 2.1). B, spheroid growth was monitored in cells stably expressing control shRNA (C1.1) or shRNA-targeting eIF4E2 (1.1, 2.1, and 2.2). Data, mean ± SEM of three independent experiments. Significance measured by the Student t test; *, P < 0.05; **, P < 0.01; *** P < 0.001. C, Western blot analysis of 4-day-old spheroids stably expressing control shRNA or shRNA-targeting eIF4E2. HIF-1α was used as a marker for hypoxia. GAPDH was used as a loading control. D and E, immunolabeling of a 4-day-old glioblastoma spheroid stably expressing shRNA-targeting eIF4E2 incubated with hypoxyprobe (D) or anti-P4E-BP (E), revealing active mTORC1 throughout. Hoechst was used as a DNA counterstain. Solid line, spheroid border. Scale bar, 100 μm.
Reintroduction of exogenous eIF4E2 restores tumor cell characteristics

To substantiate that the observed phenotypes were specific to eIF4E2 knockdown, eIF4E2 was stably reintroduced into two glioblastoma cell lines stably expressing shRNA targeting the eIF4E2 3’UTR (clones 2.1 and 2.3). Because the exogenously expressed eIF4E2 harbors the vehicle-derived 3’UTR, only the endogenous eIF4E2 is targeted by the stably expressed shRNA. Western blot analysis revealed the reappearance of eIF4E2 in knockdown cells stably expressing exogenous eIF4E2 (Y and Z), but not in vehicle control cells (v1 and v2; Fig. 6A). Furthermore, these rescued cells regained the ability to induce EGFR in hypoxia, whereas vehicle control cells did not (Fig. 6B and Supplementary Fig. S7A). Knockdown cells stably expressing exogenous eIF4E2 also recovered the ability to divide in hypoxia. The cell number significantly increased for rescued cells in hypoxia relative to eIF4E2-depleted vehicle control cells (Supplementary Fig. S7B). Conversely, cell numbers did not change during normoxic exposure regardless of eIF4E2 protein levels (Supplementary Fig. S7B). Cellular proliferation was also rescued by eIF4E2 reintroduction in cells cultured in hypoxia, but there was no difference in normoxia (Fig. 6C and Supplementary Fig. S7C). To address whether the reintroduction of eIF4E2 reduced the initiation of apoptosis under low oxygen tension, the percentage of cells with active caspase-3 was compared between hypoxia and normoxia. Indeed, significantly less active caspase-3-positive cells were observed in hypoxic rescued cells relative to vehicle control cells, but there was no change in normoxic conditions (Fig. 6D and Supplementary Fig. S7D). Importantly, larger (Fig. 6E and Supplementary Fig. S7E), more hypoxic (Fig. 6F and Supplementary Fig. S7F) spheroids were observed when eIF4E2 was stably reintroduced. Finally, the ability of eIF4E2 reintroduction to restore tumor cell growth in an in vivo setting was measured. Nude mice were injected with vehicle control eIF4E2 knockdown cells on one flank and rescues on the other flank. Significantly larger tumors were observed as early as 2 weeks after injection of rescued cells relative to vehicle control cells (Fig. 6G and Supplementary Fig. S7G). These results establish a critical role for eIF4E2 and hypoxic protein synthesis in the early stages of tumor formation and highlight the central role of this alternative translation initiation pathway regardless of the tissue of origin or the mutational profile of the cancer cells.

Discussion

Prokaryotic infections such as pneumonia and tuberculosis were the leading cause of death before the advent of antibiotics. Many antibiotics are protein synthesis inhibitors that selectively inhibit the bacterial machinery, but not that of the host, providing a window for effective treatment. It is generally believed that cancer cells exploit the same protein synthesis machinery as normal cells. This has hampered efforts in identifying compounds that would selectively prevent the protein synthesis of cancer cells with limited toxicity to normal cells. The data shown here demonstrate that tumor cells exploit a parallel alternative cap-dependent protein synthesis machinery that functions in hypoxia and relies on eIF4E2. This occurs when cancer cells rapidly encounter hypoxia en route to forming a tumor requiring eIF4E2-directed translation for survival. This dependence on eIF4E2 provides a unique window.
to develop anticancer agents that prevent the protein synthesis of tumor cells without affecting normoxic somatic cells, analogous to the function of antibiotics.

Our data demonstrate that as tumors grow, and their cores become increasingly hypoxic, eIF4E2 depletion results in less proliferation (Fig. 2E and Supplementary Fig. S2C) and an increase in cell death (Fig. 2F and Supplementary Fig. S3). Tumors lacking eIF4E2 are too small to notice beneath the skin of a nude mouse, whereas a parallel control reaches an ethical endpoint in the same time frame (Fig. 3A). Furthermore, in vitro eIF4E2-depleted spheroids could not exceed a diameter of 500 μm and were loose, fragile, and fully oxygenated (Fig. 4). The data also indicate that several genetically diverse cancer cells in a physiologic tumor setting do not solely exploit the eIF4E-dependent translation system, even though it is sometimes hyperactive or functionally deregulated compared with normal cells. Indeed, eIF4E is not as strongly associated with polysomes as eIF4E2 in spheroids and xenografts (Fig. 1B). In addition, eIF4E-mediated translation cannot sustain the formation of hypoxic spheroids or detectable tumors in xenograft assays in the absence of eIF4E2. Thus, cancer cells must activate the eIF4E2-directed hypoxic translation system to adapt to hypoxia and to form tumors independently of the functional attributes or expression profiles of eIF4E.

The link between eIF4E2 and cancer is not unprecedented. One study identified candidate polymorphisms that influence overall survival in patients with advanced non–small cell lung cancer (34). In this genome-wide association study, the strongest predictor of shortened overall survival was a single-nucleotide polymorphism (SNP) in eIF4E2. Interestingly, the frequency of this SNP differed across ethnic backgrounds (rs1656402). Another study described a molecular signature in primary solid tumors that is a predictor of...
metastatic potential (35). Eight genes made up the strongest predictors of metastasis, including eIF4E2 referred to by its alias elf4E2L3.

eIF4E is overexpressed in a variety of cancers and is a target of modern therapies (36–41). Furthermore, elf4E contributes to tumorsphere growth in hypoxic breast cancer cells (42). Indeed, elf4E remains an attractive target to inhibit the growth of cancer cells. According to our model, the more oxygenated edge of tumors would be more susceptible to elf4E inhibitors because it has more translation competent elf4E due to active mTORC1 (Fig. 1C–F and Supplementary Fig. S1A–S1D). Interestingly, studies are emerging that directly target elf4E causing smaller tumors in mouse models. One study uses antisense oligonucleotides (ASO) targeting elf4E and is the first elf4E-specific therapeutic to advance to clinical trials (36). These authors report similar effects of silencing elf4E as we do with elf4E2, such as a reduction in tumor growth in mice, a reduction in cellular proliferation and an increase in apoptosis. However, the observed elf4E silencing is incomplete and the phenotype is not rescued by reintroduction of elf4E. The cell lines produced in our study display a strong reduction of elf4E2 and, more importantly, the phenotype was rescued by the expression of exogenous elf4E2 that evaded shRNA recognition. We attempted to create cancer cell lines stably expressing shRNA-targeting elf4E, but, as silencing elf4E significantly hampers normoxic protein synthesis, clones were not viable in multiple attempts. The clones that we did obtain retained levels of elf4E protein and normoxic protein synthesis that we found too high and, thus, unsatisfactory to further investigate in tumor assays. Therefore, it seems that there is a limited window in which elf4E can be reduced enough to affect the ability of cancer cells to form tumors, but have minimal toxic effects to the organism. Many questions remain for such a clinical trial, however, as the toxicity of targeting a ubiquitous and important protein synthesis component, and the dosage of ASOs required to achieve the desired effect in humans. In conclusion, we propose that targeting elf4E2-directed hypoxic translation would be a less toxic and more direct alternative to selectively inhibit the protein synthesis machinery of tumor cells regardless of their tissue of origin or mutation profile.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: J. Uniacke, J.K. Perera, G. Lachance, S. Lee

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Tumorigenesis Requires elf4E2-Directed Translation

Cancer Cells Exploit eIF4E2-Directed Synthesis of Hypoxia Response Proteins to Drive Tumor Progression

James Uniacke, J. Kishan Perera, Gabriel Lachance, et al.

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