The eIF4E2-mediated hypoxic protein synthesis complex permits tumourigenesis in several genetically distinct cancers

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Abstract

Identifying exploitable differences between cancer cells and normal cells has been ongoing since the dawn of cancer therapeutics. This task has proven difficult due to the complex genetic makeup of cancers. Tumours, however, share a low oxygen (hypoxic) microenvironment that selects for malignant cancer cells. It has recently been shown that cells switch from eIF4E to eIF4E2-mediated protein synthesis during periods of hypoxia, similar to those found in tumour cores. We hypothesize that this hypoxic translation complex is required for cell survival in hypoxia and can be targeted by inhibiting the eIF4E2 cap-binding protein. Here, we show that genetically diverse cancer cells require the cap-binding protein eIF4E2 for their growth, proliferation, and resistance to apoptosis in hypoxia, but not in normoxia. Furthermore, *in vitro* and *in vivo* eIF4E2-depleted tumour models cannot grow or sustain hypoxic regions without the reintroduction of exogenous eIF4E2. Thus, tumour cells could be targeted over somatic cells by selectively inhibiting their protein synthesis machinery, much like the function of antibiotics that revolutionized medicine.

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List of Abbreviations

4E-T	4E-Transporter
4EBP	4E-Binding Protein
CA	Carboanhydrase
ccRCC	Clear Cell Renal Cell Carcinoma
Cul-2	Cullin-2
EGFR	Epidermal Growth Factor Receptor
eIF	Eukaryotic Initiation Factor
EPO	Erythropoietin
EPRI	Electron Paramagnetic Resonance Imaging
ER	Endoplasmic Reticulum
FIH	Factor Inhibiting HIF
GDP	Guanosine Diphosphate
GLUT	Glucose Transporter Protein
GTP	Guanosine Triphosphate
GβL	G protein β-subunit like protein
HIF	Hypoxia Inducible Factor
HRE	HIF-Responsive Elements
HRI	Heme-Regulated Inhibitor of Translation
IGF-2	Insulin-like Growth Factor Receptor
IRES	Internal Ribosome-Entry Site
MET-tRNA	Methionine transfer RNA

MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
mTOR	Mammalian Target of Rapamycin
N-TAD	Amino-Terminal Transactivating Domain
NO	Nitric Oxide
ODD	Oxygen Dependent Degradation Domain
PABP	Poly-(A)-Binding Protein
PERK	RNA-like Endoplasmic Reticulum Kinase
PET	Positron Emission Tomography
PHD	Prolyl Hydroxylases
PKR	RNA-activated Protein Kinase
pO2	Oxygen Pressure
rHRE	RNA Hypoxic Response Elements
S6K1	S6 Protein Kinase 1
SEM	Scanning Electron Microscopy
UTR	Untranslated Region
VEGFR	Vascular Endothelial Growth Factor Receptor
VHL	von Hippel-Lindau Protein

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***See Appendix at end of thesis for table of contributions**

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1.0 INTRODUCTION

1.1 The Nature of Cancer

The eukaryotic cell is the fundamental unit that composes multicellular organisms. Cell life is strictly controlled through numerous molecular pathways that respond to the chemical and physical environment. In multicellular organisms these environments can affect such events as cell differentiation, enzyme release and cell division, to name a few. Cell division, known as mitosis in mammalian cells, is the division of one parent cell into two daughter cells (Gall and McIntosh, 2002). A set of diseases exists which are defined by uncontrolled cell division in an abnormal cell. The generic term cancer, describes these diseases. Cancer is a leading cause of death world wide, accounting for 13% of deaths in 2008 (Ferlay J, 2008). It is estimated that 75,000 Canadians died due to cancer in 2011, and that 25% of women and 29% of men in Canada will die from cancer in 2012 (Statistics, 2012).

1.2 Genetic Alterations Lead to Cell Transformation

Like a car, cells have a gas pedal (oncogenes) and brakes (tumour suppressors) that regulate cell proliferation. An oncogene is defined as a gene that promotes cell division and, when mutated, will constantly order cells to divide, which can transform a cell into a cancer cell (Croce, 2008; Rous, 1979).

1.2.1 Oncogenes

In 1970, G. Steven Martin discovered the first oncogene while studying the rous sarcoma virus (Martin, 1970; Rous, 1979). The Rous sarcoma virus was initially studied for its ability to produce tumours in chickens. After further investigation, it was soon learned that a virus affecting the c-src proto-oncogene produced the tumours (Dorai et al., 1991). Before an oncogene becomes overactive due to a mutation, it is known as a proto-oncogenes (Todd and Wong, 1999). Proto-oncogenes generally encode growth factors, growth factor receptors as well as proteins in signal transduction cascades (Antonicelli et al., 2013; Geletu et al., 2012; Goodsell, 1999a; Malumbres and Barbacid, 2003; Nilsson and Cleveland, 2003). As an example, the *Ras* oncogene produces proteins that are involved in intracellular signaling from cell surface receptors, such as growth factor receptors and G-coupled protein receptors (Goodsell, 1999b). These intracellular protein cascades have effects ranging from lipid metabolism, to DNA synthesis (Goodsell, 1999b). Ras protein acts as a messenger and is regulated through phosphorylation. Specifically, Ras protein incorporates guanosine diphosphate (GDP) in its inactive form, which is exchanged for guanosine triphosphate (GTP) in its active form. Ras-GTP has a short half-life so unless it is reactivated it will become inactive after delivering its intracellular message. *Ras* mutations can produce a Ras protein that is insensitive to the phosphorylation of guanosine, and remain active at all times. This can have drastic effects on malignancy of cancers by giving mutated cells a proliferative advantage compared to non-mutated cells (Malumbres and Barbacid, 2003).

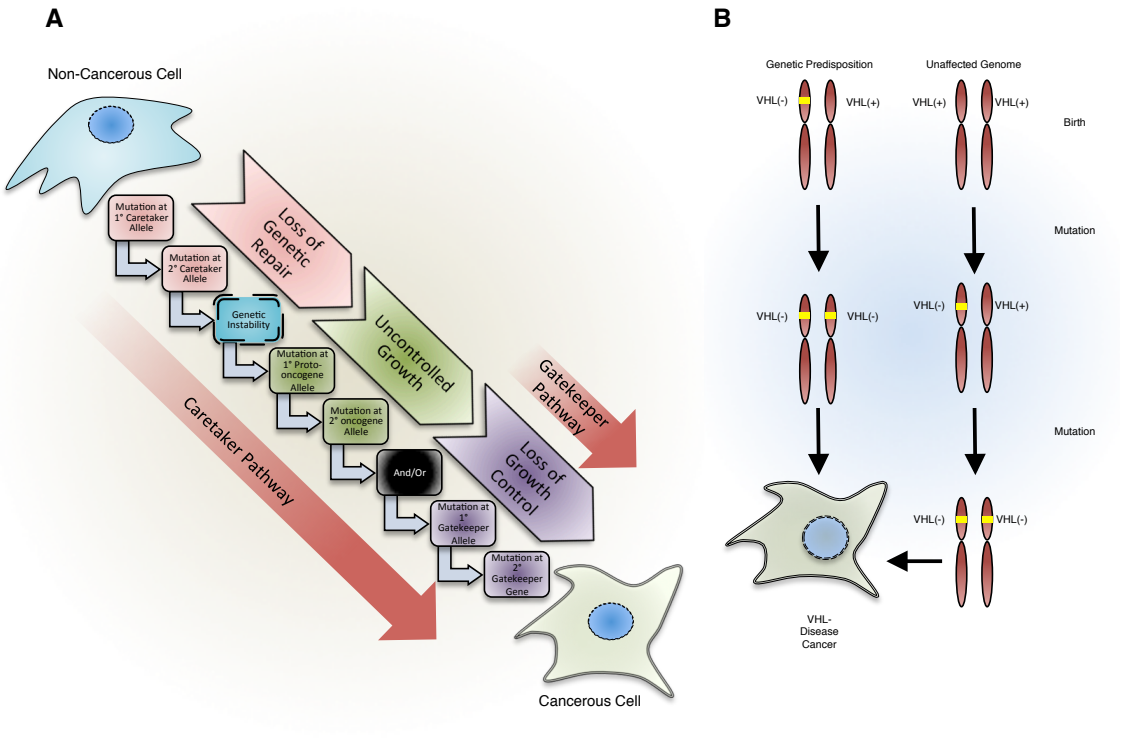
1.2.2 Tumour Suppressors: Gatekeepers, Caretakers, Landscapers

The cellular brake, known as tumour suppressor proteins, signals to stop cell division or cause programmed cell death in order to inhibit abnormal cell growth (Knudson, 1971). Tumour suppressors are mutated at the gene level, and although single gene mutations have been shown to produce dysfunctional tumour suppressors (Amos-Landgraf et al., 2012; Salmena and Narod, 2012; Santarosa and Ashworth, 2004; Su et al., 2012), most require a mutation at both alleles (Figure 1). For instance, these detrimental mutations may occur through deletion of an entire chromosome (Lewis, 2005).

Tumour suppressor proteins can be further subdivided into caretaker, gatekeeper, and landscaper genes. Caretaker proteins maintain genome stability and help stop mutations in genes that can lead to cancer (Kinzler and Vogelstein, 1997). Gatekeeper proteins, on the other hand, help to monitor cell division and under certain circumstances promote cell death. Cellular transformation towards neoplasia is not possible without the deregulation of, or mutations to, gatekeeper proteins (Figure 1A) (Michor et al., 2003). Mutations in both maternal and paternal genes are necessary for transformation (Vogelstein and Kinzler, 2004). If the cell has a pre-existing mutation which occurred in the parental germ line cell, known as a genetic predisposition, then only one mutation is needed, otherwise two somatic mutational events are necessary, see Figure 1B (Kinzler and Vogelstein, 1997). Typically, abnormal cell growth is inhibited through programed cell death, however since gatekeeper proteins regulate apoptosis, this can be bypassed through gatekeeper gene mutations leading to cancer. A new subdivision of tumour suppressor genes has also been recognized and

Figure 1. Genetic Alterations Lead to Abnormal Cell Proliferation and Tumourigenesis

[A] Transformation into a cancer cell requires several genetic mutations. According to the Caretaker pathway, mutations first occur at the caretaker alleles, which increases the probability of mutations in tumour suppressor or oncogene alleles. Tumour suppressors are required for tumourigenesis and the gatekeeper pathway requires mutations at only the tumour suppressor alleles. **[B]** *VHL* mutations illustrate an example of a tumour suppressor gene mutation. Patients who inherited a genetic predisposition due to a mutation in the parent's germ line cell requires fewer mutations to develop cell transformation than a patient without a genetic predisposition.


A
Non-Cancerous Cell
Mutation at 1° Caretaker Allele
Mutation at 2° Caretaker Allele
Genetic Instability
Mutation at 1° Proto-oncogene Allele
Mutation at 2° oncogene Allele
And/Or
Mutation at 1° Gatekeeper Allele
Mutation at 2° Gatekeeper Gene
Loss of Genetic Repair
Uncontrolled Growth
Loss of Growth Control
Caretaker Pathway
Gatekeeper Pathway
Cancerous Cell
B
Genetic Predisposition
Unaffected Genome
VHL(-)
VHL(+)
VHL(+)
VHL(+)
Birth
Mutation
VHL(-)
VHL(-)
VHL(-)
VHL(+)
Mutation
VHL(-)
VHL(-)
VHL-
Disease
Cancer

named the landscaper genes. These genes generate products that bind to the extracellular matrix or neighboring cells. As a result, mutations in these genes lead to a development of a growth promoting environment (Schneider, 2011). It is thought that these proteins can clear extracellular matrix molecules leading to invasion by human immune cells, which can release growth factors that further stimulate growth of mutant cells (Bissell and Radisky, 2001). Landscaper gene mutations do not seem to be vital for malignancy, however, they show strong support for the mutant phenotype (Schneider, 2011).

Tumour suppressor gene and oncogene mutations give mutant cells a selective advantage through increased cellular proliferation, decreased cell cycle arrest, and ability to evade apoptosis. As a result of mutations, affected cells can abnormally outgrow their non-mutated counterparts. These numerous mutations originate in a variety of tissues, making cancer a very complex disease. However, cancers do share some physiological attributes, such as the tumour microenvironment and the hallmarks of cancer.

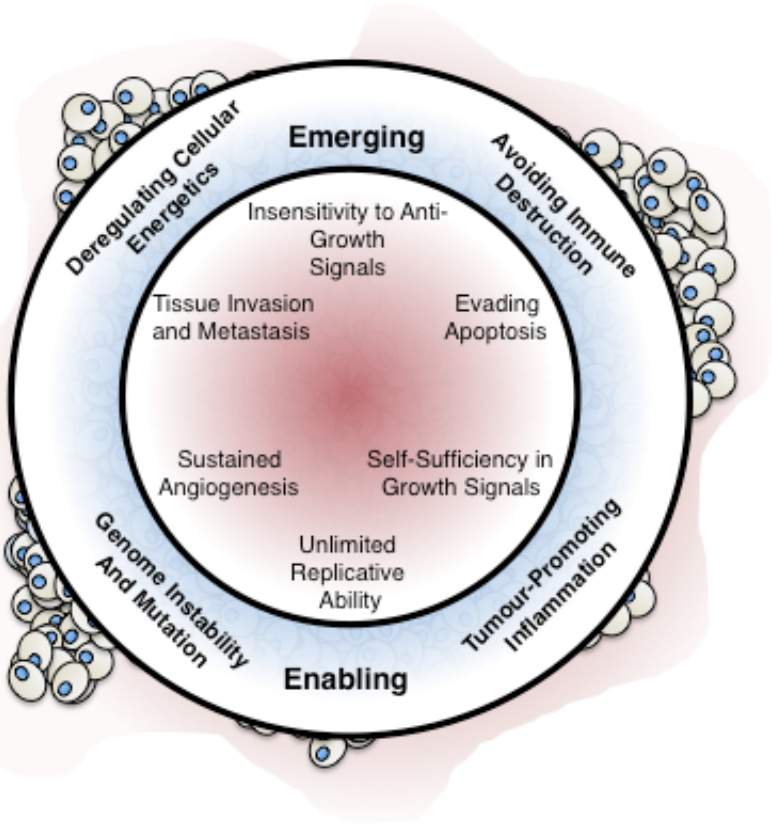
1.2 The Hallmarks of Cancer

Cancers are complex genetic diseases and many distinct cancer types exist. Our body of knowledge has helped identify several shared traits, known as the hallmarks of cancer (Figure 2). These traits include the ability to sustain angiogenesis, grow autonomously, evade anti-growth signals, invade and metastasize, evade apoptosis and produce growth signals self-sufficiently (Hanahan and Weinberg, 2000). Recently, emerging hallmarks such as deregulated energetics and evasion of immune destruction have been added to this list (Hanahan and Weinberg, 2011). An increasingly recognized component to tumourigenesis is the tumour microenvironment, which produces enabling characteristics

Figure 2. The Hallmarks of Cancer.

Cancers are defined by several traits, which include: insensitivity to growth signals, evasion of apoptosis, self-sufficiency in growth signal production, unlimited replicative potential, sustained angiogenesis, and tumour invasion and metastasis.

Hallmarks of Cancer



Modified from: Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell, 144*(5), 646-674. doi: 10.1016/j.cell.2011.02.013

such as genome instability and mutation, and tumour promoting inflammation (Hanahan and Weinberg, 2011). The hallmarks are summarized in Figure 2.

1.3 The Tumour Microenvironment is a Shared Physiological Trait

Malignant tumours are highly complex due to the many genetic differences that lead to tumourigenesis. However, solid tumours form organ-like structures that are similar in their physical and biochemical microenvironment. Metabolic alterations, specifically aerobic glycolysis (Hanahan and Weinberg, 2011) and carbonic anhydrase activity (Helmlinger et al., 2002), are a defining characteristic in cancer cells that lead to low extracellular pH. Alternatively, low tumour pH may result from vasculature inadequacies that poorly transport extracellular acidic molecules from tumours (Fukumura and Jain, 2007). Insufficient vasculature is a major cause of low oxygen availability in developing tumours. The resulting tumour hypoxia has become a serious challenge for chemo- and radiotherapy.

1.4 Tumour Hypoxia: Development and Measurement

1.4.1 Development of Hypoxia in Tumours

Hypoxia is a major component of the tumour microenvironment, and can be achieved through:

a) Defects in development of vasculature

b) Outgrowth of oxygen diffusion limit

c) Reduced oxygen transport capacity of blood

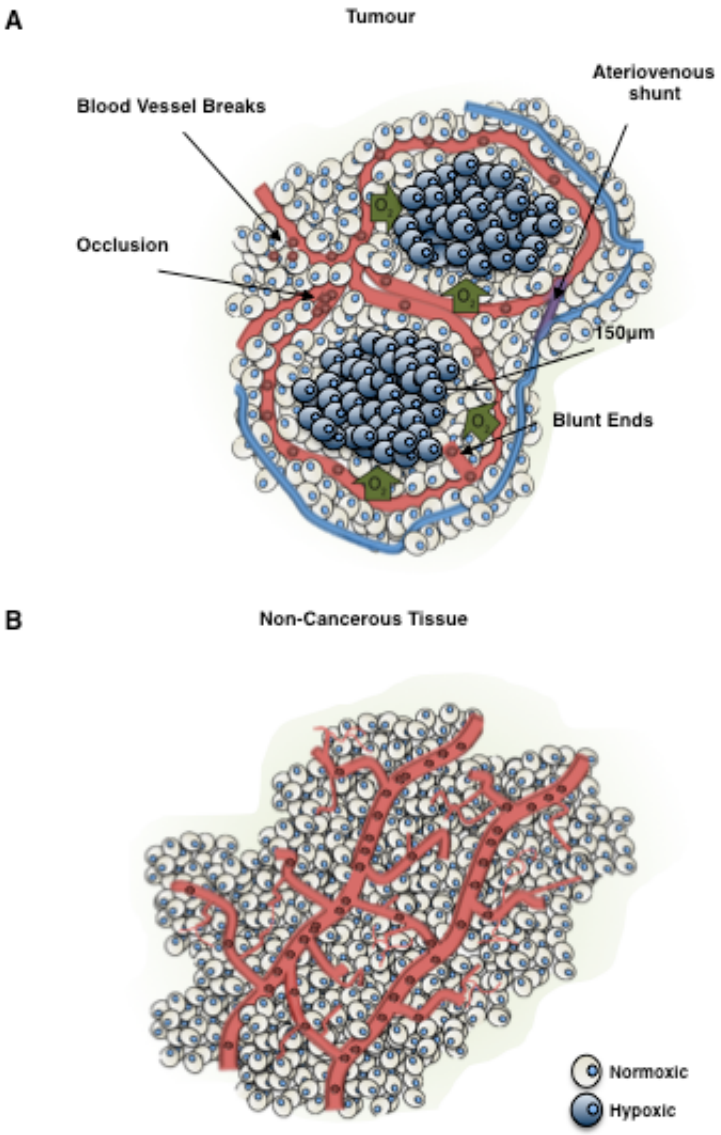
Tumours grow at an abnormal rate, which often results in severe structural deformities in vasculature (Fukumura and Jain, 2007). The resulting deformities cause fluctuations in blood perfusion through the tumour with varying periods of oxygen availability (Yuan et al., 1994). Using light microscopy and scanning electron microscopy (SEM) it has been shown that tumours have poor blood vessel development leading to blunt-endings of blood vessels, as illustrated in Figure 3A (Grunt et al., 1985). Additionally, while comparing tumours to granulating tissues, it was noticed that tumours had four times less vessel density (Dewhirst et al., 1989). Furthermore, 3-dimensional casting techniques have revealed that tumours experience leaky blood vessels (Figure 3A) that are highly irregular and not adequate for the tissue size (Shah-Yukich and Nelson, 1988). Together, these studies reveal the poor vasculature development leading to acute and chronic hypoxia in tumours. Tumours may experience chronic hypoxia if a region is beyond the diffusion limit of oxygen, approximately 150 μm (Groebe and Vaupel, 1988; Olive et al., 1992). The tumour vasculature abnormalities are summarized in Figure 3. Lastly, hypoxia can result from chemotherapy, non-chemotherapeutic drugs, or tumour adaption through the reduction in the ability of blood to transport oxygen. This condition is known as anemic hypoxia. These regions of low oxygen availability appear to be physiologically rare (Carreau et al., 2011).

1.4.2 Advances in pO_2 Measurement Has Enhanced Our Understanding of Cancer

Several methods have been used to measure oxygen pressure in tumours, however the use of less invasive methods have not only made the measurement of hypoxia easy but have become important in patient prognosis. Thomlinson et al. (1955) were the first to suggest that hypoxia could help tumour cells evade radiotherapy. They demonstrate through

Figure 3. Abnormal Vasculature Development is A Contributor to Tumour Hypoxia.

[A] Tumours have characteristically poor vascular development with distinguishable traits such as: blood occlusions from constricted blood vessels, random breaks in vessel walls, capillary-less vessel endings, and arteriovenus shunts. Chronic hypoxia can occur in regions past the oxygen diffusion limit (≈150μm). [B] Alternatively, non-cancerous tissues typically show a strong network of vasculature.


A
Tumour
Ateriovenous shunt
Blood Vessel Breaks
Occlusion
150µm
Blunt Ends
B
Non-Cancerous Tissue
Normoxic
Hypoxic

histological techniques, that areas of a tumour found ≈150 μm from stroma or vascularization were in a state of near zero oxygen levels (Clarke and Pallister, 2005; Groebe and Vaupel, 1988; Olive et al., 1992). Hypoxia in tumours is measured using various scientific techniques, the first of which was completed in 1985. Polarographic measurements of O_2 pressure (pO_2) is the most direct way of calculating tumour pO_2, and is completed through the use of oxygen microsensors. Through this method, pO_2 has been graphed for various cancers including, but not restricted to: glioma (Mendichovszky and Jackson, 2011), lung (Graves et al., 2010), breast and cervical cancer (Lartigau, 1998). Additionally, tumour sections can be biopsied, sliced and stained for endogenous and exogenous hypoxic markers (Li et al., 2012; Mandeville et al., 2012; Perez-Sayans et al., 2013). However, both techniques require the tumour to be accessible, and neither give an adequate gradient of tumour hypoxia. As a result, three main non-invasive methods are currently used for research and diagnosis: nuclear Magnetic Resonance Imaging (MRI), Positron Emission Tomography (PET), and Electron Paramagnetic Resonance Imaging (EPRI). These techniques allow for the detection of oxygen in deeply covered tumours. Due to pO_2 measurement advances, hypoxia has been implicated in the development of tumourigenesis and chemo and radioresistance.

1.5 Tumour Hypoxia is Detrimental to Patient Prognosis

1.5.1 Hypoxic Tumour Cells Evade Radiation Therapy

It has been shown that the extent of hypoxia in tumour cores enable evasion from radiation therapy (Fowler, 1981). Although data has not specifically concluded the reasons why radiation therapy is ineffective in hypoxia, several theories have been

suggested. Firstly, oxygen separates into free radicals under radiation catalysis (Riley, 1994). Radiation therapy may, therefore, be partially dependent on the availability of oxygen in the cell to promote cell death. Additionally, oxygen may prevent the restoration and repair of fragmented DNA that resulted from radiation therapy, thereby making radiation damage permanent (Vaupel et al., 2001). HIF-1α has been shown to mediate cell cycle arrest when activated in hypoxia (Goda et al., 2003). Since radiotherapy is predominantly effective on dividing cells, hypoxic cell cycle arrest may contribute to radiotherapy evasion in tumours. The tumour microenvironment stressors result in proteome changes that help radioresistance, partially through the HIF hypoxic response (Figure 4) (Blouw et al., 2003). These stressors produce genetic disruptions and selective pressures that promote highly abnormal and aggressive cells. Additionally, the tumour microenvironment contributes to a 5-fold increase in genetic instability (Reynolds et al., 1996). An increase in genetic variations produces heterogeneity in the tumours. The selective pressures of the tumour microenvironment produce highly malignant cells that are difficult drug targets and cause post-surgical complications.

1.5.2 Hypoxic Tumour Cells are Difficult Chemotherapy Targets

As discussed, abnormal growth of cancer cells leads to poor vascular design and regions of low oxygen availability. This presents a unique problem for chemotherapeutics that have a poor diffusion distance through tissue, since they would not be able to penetrate non-vascularized regions. There are, however, additional problems including drug inactivity in low oxygen and hypoxic molecular mechanisms leading to drug ineffectiveness (Chen et al., 2008). A decrease in the generation of oxygen free radicals due to lower oxygen availability reduces the effectiveness of these drugs (Kuffel et al.,

1992). Chemotherapy ineffectiveness expresses the difficulty of targeting hypoxic tumour cores, however, these regions may also contribute to severity of malignancy.

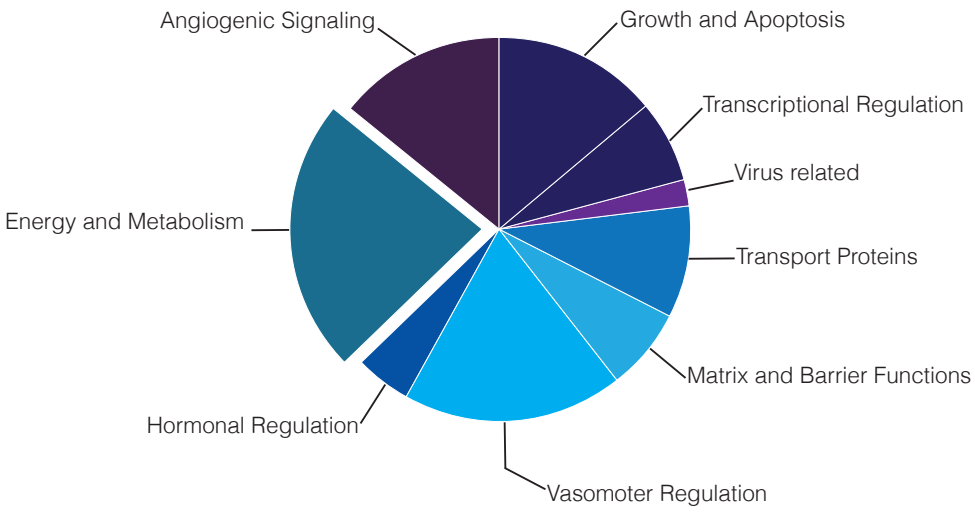
1.5.3 Hypoxia Has Been Linked to a Malignant Phenotype

Cellular adaption to low oxygen conditions (≤ 1% O_2) involves the upregulation of hypoxia specific proteins through transcriptional regulation and post-translational modifications. Transcriptional activity of hypoxic response transcripts is often regulated through the HIF heterodimeric transcription factors. These factors consist of an oxygen-regulated alpha subunit either HIF-1α (Wang et al., 1995), or HIF-2α (Makino et al., 2002), and a constitutively-expressed HIF-1β (also known as ARNT) subunit (Greer et al., 2012). Each alpha subunit contains an oxygen-dependent degradation (ODD) domain, which is targeted and hydroxylated by prolyl-hydroxylases (PHDs) (Epstein et al., 2001). The E3 ubiquitin ligase complex, containing von-Hippel Lindau (VHL) protein, detects post-translational hydroxylation. This secondary post-translational modification marks HIFα subunits for degradation through the 26S proteasome (Figure 5) (Maxwell et al., 1999). PHDs require oxygen as a chemical catalyst and therefore cannot hydroxylate HIFα subunits in low oxygen conditions. Without hydroxylation, HIFα subunits can, consequently, avoid degradation and activate their genetic targets. The resulting hypoxic response promotes the production of erythropoietin (EPO) for erythropoiesis, glucose transporters GLUT 1 and GLUT 3, angiogenic promoters such as vascular endothelial growth factor (VEGF) and VEGF-receptor (VEGFR), carboanhydrase (CA) protein family components to adapt to acidosis, as well as growth promoting proteins such as insulin-like growth factor-2 (IGF-2) and epidermal growth factor receptor (EGFR)

Figure 4. Cells respond to hypoxia by upregulating hypoxic response proteins.

HIF transcription factors upregulate transcription of hypoxic response proteins required for hypoxic survival. Graph illustrates the distribution of HRE target genes.

Hypoxic Response Proteins
HIF Gene Targets



Modified from: Schofield, C. J., & Ratcliffe, P. J. (2004). Oxygen sensing by HIF hydroxylases. Nat Rev Mol Cell Biol

(Ke and Costa, 2006). This proteome response to extracellular stimuli can become permanent through clonal selection of tumour cells, resulting in a more malignant phenotype.

1.5.4 Hypoxia and The Post-Surgical Nightmare

The enhancement of the malignant phenotype by hypoxia is seen in post surgical reoccurrence of hypoxic cancers. Höckel et al. (1996) used polarographic techniques to determine the pre-surgical pO_2 of cervical cancers in 47 patients. Interestingly, there was no significant relationship between the extent of tumour hypoxia and the tumour size, grade, clinical stage or histological type; however, post-surgery, tumours that were more hypoxic (containing a mean pO_2 below 10 mmHg) were more aggressive due to tumour reoccurrence, higher parametrial infiltration and ability to form larger tumour extensions (Hockel et al., 1996). Part of this post-surgical phenotype may result from the reduction of radiotherapy effectiveness on hypoxic tumours. A second study showed the same result in head and neck cancer after staining for endogenous markers of hypoxia (Winter et al., 2006). Tumour hypoxia is a proven detriment to patient prognosis; however, the molecular events that lead to cell survival during hypoxia may provide an interesting target for cancer therapy. Hypoxic exposure results in cellular responses at the metabolic, transcriptional and translational levels that promote survival and tumourigenesis.

1.6 The Hypoxic Response

Cells adapt to hypoxia by activating gene expression changes known as the hypoxic response, mediated by the HIF family of proteins. HIF-1α and HIF-2α, although homologous, have some unique functions in the cell. Unlike HIF-1α (produced from the

HIF1A gene), HIF-2α is a product of the *EPAS1* gene (Tian et al., 1997). PHDs regulate HIF activity through hydroxylation and eventual degradation (Bruick and McKnight, 2001). As well, both basic helix-loop-helix transcription factors are regulated by factor inhibiting HIF (FIH) which destabilizes the binding of HIF cofactors, through hydroxylation (Hewitson et al., 2002; Lando et al., 2002; Mahon et al., 2001). Although HIF-1α and HIF-2α have similar transcriptional specificity residues, known as the amino-terminal transactivating domain (N-TAD), they can promote different gene targets. This suggests that the N-TAD regions confer some specificity or that different coactivators may be involved (Hu et al., 2007). In fact, some of the differences in gene activation lead to opposite effects.(Takeda et al., 2010). HIF-1α gene targets are important factors in glucose transport, angiogenesis, glycolysis and NO production. Similarly, HIF-2α also targets some of the same genes, however, it acts to decrease NO, promote erythropoiesis, growth factor production, cell cycle progression and vascular remodeling (Keith et al., 2012). Expression of HIF-1α or HIF-2α is generally detrimental for patient prognosis; however, several studies in genetically unique cell lines have varying tumourigenic results when only one of the two is knocked down. Distinct gene targets may explain this result (Keith et al., 2012). The differences between HIF-1α and HIF-2α continue as the latter is implicated in a role outside of transcription.

Interestingly, 80-90% of clear cell Renal Cell Carcinomas (CCRCCs) have mutations in the tumour suppressor von Hippel-Lindau (*VHL*) genes, which lead to malignant cell transformation. VHL is a multifunctional protein most known for its interaction with ElonginB, ElonginC, Rbx1, and Cullin-2 (Cul-2), forming an E3 ubiquitin ligase complex (Iwai et al., 1999; Kibel et al., 1995; Lonergan et al., 1998;

Pause et al., 1997). Hydroxylated HIFα is recognized by the ElonginB/C/Rbx1/Cul-2/VHL complex, which then marks it for degradation through the 26S proteasome (Figure 5) (Hershko and Ciechanover, 1992, 1998; Varshavsky, 2012; Weissman, 2001). In hypoxia, PHDs are inactive leading to the stabilization of HIFs, their binding to HIFβ subunits, and the increased transcription of hypoxic response mRNA (Figure 4). Proteins that aid in glycolysis, glucose transport, angiogenesis, and erythropoiesis are among the targets of HIFs and aid in the hypoxic response (Goda and Kanai, 2012; Mason and Johnson, 2007). Mutations in *VHL* result in stabilization of a hypoxic response in normoxic conditions, which can contribute to the regulation of metastatic potential in CCRCC (Brauch et al., 2000). HIF-2α stabilization may contribute to CCRCC transformation by promoting the translation of proteins in normoxia including EGFR.

1.7 Hypoxia Inducible Factor 2α (HIF-2α) Is More Than a Transcription Factor

As shown through a plethora of studies, EGFR overexpression is a common feature in many cancer types (Salomon et al., 1995). EGFR activity without stimulus promotes autonomous proliferation, through internal signaling, which is a hallmark of cancer (Hanahan and Weinberg, 2011; Oda et al., 2005). Our group has shown that the tumour microenvironment can upregulate EGFR (Franovic et al., 2007; Oda et al., 2005). The microenvironment in tumours often experiences hypoxia due to outgrowth of available vasculature, which can be simulated through *in vitro* tumour assays (Hockel et al., 1996; Vaupel et al., 2001). Interestingly, EGFR upregulation is inhibited in a HIF-2α dependent fashion (Franovic et al., 2007; Liu et al., 2006). The renal adenocarcinoma

Figure 5. HIFα is stabilized in low oxygen conditions.

In normoxia, Hypoxia Inducible Factor (HIF)α subunits are hydroxylated by prolyl hydroxylases (PHDs), and subsequently ubiquitinated by an E3 ubiquitin ligase complex containing von Hippel-Lindau (VHL) protein, Cullin-2 (Cul-2), Rbx1, and Elongin B and C, which marks HIFα subunits for degradation through the 26S proteasome. In hypoxia, PHDs are inhibited and VHL cannot bind and mark HIFα proteins for degradation. HIFα and HIFβ subunits can then interact and cause the upregulation of specific mRNAs.

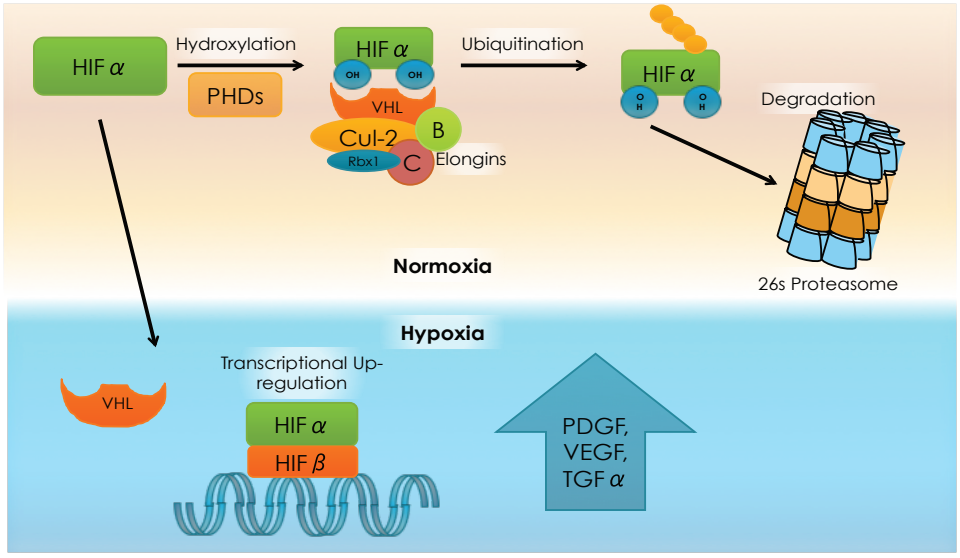


Image modified from: Balligand, J.L., Feron, O., and Dessy, C. (2009). eNOS activation by physical forces: from short-term regulation of contraction to chronic remodeling of cardiovascular tissues. Physiol Rev 89, 481-534.

(786-0) cells lack functional VHL and therefore have high levels of HIF-2α, and subsequently EGFR, in normoxic conditions (Lee et al., 2008). When VHL is reintroduced in these cells there is no change in EGFR mRNA, but a reduction in EGFR protein levels (Franovic et al., 2007). Therefore, HIF-2α may play a role in synthesis or stability of EGFR protein (Franovic et al., 2007).

1.8 Human Cancers Converge at The HIF-2α Oncogenic Axis

Although HIF-1α has been shown to be an important transcription factor in the hypoxic response, the knockdown of HIF-1α protein does not inhibit the formation of tumours through *in vivo* mouse xenografts (Franovic et al., 2009; Mazumdar et al., 2010), on the other hand cells depleted of HIF-2α form significantly smaller tumours (Franovic et al., 2009; Kondo et al., 2003; Li et al., 2012; Mazumdar et al., 2010). Additionally, after inhibiting all HIFα subunits, rescue of HIF-1α was not sufficient to return tumourigenesis, however, rescue of HIF-2α was enough (Kondo et al., 2002; Maranchie et al., 2002). Furthermore, when serum is removed from media, cancer cells deficient in HIF-2α protein are incapable of autonomously proliferating to the same extent as parentals, even in normoxia. Only HIF-2α-dependent transcription targets had a significant reduction in mRNA in shHIF-2 expressing cells, therefore an alternative role for HIF-2α during hypoxia could explain the proliferative phenotype this protein promotes (Franovic et al., 2009). Although HIF-2α had been implicated as a major factor in tumourigenesis and EGFR translation, the translational machinery that is typically used

is inhibited in hypoxia. This poses the question as to what molecular mechanisms and components allow for hypoxic protein synthesis?

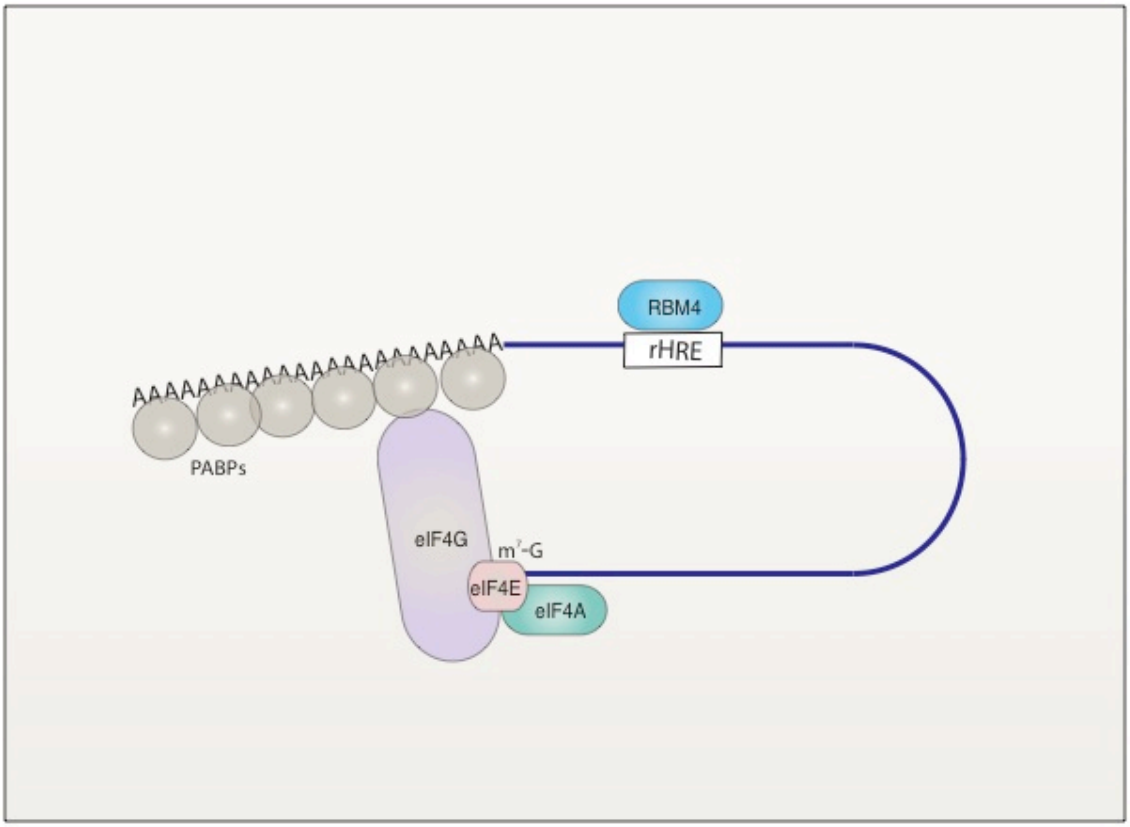
1.9 Cap-Dependent Protein Synthesis is Regulated at Many Levels During Stress

1.9.1 Cap-dependent Protein Synthesis

Protein synthesis involves the interaction of specific proteins, such as the eukaryotic initiation factors, which have been analyzed in depth (Aitken and Lorsch, 2012; Grzmil and Hemmings, 2012; Pichon et al., 2012). When mature mRNAs are produced they contain a 5' 7-methylguanosine cap structure (m^7GpppN) and a polyadenylated tail. These unique structures are required for the binding of poly-(A)-binding proteins (PABPs) and the eukaryotic initiation factor 4F (eIF4F) cap-binding complex. eIF4F consists of three subunits; eIF4E, eIF4A, and eIF4G. eIF4E is responsible for binding the 5'cap of mRNA. eIF4G plays a scaffolding role due to its ability to bind eIF4E, PABPs and eIF3 (Figure 6) (Schutz et al., 2008). eIF4A is an adenosine triphosphate (ATP) – dependent RNA helicase that belongs to the DEAD-box helicase family. Additionally, eIF4A is important for 40S ribosomal recruitment to mRNA (Rogers et al., 2002; Schutz et al., 2008). When bound to mature mRNA in the cytoplasm, these proteins aid the formation of the mRNA closed-loop structure (Kahvejian et al., 2001). This structure is thought to increase translation efficiency because after termination, ribosomes will be in close proximity to the start sequence, which would increase the probability of re-binding for further rounds of translation.

Figure 6. Cap-dependent protein synthesis in normoxic conditions (21% O_2).

When oxygen is available, protein synthesis is initiated through the eIF4Fcomplex, which consists of the cap-binding protein eIF4E, eIF4G and eIF4A. Together, eIF4G, eIF4E, and eIF4A form the eIF4F complex that helps form mRNA secondary structure. Poly-A-binding proteins (PABPs) attach to the poly-adenylated tail and interact with eIF4G. RBM4 may still bind to rHRE regions during normoxia.



Modified from: Uniacke, J., Holterman, C.E., Lachance, G., Franovic, A., Jacob, M.D., Fabian, M.R., Payette, J., Holcik, M., Pause, A.M., and Lee, S. (2012). An oxygen-regulated switch in the protein synthesis machinery. Nature 486, 126-129.

1.9.2 mTORC1 is a Major Regulator of Cap-Dependent Protein Synthesis

The implication of HIF-2α in translation and tumourigenesis is interesting because translation is regulated in response to many stresses. Primarily, mRNA translation is regulated at the rate-limiting step of initiation through the eukaryotic initiation factors. A major regulator of translational control is the mammalian target of rapamycin (mTOR). Protein synthesis is a fundamental requirement for mammalian cell survival (Gerschenson and Rotello, 1992; Martin et al., 1988; Martin et al., 1990; Polunovsky et al., 1994; Whyte et al., 1997). Direct eIF4E inhibition is through 4E-binding protein (4E-BP) sequestration. Both 4E-BP1 and its homologue 4E-BP2 bind to eIF4E with a domain similar to eIF4G (Mader et al., 1995). 4EBPs are functionally active when hypophosphorylated but are inhibited when hyperphosphorylated by their protein kinase, mTOR (Figure 7). Specifically, mTOR complex 1 (mTORC1) stimulates mRNA translation by acting as a direct or indirect kinase of protein synthesis stimulators and inhibitors. Consisting of 2549 amino acids, the large mTOR molecule contains a kinase domain in its C-terminal region (Hay and Sonenberg, 2004). mTOR interacts with several different proteins that do not seem to be affected by rapamycin, the first known inhibitor of mTOR (Hay and Sonenberg, 2004). In mammals, mTOR complex 2 (mTORC2) is bound to mammalian AVO3 (mAVO3) also known as Rictor and is insensitive to rapamycin (Sarbassov et al., 2004). On the other hand, mTORC1 is bound to regulatory associated protein of TOR (Raptor), which is capable of binding established mTOR downstream targets such as S6 Kinase 1 (S6K1) and 4E-BP1 (Hara et al., 2002). mTOR also complexes with G protein β-subunit like protein (GβL) which has been linked to the stability of mTOR binding to S6K1 and 4E-BP1 substrates as well as mTOR binding to

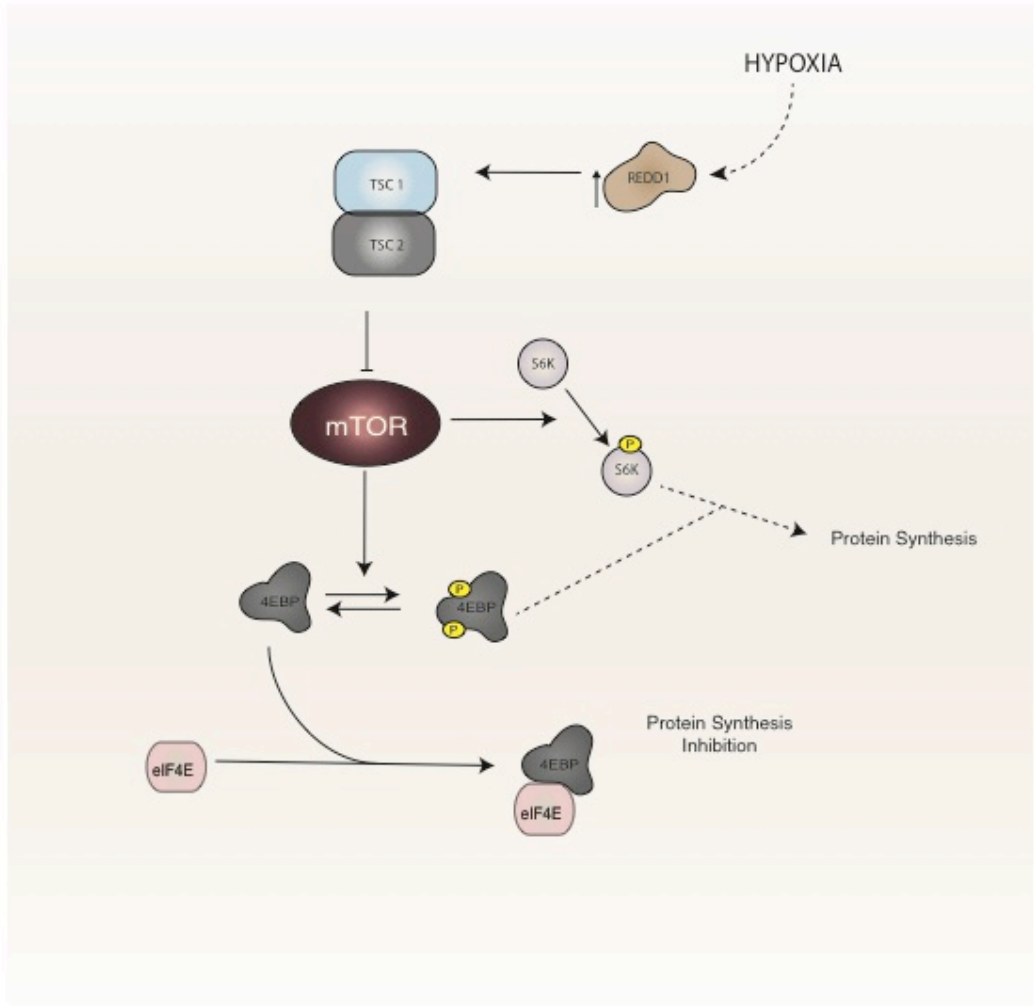
Raptor (Kim et al., 2003). Interestingly, mTORC1 seems to be sensitive to rapamycin only in the presence of GβL, which emphasizes its importance. Outside of hypoxia, mTOR is upregulated by growth factors and can be inhibited through amino acid starvation (Hay and Sonenberg, 2004). Inhibition of mTORC1 is thought to be a method of reducing cell energy demand, since mTORC1 plays a significant role in the regulation of protein synthesis and ribosomal biogenesis; two energy-costly processes. mTORC1 also regulates translation through the eIF4A protein. eIF4A is stimulated by the activity of eIF4B (Rozen et al., 1990) and can be inhibited from binding to eIF4G by Pdcd4 (Yang et al., 2003). eIF4B is activated (Shahbazian et al., 2006), and Pdcd4 is inactivated (Dorrello et al., 2006), after phosphorylation by S6 Kinase, specifically $p70^{rsk}$, which in turn is activated through mTORC1 phosphorylation.

1.9.3 Alternative Methods of eIF4F Regulation

As mentioned previously translation initiation is a rate-limiting step in protein synthesis and is a site of regulation during stress. Inhibiting protein synthesis can be mediated through proteolytic cleavage of eIF4G during virus infection (Gradi et al., 1998; Svitkin et al., 1999), apoptosis (Marissen et al., 2000), and sequestration during heat shock (Cuesta et al., 2000). Likewise, eIF4E is also a site for protein synthesis inhibition. An indirect method of inhibiting eIF4E function is through nuclear import by the 4E-transporter (4E-T) thereby dissociating it from its cap-binding partners (Dostie et al., 2000). Transcriptional control of eIF4E mRNA occurs through myc protein, as eIF4E gene promoters contain classic myc binding sites (Raught and Gingras, 1999).

Figure 7. The mTORC1-4EBP regulation pathway.

The general mTORC1-4EBP pathway is outlined for the activation of mTOR kinase in mTORC1 and subsequent phosphorylation (P) of 4E-BP and S6K. Hypoxia inhibits mTOR activity through a mechanism that depends on the TSC. TSC, tuberous sclerosis complex; mTORC1, mammalian target of rapamycin complex 1; 4E-BP, eIF4E binding protein; S6K, p70 S6 kinase.


HYPOXIA
REDD1
TSC 1
TSC 2
mTOR
S6K
P
S6K
Protein Synthesis
4EBP
P
4EBP
P
Protein Synthesis
Inhibition
eIF4E
4EBP
eIF4E

1.9.4 Stress-Specific Molecules Regulate Translation at eIF2

A second level of translation regulation occurs through inhibition of eIF2. This protein is required for directing methionine-transfer RNA (Met-tRNA) to the 40S ribosomal subunit. eIF2 activity is regulated by eIF2B which exchanges GDP in eIF2-GDP for GTP, thereby activating eIF2 (Wouters et al., 2005). The α subunit of eIF2 can be phosphorylated, which inhibits the activity of eIF2B resulting in a general decrease in mRNA translation. Four proteins have been identified in the phosphorylation of eIF2α; RNA-activated protein kinase (PKR) (Berry et al., 1985); Heme-regulated inhibitor of translation found in erythrocyte cells (HRI) (Chen and London, 1995); an amino-acid concentration sensitive kinase, GCN2 (Cherkasova and Hinnebusch, 2003); an endoplasmic reticulum protein involved in the unfolded protein response, protein kinase RNA-like endoplasmic reticulum kinase (PERK) (Harding et al., 1999). Each of the above responds to stresses experienced by the cells. PKR is responsive to interferon and upregulated during exposure to dsDNA, and therefore thought to be important during viral infections (Berry et al., 1985). Amino acid starvation can lead to GCN2 upregulation and low cellular ATP levels can result in misfolded proteins in the endoplasmic reticulum (ER) resulting in PERK autophosphorylation. Interestingly, complexes lacking eIF2 are translationally incompetent and are localized to discrete cytoplasmic domains known as stress granules (SGs) (Anderson and Kedersha, 2002). SG formation has also been shown to suppress HIF-1α expression in hypoxia (Gottschald et al., 2010) and prevent apoptosis in cancer (Thedieck et al., 2013).

1.9.5 Alternative, Cap-Independent Methods Of Protein Synthesis Cannot Explain the Quantity of Proteins Synthesized in Hypoxia

Known alternative methods of hypoxic protein synthesis cannot account for the large number of proteins produced in the tumour microenvironment. Proteins can also be synthesized through a cap-independent pathway involving internal ribosome-entry sites (IRES) located in the 5' untranslated region (UTR) of mRNA that allow for mRNA translation without the eIF4E cap-binding protein (Hellen and Sarnow, 2001). Although no specific characteristics can describe IRES, mRNAs that contain them typically have multiple AUG start regions and long 5'UTRs that form secondary structures to promote translation (Balvay et al., 2009). Some IRES-containing mRNAs can promote the binding of ribosomes, and translation, without eIFs; however, many require eIF involvement (Komar and Hatzoglou, 2011). IRES-mediated translation is implicated in translating between 3-5% of cellular mRNAs (Holcik and Sonenberg, 2005). Although this is significant, IRES-mediated translation cannot account for the relatively high amount of protein synthesis that occurs during hypoxia. Therefore, there must be another mechanism outside eIF4E and IRES-dependent mRNA translation in hypoxia.

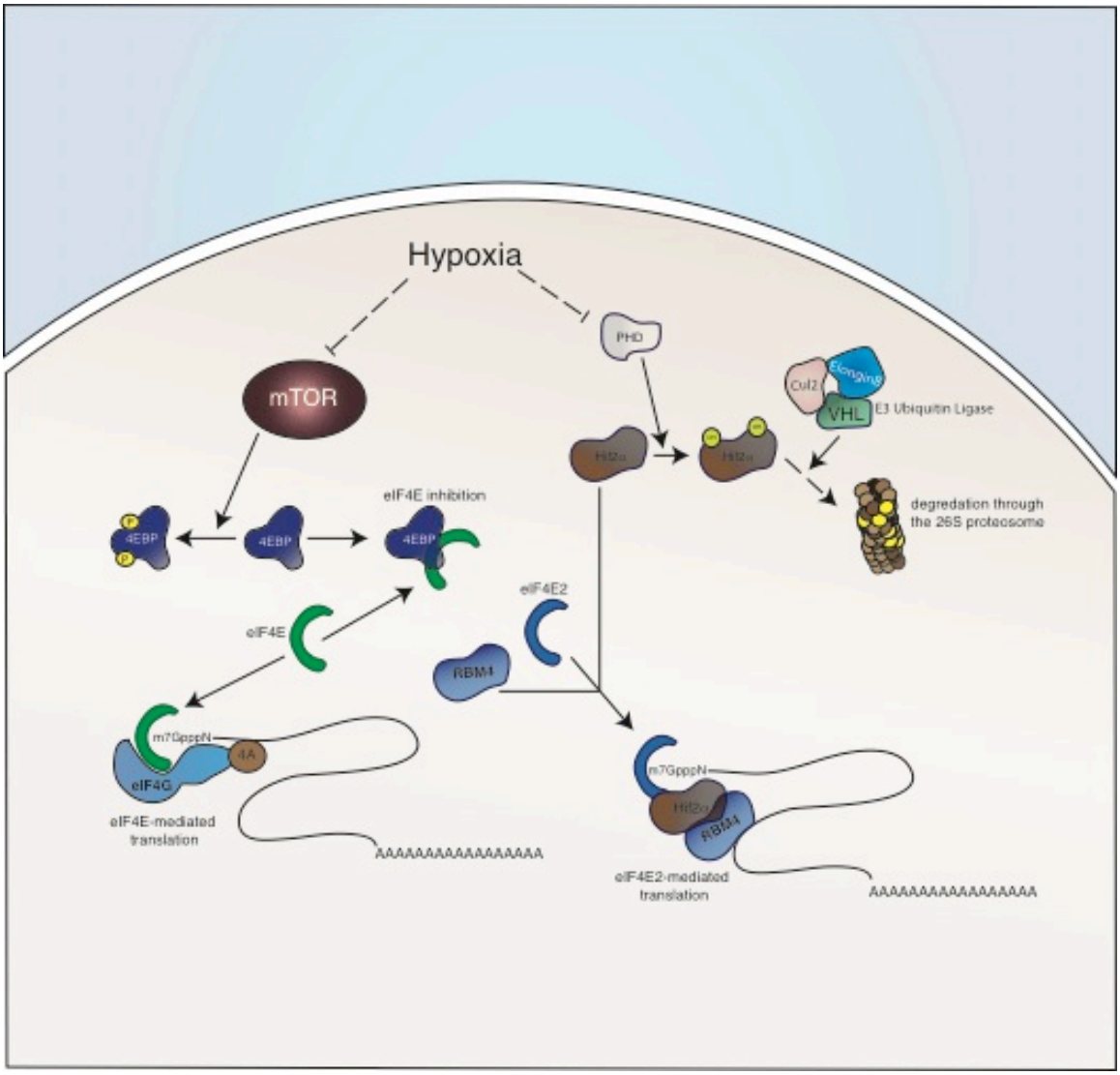
1.9.6 An Alternative Cap-Dependent Protein Synthesis Pathway is Activated in Hypoxia

Recent data by Uniacke et al. (2012) have shown that a switch in cap-binding proteins occurs during hypoxia, which regulates selective protein expression. While studying HIF-2α, it was found that EGFR mRNA was captured by polysomes in a HIF-2α-dependent manner even during treatment with a transcription inhibitor. This data would implicate

that HIF-2α regulates EGFR production through a non-transcriptional pathway. In fact, HIF-2α protein was associated with heavier polysome fractions suggesting it might have a role in the translation complex (Uniacke et al., 2012). HIF-2α associates with EGFR mRNA at the 3' untranslated region (3'UTR). Interestingly, the addition of this 3'UTR sequence was the only requirement for translation of a luciferase reporter in hypoxia (Uniacke et al., 2012). This set of nucleotides was named the RNA Hypoxia Response Element (rHRE). HIF-2α is linked to EGFR mRNA through an RNA binding protein, RBM4, at the rHRE (Uniacke et al., 2012). RBM4 has been linked to several functions in the cell such as; pre-mRNA splicing, translation inhibition and silencing of RNA (Markus and Morris, 2009). The rHRE contains a CGG motif, that when disrupted affects the translation of mRNA. Consequently, loss of RBM4 produces a drop in hypoxic protein synthesis similar to HIF-2α loss. This sequence of nucleotides is contained in many mRNAs expressed during hypoxia. The HIF-2α/RBM4 complex immunoprecipitated with the cap binding protein eIF4E2, a homologue of eIF4E. This homologue can evade global translation repression because it is weakly targeted by 4E-BP during hypoxia due to a slight amino acid change at the 4E-BP binding site (Tee et al., 2004). Therefore, its activity allows for translation of rHRE containing mRNAs in hypoxia, when eIF4E is inhibited (Figure 8). Consequently, when eIF4E2 is knocked down in low oxygen conditions, a massive loss in protein synthesis is observed. Furthermore, cells incubated in hypoxia have eIF4E2 protein associated with heavy polysome fractions suggesting that it plays a role in the translation machinery. On the other hand, eIF4E protein is associated with polysomes only when incubated in normoxic conditions. Hypoxic incubation leads to eIF4E dissociation from these fractions, which is

Figure 8. An Alternative Method of Cap-Dependent Protein Synthesis.

Under normal oxygen conditions the eIF4F complex (eIF4E, eIF4A, and eIF4G) binds the 5' cap of mRNA and initiates protein synthesis. Under these same conditions HIF2α is degraded by the 26S proteasome. Under low oxygen conditions, the inhibitor of eIF4E (4E-BP) is stabilized resulting in protein synthesis inhibition through the eIF4F complex. HIF2α, is also stabilized, and binds to a homologue of eIF4E, eIF4E2, which can bind to the 5' cap of specific mRNA and promote their translation.


Hypoxia
mTOR
PHD
Cul2
Elongin
VHL
E3 Ubiquitin Ligase
HIF-α
HIF-α
degredation through
the 26S proteosome
eIF4E inhibition
4EBP
4EBP
4EBP
P
P
eIF4E
eIF4E2
RBM4
m7GpppN
4A
eIF4G
eIF4E-mediated
translation
AAAAAAAAAAAAAAAA
m7GpppN
HIF-α
RBM4
eIF4E2-mediated
translation
AAAAAAAAAAAAAAAA

the opposite as observed with eIF4E2 protein (Uniacke et al., 2012). This data would suggest that cells switch from eIF4E to eIF4E2-mediated translation. Further investigation shows that rHRE containing mRNAs are translated by eIF4E only under normoxic conditions. When eIF4E is knocked down in hypoxia there is a non-significant effect of protein synthesis potential. Therefore, the eIF4E2-mediated protein synthesis complex is a major player in hypoxic translation.

2.0 Rationale, Hypothesis and Objectives

2.1 Rationale

The use of eIF4E-mediated translation inhibitors as chemotherapeutics requires tumours to predominantly use eIF4E for protein synthesis. However, it is well described that eIF4E is inhibited in hypoxia, which is a common feature of tumours (Raught and Gingras, 1999). The persistence of protein synthesis in this condition suggests that an alternative pathway is compensating for the inhibition of eIF4E protein. Recent conceptual advances in protein synthesis have implicated eIF4E2 in producing a substantial amount of protein under low oxygen conditions. Therefore we propose that targeting, and inhibiting, the eIF4E2 protein synthesis complex may be a superior method of preventing tumourigenesis. If cancers harness this alternative protein synthesis complex for tumourigenesis, than several new targets may be identified for combating cancer. Further study into this field could also explain why hypoxic tumour cores promote aggressive and metastatic cells. By characterizing the importance of the eIF4E2-mediated protein synthesis pathway the cancer translation field may finally understand what makes cancers aggressive, and how to attack this attribute. We have set out several objectives to accomplish this task.

2.2 Hypothesis

The eIF4E2-mediated protein synthesis complex allows for tumourigenesis by facilitating hypoxic survival and proliferation.

2.3 Research Objectives

Objective 1: Develop an *in vitro* system to study eIF4E2-mediated translation in cancer cells

An oxygen-regulated switch in cap-binding protein results in an alternative translation complex that synthesizes the bulk of proteins in hypoxic monolayers. I will provide evidence that small tumour masses experience hypoxia and a switch in translation machinery with the use of spheroids, an *in vitro* tumour model. Spheroids are agglomerates of cancer cells that have been shown to mimic the tumour microenvironment. I will verify this hypoxic model through immunohistochemistry and western blot. Finally, by determining the localization of eIF4E2 in polysome fractions we can provide evidence for the presence or lack of eIF4E2 activity.

Objective 2: Stably knock down eIF4E2 protein and determine the effects on cell proliferation and death

Since a large fraction of hypoxic protein synthesis is dependent on eIF4E2, we hypothesize that the stable loss of this protein would be detrimental in hypoxic adaption by cells. To determine the importance of eIF4E2 in cancer I will stably knockdown eIF4E2 protein in several genetically unique cell lines (HCT116, 786-0, U87MG, Hela, and A549). Firstly, Lentivirus will be prepared containing either and shRNA targeting eIF4E2 or a scramble control. Cells will be infected and selected, using antibiotics. Screening will be completed by western blot to indicate depletion of eIF4E2 protein, which we associate with the stable expression of shRNA. The proliferative capacity in hypoxia of these stably cell lines will be measured through BrdU incorporation and basic

cell counts using the appropriate controls. Also, early apoptosis detection through active caspase-3 staining will identify the importance of eIF4E2 in survival during normoxic and hypoxic conditions. Lastly, Measuring spheroid growth over time in eIF4E2-depleted cells will determine if this alternative protein synthesis machinery is important during early tumourigenesis.

Objective 3: Determine if eIF4E2 confers an aggressive phenotype

Several proteins thought to be involved in tumourigenesis (EGFR, PDGFRA, and IGF1R) are translated in an eIF4E2-dependent manner. Thus we wonder if eIF4E2 is associated with a proliferative phenotype. To identify the role of eIF4E2 in the cancer phenotype I will also develop U87MG and 786-0 stable cell lines lacking eIF4E protein. Using these cell lines we can compare the effects of depleting this cap-binding homologue on tumourigenesis and whether eIF4E2 can rescue eIF4E inactivity in normoxic conditions.

Objective 4: Test the tumourigenic ability of silenced eIF4E and eIF4E2 cells *in vivo*

Complete *in vivo* analysis with cells lacking eIF4E and eIF4E2 protein to determine their importance in tumourigenesis.

3.0 Materials and Methods

3.1 Cell culture and cell lines.

The U87MG glioblastoma, 786-O renal cell carcinoma, and HCT116 colorectal carcinoma were obtained from the American Type Culture Collection and maintained in DMEM (Thermo Scientific), RPMI (Wisent), and McCoy's 5A (Wisent), respectively. Media was supplemented with 5% Fetal Bovine Serum (FBS). Cells were incubated at 37 ^{o}C in ambient O_2 levels and a 5% CO_2 environment. Hypoxia was induced by incubating at 37 ^{o}C in a 1% O_2, 5% CO_2 and N_2-balanced atmosphere for 24 h unless otherwise indicated. Cell growth assays were performed by plating 10^5 cells in 6-cm plates and recording the cell number after 48 h.

3.2 Polysomal analysis.

Cells (2.5 X 10^6) were cultured in 15 cm plates under normoxic or hypoxic conditions for 24 hours. Spheroids were grown for 6 days and tumour xenografts were grown for 4-6 weeks. For isolation of intact polysomes, 100 mg/ml of cycloheximide was added to cells for 5 min. at 37°C before harvesting. Polysome lysates were prepared in RNA lysis buffer [15 mM Tris·HCl (pH 7.4])/15 mM $MgCl_2$/0.3 M NaCl/1% Triton X-100/0.1 mg/ml cycloheximide/100 units/ml RNasein), and subjected to sucrose gradient (10-50%) centrifugation at 39,000 rpm with a SW-41-Ti Rotor (Beckman Coulter, Fullerton, CA) for 90 min. at 4°C. Gradients were then collected into 10 equal fractions while the absorbance at 254 nm was continuously monitored. Proteins from each fraction were concentrated by trichloroacetic acid (TCA) precipitation. 1:4 volume of TCA was added

to each fraction and incubated for 10 min at 4 ^{o}C. Samples were centrifuged at 12,000 g for 5 min and pellets washed twice with 200 µL cold acetone with centrifugation at 12,000 g for 5 min in between. Pellets were dried at 95 ^{o}C to evaporate acetone. Pellets were resuspended in 2X SDS-PAGE sample buffer and boiled before performing western blot analysis. Protein integrity was verified by blotting for ribosomal proteins L5 and S13.

3.3 In Vitro Spheroids.

Cells (2 X 10^5) were plated in 24-well plates coated with 1% Seaplaque agarose (Cambrex). Plates were swirled twice in a circular motion for 30 min after plating, with a 1 h rest in between, to promote cell–cell adhesion. Multicellular spheroids were grown for 4 days. For spheroid growth assays, size was monitored 24 h after plating to give time for spheroids to form. Size was monitored daily by photographing spheroids in the 24-well plates with a Zeiss Axiovert S100 TV microscope. Spheroid area was measured by calculating the pixel area of each image with Adobe Photoshop CS5.1.

3.4 Xenograft tumours.

Female CD-1 nude mice (Charles River) were injected in their flanks with 10^7 cells diluted in 200 µL sterile 1X PBS. Mice were killed 6–8 weeks post-injection according to facility protocols (University of Ottawa) or earlier in cases of significant morbidity. Tumour dimensions were recorded weekly and final volumes measured and photographed at the time of kill. In order to determine tumour volume by external caliper, the greatest longitudinal diameter (length) and the greatest transverse diameter (width)

were determined. Tumour volumes based on caliper measurements were calculated by the modified ellipsoid formula 1/2 (length x width^2). All experiments were performed double-blinded.

3.5 Immunohistochemistry.

Spheroids or biopsied xenografts were frozen in O.C.T. media (Tissue-Tek) and were sectioned into 10 μm slices by a Microm HM 500 M Cryostat. Samples were fixed in acetone for 10 min at -20 ^{o}C, and then blocked with 10% goat serum in 1X PBS for 1 h at RT followed by incubation overnight at 4 ^{o}C with primary antibody (1:300) diluted in 5% goat serum in 1X PBS. Three 5 min washes in 1X PBS were performed followed by incubation with secondary antibody (1:200) diluted in 5% goat serum in 1X PBS for 1-3 h at RT. After three 5 min washes in 1X PBS, slides were mounted onto coverslips with Fluoromount G (Electron Microscopy Sciences). Images were acquired with a Zeiss Axiovert S100 TV microscope. Antibodies used for immunohistochemistry were P4E-BP (Cell Signaling), 4E-BP (Cell Signaling), and S6-P (Cell Signaling). Hypoxia within spheroids was determined by labeling with Hypoxyprobe^{TM} according to manufacturer's protocol. All specimens were counterstained with Hoechst 33258 reagent (Sigma) to identify total cells. Blood vessels were visualized with H&E (Thermo Scientific) as previously described (Franovic et al., 2009).

3.6 Western Blot analysis.

Cells were washed with PBS and harvested in 4% SDS in PBS. Protein concentrations were determined by bicinchoninic acid protein assay, and samples were separated on

denaturing polyacrylamide gels. Membranes were incubated with primary antibodies anti-EGFR (Ab-12; LabVision), anti-GAPDH (Genetex), anti-HIF-2α (Novus), anti-Actin (Sigma), anti-L5 (abcam), anti-S13 (abcam), anti-eIF4E (Genetex), and anti-eIF4E2 (Genetex). Secondary antibodies were HRP-conjugated anti-mouse (Amersham Biosciences) or anti-rabbit (Jackson ImmunoResearch Inc.). Bands were detected by enhanced chemiluminescence (Pierce).

3.7 Constructs, short hairpin RNAs, and transfections.

Commercially available GIPZ Lentiviral Human EIF4E2 shRNAmir (Thermo Scientific) was used to target the eIF4E2 CDS (V2LHS_68041 shRNA sequence TGAACAGAATATCAAA) or the 3'UTR (V3LHS_405000 shRNA sequence CAGCTGAGATCACTTAATAA). A non-targeting shRNA in a pGIPZ vector was used as a control (Thermo Scientific). Constructs were transfected along with lentiviral packaging particles (Invitrogen) into HEK293T cells using lipofectamine reagent (Invitrogen) according to manufacturer's protocol. After 48 h, the supernatant was collected as used as the infection mix for infecting U87MG, 786-O, or HCT116 for eIF4E2 silencing. 1.5 mL of infection mix was added per 10-cm plate and incubated for 24 h. Cells were split 1:4 and fresh media containing 1 μg/mL puromycin was added for selection. After 1-2 weeks, clonal populations were picked and screened by western blot for the identification of cells stably-expressing eIF4E2 shRNA. 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 eIF4E2 knockdown cells (clones 2.1 and 2.3) with an eIF4E2 ORF cDNA construct (Genecopoeia) containing a Neomycin

resistance gene. Stable clones were selected over a two week period in media containing 100 µg/mL Neomycin/G418 (Wisent).

3.8 Cell proliferation and activated programmed cell death assays.

For measuring proliferation or the activation of apoptosis in culture, cells were plated at low density on coverslips and incubated for at least 16 h in DMEM plus 5% FBS. Cells were washed in 1X PBS and incubated for 24 h in normoxia or hypoxia in DMEM plus 5% FBS. For measuring proliferation, cells were incubated in the presence of 10 µM 5-bromo-2′-deoxy-uridine (BrdU; Jackson ImmunoResearch) for 3 h, then fixed in 70% ethanol in 50 mM glycine (pH 2.0) for 30 min at RT. Cells were washed three times in 1X PBS and incubated with a solution containing an anti-BrdU antibody (1:40) for 1 h at 37 °C. Cells were then washed three times in 1X PBS and incubated with an anti-mouse Alexa594-conjugated secondary antibody (1:200) (Invitrogen). For measuring the activation of apoptosis, cells were washed in 1X PBS and fixed at -20 ^{o}C in methanol for 10 min followed by cold acetone for 1 min. Cells were washed three times in 1X PBS and then incubated overnight in 10% FBS blocking solution in 1X PBS. Cells were then incubated with anti-active caspase-3 primary antibody (abcam) (1:300) diluted in blocking solution with 1% Triton X-100 for 2 h at 37 ^{o}C. A second wash in 1X PBS was performed followed by a 2 h incubation with an anti-rabbit Alexa594-conjugated secondary antibody (1:200). Coverslips were mounted onto slides with Fluoromount G. All coverslips were counterstained with Hoechst 33242 reagent (Sigma) to identify nuclei. The percentage of BrdU-labeled or active caspase-3-labeled cells (Alexa594-stained cells/Hoechst-stained cells) was determined with a Zeiss Axiovert S100TV

microscope and digital imaging. To quantify these parameters at least five representative fields were counted at 400X magnification per triplicate experiment. For measuring proliferation in a spheroid section, acetone-fixed slides were blocked with 5% goat serum in 1X PBS for 1 h and then washed for 5 min in 1X PBS. Slides were incubated with anti-mouse Ki-67 antibody (Dako) (1:150) for 1 h followed by three washes of 1X PBS. Slides were incubated with anti-mouse Alexa594-conjugated secondary antibody (1:200) for 1 h at RT. Three final 10 min washes in 1X PBS were performed followed by mounting with Fluoromount G. For measuring the activation of apoptosis in a spheroid section, follow the same procedure as described above for Immunohistochemistry.

Statistical analysis. *P* values associated with all comparisons were based on two-tailed Student's t tests. Results are mean ($n \geq 3$) ± standard error of the mean (s.e.m.).

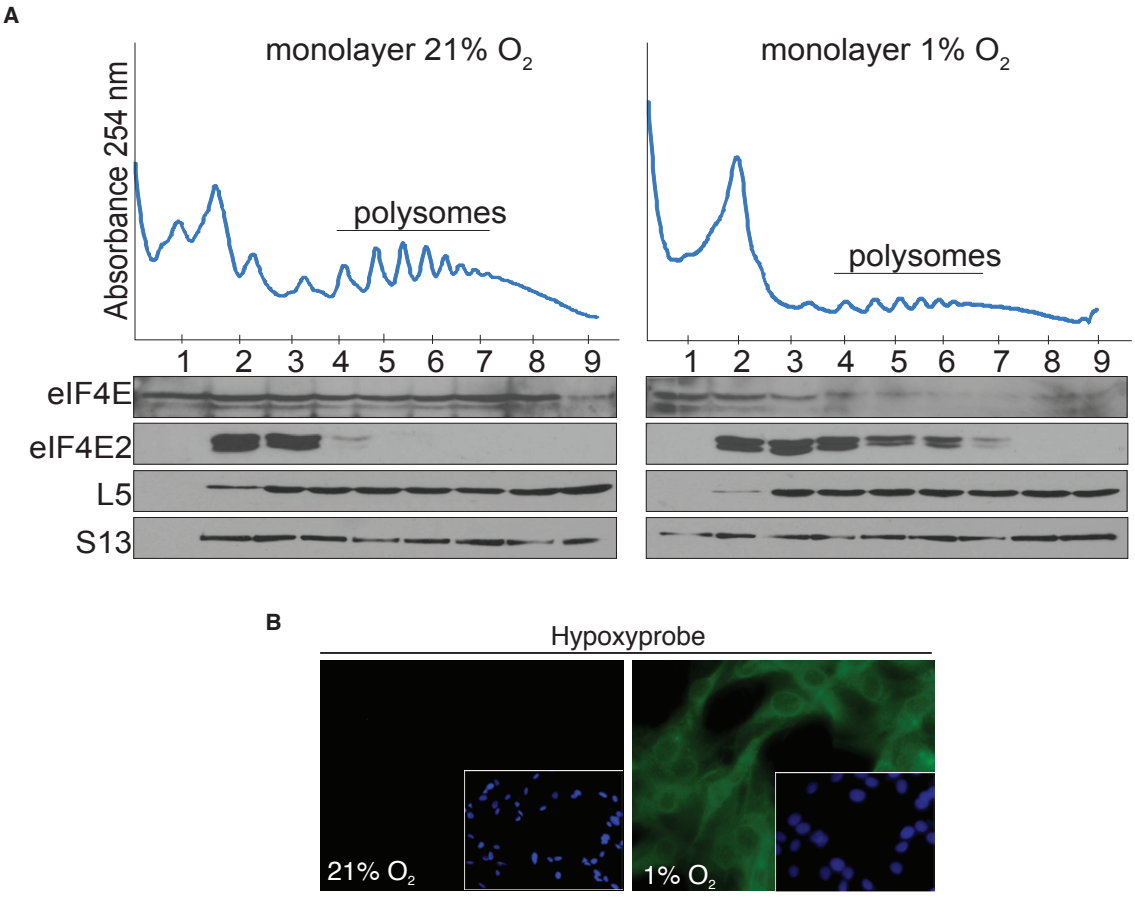
4.0 Results

4.1 Hypoxic incubation of U87MG monolayers leads to a switch in cap-binding protein

A switch in cap-dependent protein synthesis machinery occurs when cells are exposed to low oxygen conditions (≤ 10 mmHg, 1% O_2). The ability of a cap-binding protein to bind to the m^7GpppN cap is suggested through protein association with polysomes. Polysomes, or polyribosomes, are formed when several ribosomes continuously bind a highly translated mRNA, producing heavy ribonucleoprotein aggregates. We reproduced previously published data, which shows a switch in cap-binding protein from eIF4E to eIF4E2 with monolayers are cultured in hypoxia (Uniacke et al., 2012). In normoxic monolayers, eIF4E is associated with polysome fractions as expected (Figure 9A). When cells are cultured in 1% O_2, eIF4E dissociates with polysome fractions. In accordance with published data, there is a shift of eIF4E2 into the heavier fractions during hypoxia (Figure 9A). Characteristic reduction in overall mRNA translation is indicated through the reduction in peak sizes from normoxia to hypoxia. Fractions 1-2 indicate monosomes (peaks distinguish 40S, 60S and 80S, from left to right). To ensure that eIF4E, eIF4E2, and control proteins do not form aggregates that have similar size to polysomes, an additional polysome profile is completed with puromycin treatment (Uniacke et al. 2012). This antibiotic dissociates polysomes resulting in protein association in only monosomes. To control for proper function of the hypoxic chamber, 10^5 cells were plated over glass coverslips and incubated in hypoxia for 24 hours. The cells were Hypoxyprobe™ positive, indicating that oxygen levels were ≤ 1.3% (Figure 9B).

Figure 9. eIF4E2 is associated with polysomes in hypoxic monolayers.

(A) Polysomal distribution of eIF4E and eIF4E2 in a cultured monolayer of normoxic (21% O_2) and hypoxic (1% O_2) U87MG glioblastoma cells (Contributed be J.U.) (B) Cells stained for the hypoxic ($\leq$ 1.3% O_2) marker, Hypoxyprobe™.


A
monolayer 21% O2
monolayer 1% O2
Absorbance 254 nm
polysomes
polysomes
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
eIF4E
eIF4E2
L5
S13
B
Hypoxyprobe
21% O2
1% O2

4.2 eIF4E2-dependent protein synthesis is active in xenografts and spheroids

4.2.1 *In vitro* spheroids have hypoxic regions that suggest mTORC1 inactivity

As the bulk of protein synthesis is eIF4E2-dependent under low oxygen conditions we hypothesized that tumours would have eIF4E2 protein associated with heavy polysome fractions. Our first objective was to develop an *in vitro* tumour model that simulates a lack of blood vessels, known as spheroids, to test our hypothesis. Spheroids were developed by plating 2×10^5 U87MG and HCT116 cells over a soft agarose base. Because our hypothesis is based on tumour cells experiencing hypoxic protein synthesis, we stained for markers of normoxic protein synthesis inhibition. We provide evidence for eIF4E inactivation by staining for mTORC1 targets and substrates that regulate eIF4E-mediated translation. Inactive 4E-BP (P4E-BP) is located on the outer regions of the spheroids, 100-200 μm from the edge (Figure 10A, 11A). Active 4E-BP staining is predominant in the core regions, 150 μm from the edge of the spheroid (Figure 10B, 11A). Furthermore, ribosomal protein S6 was phosphorylated by mTORC1 only at the exterior (Figure 2C). Spheroid cores were Hypoxyprobe™-positive approximately 100 μm from their edge (Figure 10D, 11B). Through the immunohistochemical results we reasoned that the hypoxic cores account for 80-90% of the spheroids interior in which we suspect eIF4E2-mediated translation is active.

Figure 10. U87MG Spheroids contain hypoxic cores that suggest reduced mTORC1 activity.

U87MG cells were cultured over soft agarose to form *in vitro* tumours called spheroids. Four-day-old spheroids were frozen in OCT media and stained using basic immunohistochemical techniques. Representative images are shown. [A] Immunolabeling of phosphorylated 4E-Binding Protein (P4E-BP) shows mTORC1 activity predominantly outside the core of the spheroid. [B] Immunolabeling with anti-4E-Binding Protein (4E-BP). [C] Immunolabeling for the downstream target of mTORC1 using anti-phosphorylated ribosomal protein S6 (S6-P). [D] Cells were stained after incubation in Hypoxyprobe^{TM} for 1 hr. Solid line, section border. Dotted line, border between outer rim and core. Scale bars indicates 100μm.

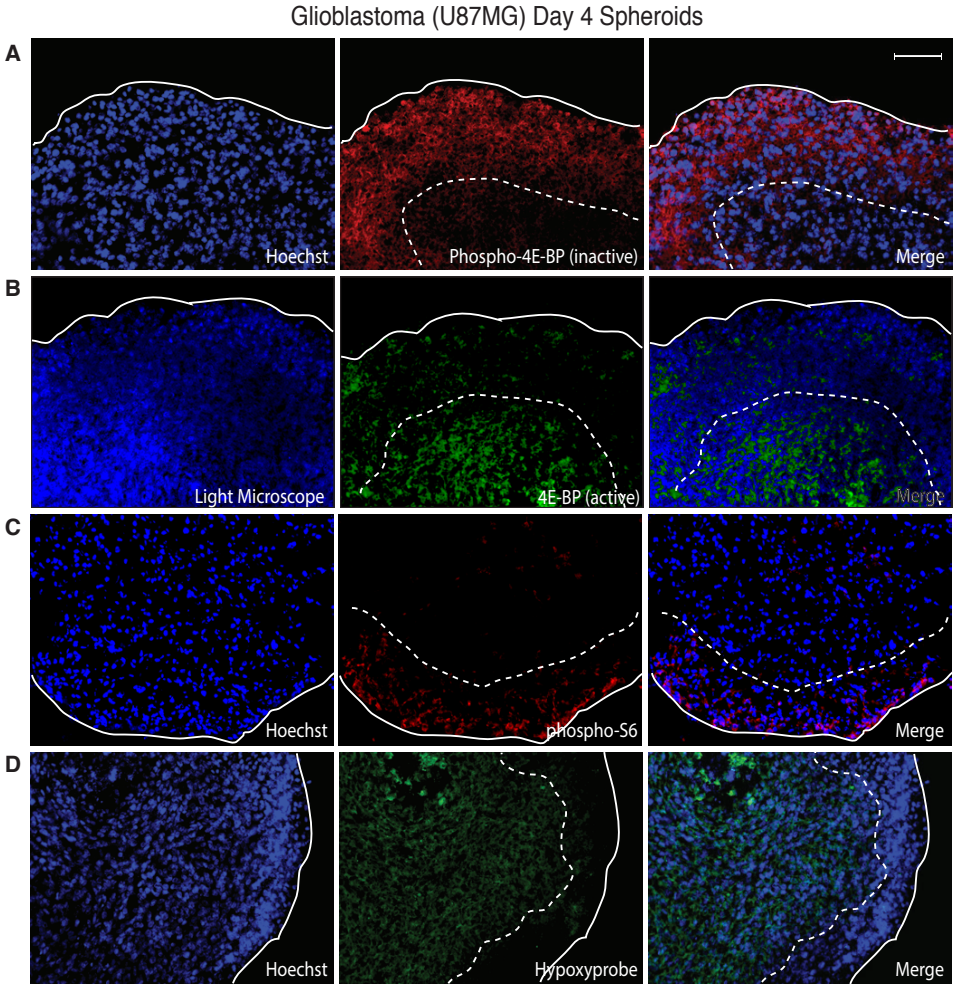
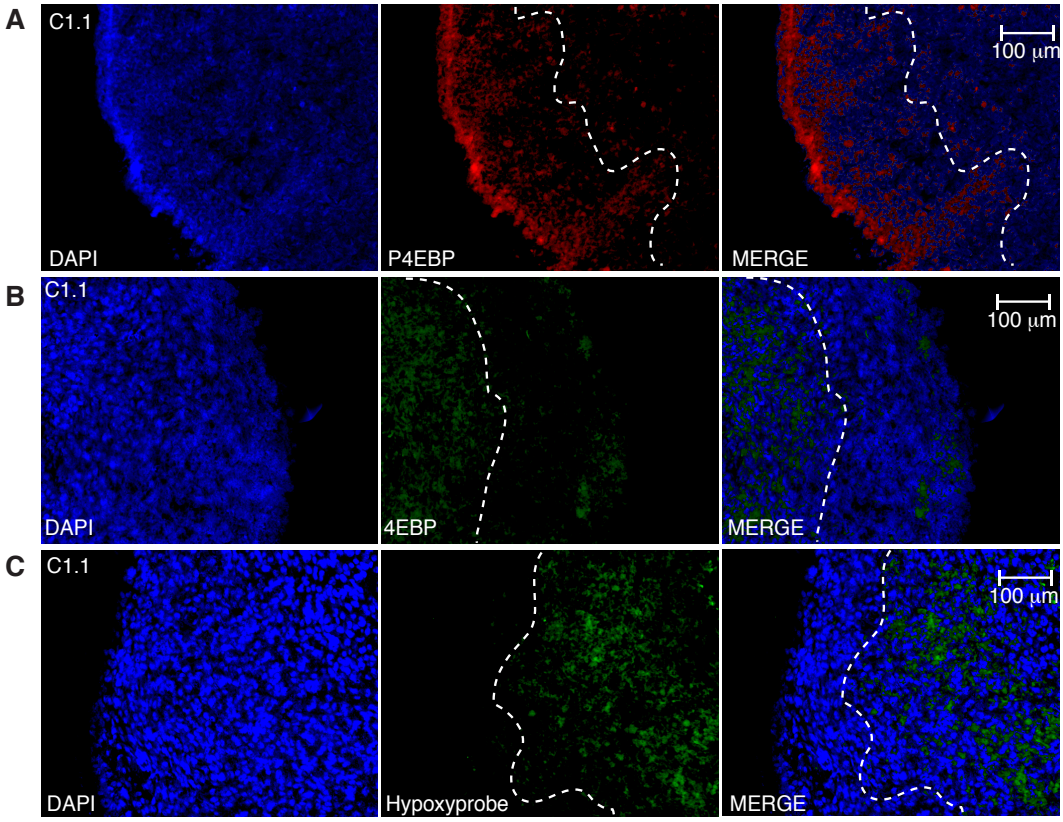

Glioblastoma (U87MG) Day 4 Spheroids
A
Hoechst
Phospho-4E-BP (inactive)
Merge
B
Light Microscope
4E-BP (active)
Merge
C
Hoechst
phospho-S6
Merge
D
Hoechst
Hypoxyprobe
Merge

Figure 11. HCT116 Spheroids contain hypoxic cores that suggest reduced mTORC1 activity

HCT116 cells were cultured over soft agarose to form *in vitro* tumours called spheroids. Four-day-old spheroids were frozen in OCT media and stained using basic immunohistochemical techniques. Representative images are shown. Immunolabeling of a four-day-old spheroid section shows [A] phosphorylated-4EBP staining or [B] unphosphorylated 4EBP. [C] Hypoxyprobe™ staining was completed after incubation in Hypoxyprobe™ for 1 hr. Solid line, section border. Dotted line, border between outer rim and core. Scale bar indicates 100μm.

Colorectal Cancer (HCT116) Day 4 Spheroids



4.2.2 Hypoxic regions have eIF4E2-active translation

Once we identified hypoxic regions in spheroid models through immunohistochemistry, we proposed that these hypoxic regions are harnessing eIF4E2-mediated protein synthesis. We determined the association of cap-binding protein with heavy polysomes in U87MG spheroids cultured for six days. Spheroids shown in Figure 10-11 have both hypoxic and normoxic regions. Consequently this was reflected in the eIF4E and eIF4E2 association in heavy polysome fractions (Figure 12). eIF4E, the normoxic cap-binding protein, was found most strongly in monosome fractions. However was still found in polysome fractions, which we reasoned is contributed by normoxic cells along the edge of the spheroids. Interestingly, eIF4E2 was strongly found in the heavy fractions of the polysome profile. The polysome profile was similar to that of a hypoxic monolayer, because it showed relatively small polysome peaks suggesting a reduction in overall mRNA translation. We suggest that the large hypoxic core is contributing to the eIF4E2 association in polysomes and the normoxic exterior is contributing to eIF4E association in polysomes.

4.2.3 Xenografts have regions of mTORC1 inactivity and eIF4E2 association with heavy polysome fractions

Tumours grown in mice expressed regions of mTOR inactivity, in which we suspected eIF4E inactivity. Based on this information, we inspected biopsied mouse tumour xenografts for evidence of eIF4E inactivation. Xenografts are complex models, compared to spheroids, because they are vascularized and contain less evident pockets of hypoxia. Tumour xenografts were developed by injecting 10×10^6 human gliobastoma

Figure 12. Spheroids have active eIF4E2 protein in polysomes.

Polysomal distribution of eIF4E and eIF4E2 in several spheroids cultured for six days. L5 and S13 are ribosomal proteins used as a control. (Contributed by J.U.)

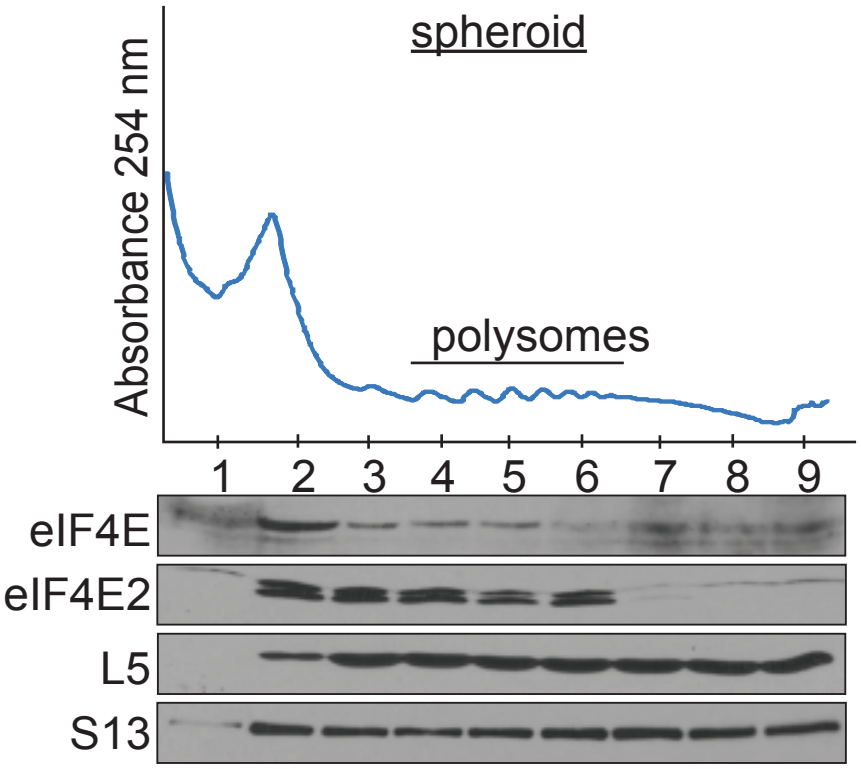
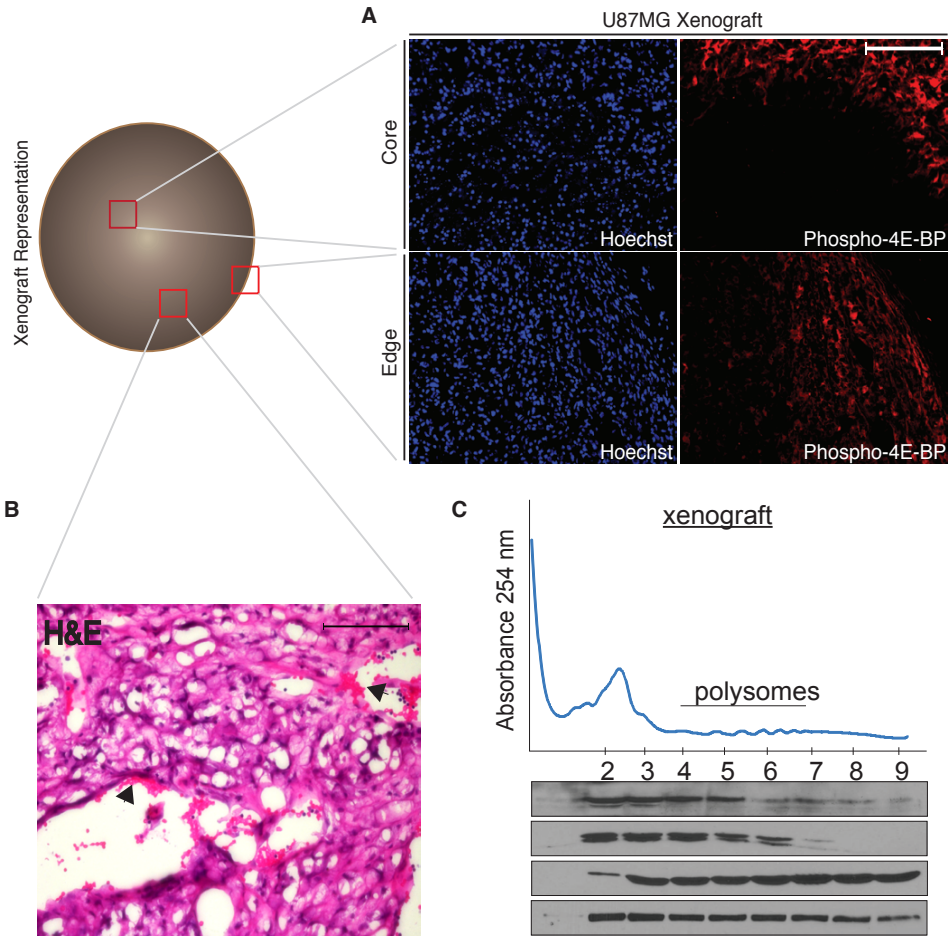

spheroid
Absorbance 254 nm
polysomes
1
2
3
4
5
6
7
8
9
eIF4E
eIF4E2
L5
S13

Figure 13. Xenografts contain cores with inactive mTORC1.

(A) Sections of an excised four-week-old nude mouse xenograft taken from the outer rim and the core. Immunolabeling with anti-P4E-BP shows that mTORC1 is active at the surface and inactive in pockets in the core. (B) Hematoxylin and eosin (H&E) stain of a four-week-old nude mouse xenograft core section. Arrows indicate blood vessels. Hoechst was used as a DNA counterstain. All experiments were performed in U87MG glioblastoma. Scale bar, 100 μm. (C) Polysome profiles of biopsied xenografts suggest eIF4E2 activity. (Polysome profile was contributed by J.U.).


A
U87MG Xenograft
Xenograft Representation
Core
Edge
Hoechst
Phospho-4E-BP
Hoechst
Phospho-4E-BP
B
H&E
C
xenograft
Absorbance 254 nm
polysomes
2 3 4 5 6 7 8 9

cells (U87MG) into the flanks of mice. Xenografts of cells expressing a scramble shRNA were biopsied at four weeks, once they grew to an ethical endpoint. Staining for P4E-BP in xenografts shows regions where mTORC1 is active. The edge region contains P4E-BP staining which decreases while moving away from available blood vessels and the core regions have pockets, which lack P4E-BP staining (Figure 13A). Blood vessels in the interior of the xenograft are indicated through Hematoxylin and Eosin staining (black arrows Figure 13B). These results confirmed that even vascularized tumours contain regions of mTORC1 inactivity.

We proposed that tumour xenografts also use eIF4E2 for hypoxic protein synthesis in these regions. Four week old biopsied xenografts were lysed and fractioned for polysome analysis. eIF4E2 activity in mRNA translation was confirmed through its association with polysomes (Figure 13C). Although eIF4E is present to a small degree in fraction 5, fractions 6 and 7 have drastically less eIF4E. Much like the spheroids and hypoxic monolayers, eIF4E2 was strongly associated with polysome fractions 5-7. Spheroids, xenografts and hypoxic monolayers are also similar in that their polysome profiles show a reduction in overall protein synthesis compared to normoxic monolayers.

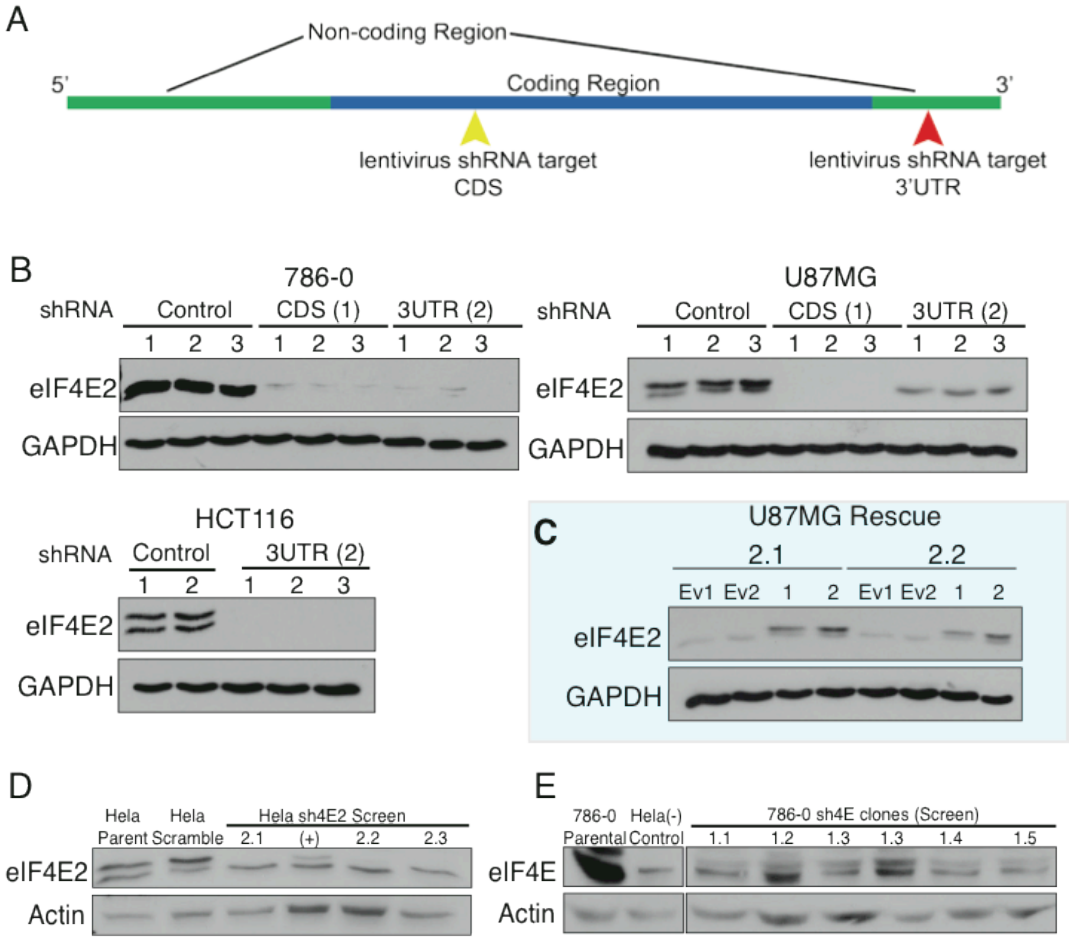
4.3 Development of cell lines that have stable knock down of eIF4E2 protein

eIF4E2 silencing experiments greatly reduce hypoxic protein synthesis (Uniacke et al., 2012). Since both *in vitro* and *in* vivo tumour models implicated regions of eIF4E2-mediated protein synthesis in their hypoxic cores, we suggested that eIF4E2 was harnessed for tumourigenesis and could be targeted to selectively kill tumour cores. To test this hypothesis, we first needed to determine the importance of eIF4E2 through the

use of *in vitro* genetic manipulations. Four genetically diverse cancer cell lines were used to show that this hypoxic mechanism of protein synthesis is a universally held system in glioblastoma U87MG (PTEN-null), Renal Cell Carcinoma 786-0 (VHL-null), Colorectal Carcinoma, HCT116 (KRAS mutation), and Cervical Carcinomas, HeLa. We used two shRNAs: one specific to the Coding Sequence (CDS) (shRNA-1) and one specific to the 3' untranslated region (3'UTR) of eIF4E2 mRNA (shRNA-2) (Diagram Figure 14A; Figure 14B). eIF4E2 knockdown was rescued using a flag-tagged eIF4E2 in 3'UTR knockdown cells (2.1Res1, 2.1Res2; 2.3Res1, 2.3Res2) (Figure 14C). As a control, these same shRNA-2 lines were transfected with a pcDNA 3.1 empty vector plasmid, from which clones 2.1EV1, 2.1EV2, 2.2EV1, and 2.2EV2 were chosen (Figure 14C). Interestingly, the antibody against eIF4E2 detected two bands by western blot. When we used a non-coding sequence knockdown in the 3'UTR of eIF4E2 mRNA only the top band is removed, whereas our coding sequence short hairpin removes both bands (Figure 14B-C). These cells, however, are similar to coding sequence knockdowns because they are unable to grow as spheroids. More importantly, the top band could be rescued, and in fact it appeared that there was less of the bottom band when we overexpressed the top band (Figure 14C). Also when we knocked down the top band only, the bottom band appears to strengthen compared to scramble shRNA expressing cells (Figure 14E, Hela knockdowns). HeLa cells with the stable knockdown of eIF4E protein were given to our lab from the Sonenberg Lab at McGill University, Montréal. To determine if eIF4E2 conferred a unique phenotype in comparison to eIF4E, we a developed HeLa cells expressing shRNA-2 (Figure 14D). Since HIF-2α is stabilized in normoxia in 786-0 cells,

Figure 14. shRNA against eIF4E2 mRNA suppresses eIF4E2 protein levels in human cancer cell lines and is rescued with reintroduction of human eIF4E2

Western blot analysis of eIF4E2 and actin levels in renal carcinoma (786-0), colorectal cancer (HCT116), and glioblastoma (U87MG) cell lines stably transfected with pGIPZ expressing scrambled shRNA (Control), or expressing one of two shRNA sequences directed against eIF4E2 (shRNA-1 or shRNA-2). [A] shRNA-1 targets the coding sequence and shRNA-2 targets the 3' untranslated region of mRNA. [B] shRNA against eIF4E2 suppressed eIF4E2 protein levels in glioblastoma, colorectal carcinoma and renal cell adenocarcinoma. Cells were infected with a pGIPZ lentiviral system and cultured in 1 μg/ml puromycin media with 5% FBS [C] Human eIF4E2 (Res) or an empty vector plasmid (EV) was transfected into U87MG clones expressing shRNA-2. [D] shRNA-2 expressing HeLa cells were developed (2.1, 2.2, 2.3), which is compared to both scramble control and HeLa parental cells. [E] eIF4E protein was stably knockdown in 786-0 cells with five clones shown (sh1.1, sh1.2, sh1.3, sh1.4, sh1.5).


A
Non-coding Region
5'
Coding Region
3'
lentivirus shRNA target
CDS
lentivirus shRNA target
3'UTR
B
786-0
shRNA
Control
CDS (1)
3UTR (2)
1 2 3 1 2 3 1 2 3
eIF4E2
GAPDH
U87MG
shRNA
Control
CDS (1)
3UTR (2)
1 2 3 1 2 3 1 2 3
eIF4E2
GAPDH
HCT116
shRNA
Control
3UTR (2)
1 2 1 2 3
eIF4E2
GAPDH
C
U87MG Rescue
2.1
2.2
Ev1 Ev2 1 2 Ev1 Ev2 1 2
eIF4E2
GAPDH
D
Hela Parent
Hela Scramble
Hela sh4E2 Screen
2.1 (+) 2.2 2.3
eIF4E2
Actin
E
786-0 Parental
Hela(-) Control
786-0 sh4E clones (Screen)
1.1 1.2 1.3 1.3 1.4 1.5
eIF4E
Actin

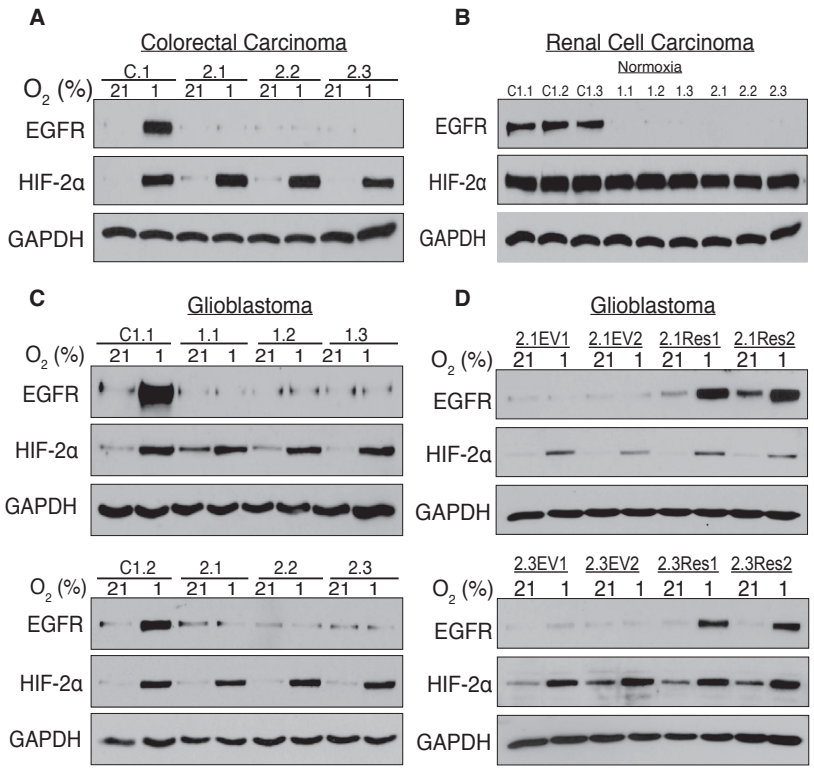
we also developed 786-0 cells expressing an shRNA that targets eIF4E protein in hopes that the eIF4E2-mediated protein synthesis machinery could rescue protein synthesis in normoxia (Figure 14E).

4.4 eIF4E2-depleted cells are unable to upregulate EGFR expression in hypoxia

After confirming eIF4E2 knockdown through western blot (Figure 14) we wanted to confirm that the eIF4E2/HIF-2α/RBM4 complex was targeted and inhibited. Epidermal growth factor receptor (EGFR) is translated in an eIF4E2-dependent fashion (Uniacke et al. 2012; Franovic et al. 2007). Therefore its translation in hypoxia was used to indicate whether shRNA-1 and shRNA-2 was targeting eIF4E2-mediated hypoxic protein synthesis. Stable silencing of eIF4E2 in HCT116 and U87MG cells was enough to disrupt the upregulation of EGFR in monolayers cultured in hypoxia for 24 h (Figure 15). Hypoxia corresponds to a drastic increase in HIF-2α protein (Figure 15, second row). HIF-2α is stabilized under low oxygen conditions and thought to be the oxygen-sensing protein that helps stabilize the eIF4E2-translation machinery in hypoxia. Since 786-0 cells have a defect in HIF-2α degradation (VHL-null), EGFR levels remained high even during normoxia. We suggest that HIF-2α is a limiting component to eIF4E2-mediated protein synthesis since eIF4E2 protein is available regardless of the conditions. 786-0 control cells express EGFR at an elevated rate as expected; however, cells lacking eIF4E2 protein did not (Figure 15B). This suggests that even in normoxia EGFR is upregulated in 786-0s in an eIF4E2-dependent manner. Reintroduction of eIF4E2 protein into 3'UTR knockdown lines rescued their ability to upregulate EGFR in hypoxia

Figure 15. Stable silencing of eIF4E2 prevents hypoxic overexpression of EGFR.

Western blot analysis of hypoxic and normoxic HIF2-α, EGFR, and GAPDH levels in [A] Colorectal Cancer, HCT116 [B] renal cell adenocarcinoma, 786-0. VHL (-/-) 786-0 cells have upregulated HIF-2α and EGFR in normoxia and no not require hypoxic treatment. [C-D] glioblastoma, U87MG cells. [A-C] cell lines stably transfected with pGIPZ expressing scramble shRNA (C1.1, C1.2, C1.3), or expressing shRNA-1 (1.1, 1.2, 1.3) or shRNA-2 (2.1, 2.2, 2.3) sequences directed against eIF4E2. [D] eIF4E2 was reintroduced in shRNA-2 expressing cells (2.1Res1, 2 and 2.3Res1, 2) and controlled with empty vector shRNA-2 cells (2.1EV1, 2 and 2.3EV1, 2). GAPDH is used as a loading control.


A
Colorectal Carcinoma
C.1
2.1
2.2
2.3
O2 (%)
21 1 21 1 21 1 21 1
EGFR
HIF-2α
GAPDH
B
Renal Cell Carcinoma
Normoxia
C1.1 C1.2 C1.3 1.1 1.2 1.3 2.1 2.2 2.3
EGFR
HIF-2α
GAPDH
C
Glioblastoma
C1.1
1.1
1.2
1.3
O2 (%)
21 1 21 1 21 1 21 1
EGFR
HIF-2α
GAPDH
C1.2
2.1
2.2
2.3
O2 (%)
21 1 21 1 21 1 21 1
EGFR
HIF-2α
GAPDH
D
Glioblastoma
2.1EV1
2.1EV2
2.1Res1
2.1Res2
O2 (%)
21 1 21 1 21 1 21 1
EGFR
HIF-2α
GAPDH
2.3EV1
2.3EV2
2.3Res1
2.3Res2
O2 (%)
21 1 21 1 21 1 21 1
EGFR
HIF-2α
GAPDH

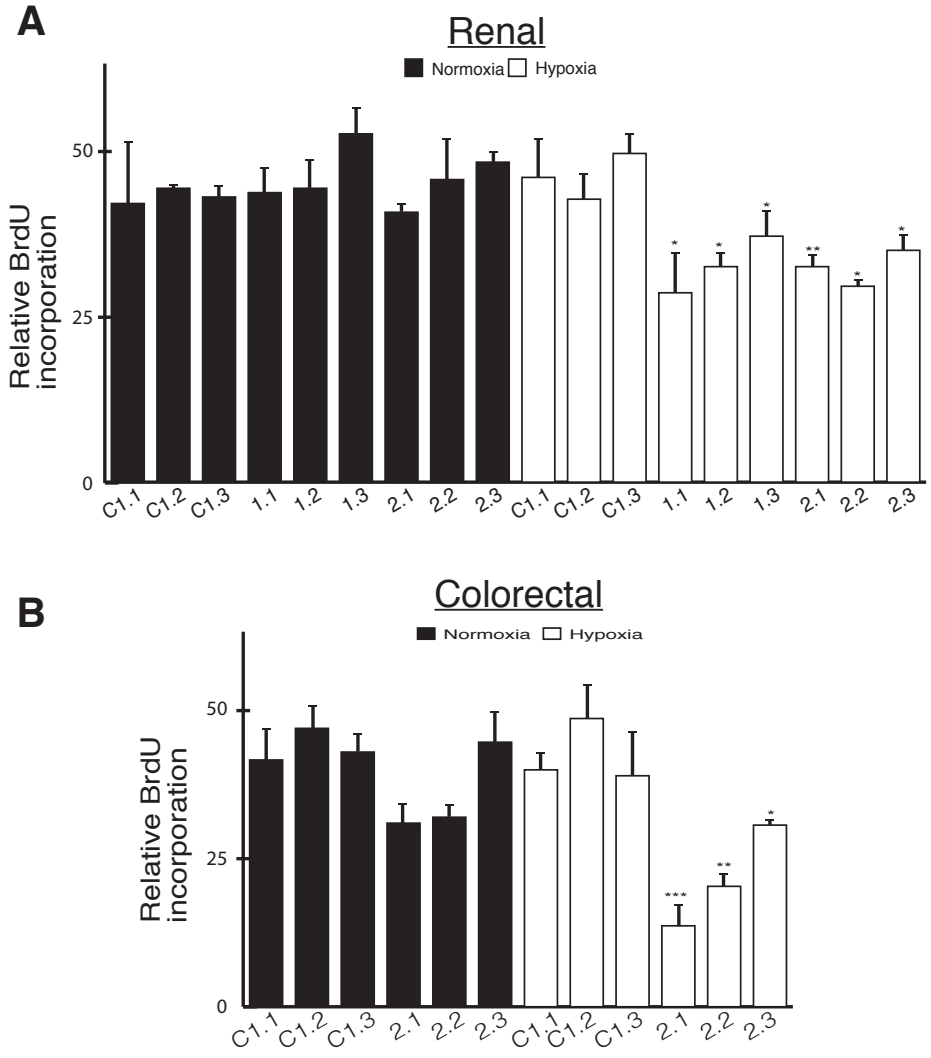
whereas empty vector controls could not (Figure 15D). The ability to reduce EGFR mRNA translation suggests that the shRNA constructs are targeting and inhibiting the hypoxic translation complex by inhibiting eIF4E2 protein, thereby acting as a secondary screen for stable line selection.

4.5 eIF4E2 is required for cellular proliferation in hypoxia

When eIF4E2 protein is transiently knocked down during hypoxia, a drastic reduction in protein synthesis is observed (Uniacke et al. 2012). Therefore we hypothesized that cells expressing eIF4E2 shRNA would be unable to adequately proliferate in hypoxia compared to cells expressing scrambled shRNA due to an inhibition of their protein synthesis machinery. To analyze the proliferating capacity in these cells, we completed a Bromo-D-Uracil (BrdU) assay of cells in normoxia and hypoxia. BrdU is incorporated into DNA during the synthesis phase of the cell cycle and can therefore can be used to mark cells that are undergoing cell division. Interestingly, renal carcinoma, colorectal carcinoma, and glioblastoma cells stably expressing scrambled shRNA did not experience a large change in percentage of proliferating cells after culturing in hypoxia for 16-24 h (Figure 16, 17A). In normoxia there was not a significant difference in BrdU incorporation between the control shRNA expressing and eIF4E2 shRNA expressing cell lines (Figures 16-17). Human renal adenocarcinomas showed around a 10-15% drop in proliferative capacity in hypoxia when eIF4E2 protein was knocked down (Figure 16). In human glioblastoma cells, BrdU incorporation decreased about 40% in knockdown cell lines that were treated with hypoxia, compared to their normoxic counterpart (Figure

Figure 16. Loss of eIF4E2-mediated protein synthesis diminishes hypoxic proliferation of 786-0 and HCT116 cells.

The ability of the [A] renal carcinoma, 786-0 and [B] colorectal carcinoma, HCT116 cell lines to proliferate in low oxygen was measured through BrdU incorporation. Stable silencing of eIF4E2 reduced proliferation of hypoxic 786-0 and HCT116 cells. 786-0 and HCT116 cell lines stably transfected with shRNA-1 (786-0 only: 1.1, 1.2, 1.3), shRNA-2 (2.1, 2.2, 2.3), or scrambled shRNA (C1.1, C1.2, C1.3) were cultured in 5% FBS medium followed by incubation in normoxic or hypoxic conditions for 24 h. Data bars depict the mean of three experiments. Error bars indicate S.E.M. Significance was determined using student's t-test. (*, $P \leq 0.05$, **, $P \leq 0.01$, ***, $P \leq 0.001$).


A
Renal
Normoxia
Hypoxia
Relative BrdU incorporation
50
25
0
C1.1
C1.2
C1.3
1.1
1.2
1.3
2.1
2.2
2.3
C1.1
C1.2
C1.3
1.1
1.2
1.3
2.1
2.2
2.3
B
Colorectal
Normoxia
Hypoxia
Relative BrdU incorporation
50
25
0
C1.1
C1.2
C1.3
2.1
2.2
2.3
C1.1
C1.2
C1.3
2.1
2.2
2.3

17A). These results would suggest that in several genetically unique cell lines, eIF4E2 protein is sufficient to allow cell proliferation in hypoxia. The requirement of eIF4E2 in hypoxic proliferation was confirmed using rescued cell lines. When eIF4E2 was reintroduced, the ability of U87MG cells to proliferate in hypoxia was also restored (Figure 17B). Even when eIF4E2 protein was severely knocked down, however, some cellular proliferation occurred. To further understand the effects on cell growth we performed basic cell counts.

In correspondence with the proliferation assays, cell doubling was affected in cells lacking eIF4E2 when incubated in hypoxia. Cells were plated in normoxia and hypoxia for 48 h. After 48 h cell numbers were compared between normoxic and hypoxic samples. In all cell types measured, HCT116, U87MG, and 786-0 each experienced varying doubling times according to their cell type. Cells lacking eIF4E2 had similar cell numbers compared to cells expressing a scrambled shRNA after 48 h in normoxia (Figure 18). In hypoxia however, renal adenocarcinoma, and colorectal carcinoma cells that had eIF4E2 protein knockdown have approximately 50% of the cells that control cells had after 48 h of hypoxic treatment (Figures 18A-B). U87MG cells behaved in a similar fashion when eIF4E2 was depleted, with a decrease to about 50% of control cells (Figure 18C). Rescued cell lines confirmed eIF4E2 dependence (Figure 18D).

Figure 17. Reintroduction of eIF4E2 protein returns proliferative potential in hypoxia.

The ability of the glioblastoma U87MG cell lines to engage in proliferation, with and without eIF4E2, under low oxygen availability was assessed as a function of their ability to incorporate BrdU. [A] stable silencing of eIF4E2 reduces the growth of U87MG cells cultured in hypoxia but not normoxia. [B] Cells expressing an shRNA against eIF4E2 but have eIF4E2 reintroduced were able to regain proliferative capacity when cultured in hypoxia. Cells were cultured in 5% FBS medium followed by incubation in normoxic or hypoxic conditions for 24 h. Data bars depict the mean of three experiments. Error bars indicate S.E.M. Significance was determined using student's t-test. (*, $P \leq 0.05$, **, $P \leq 0.01$, ***, $P \leq 0.001$).

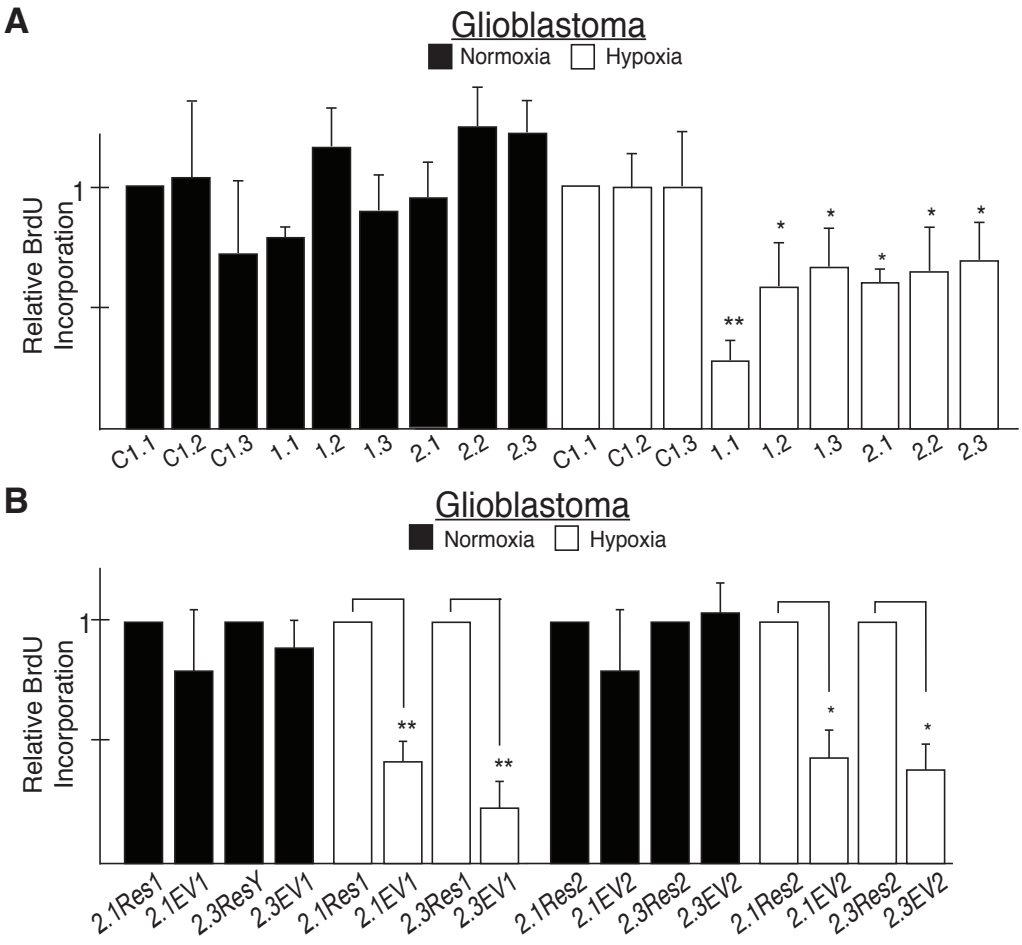
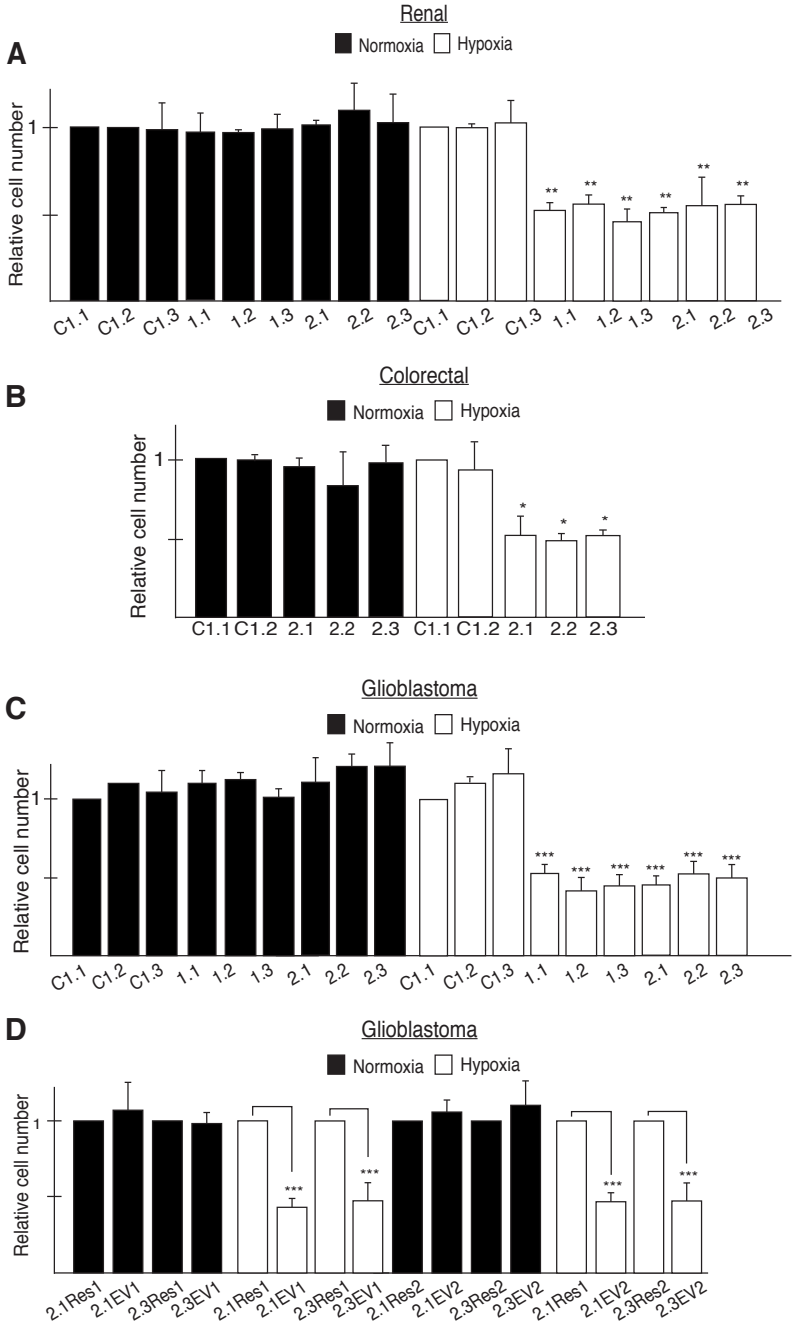

A
Glioblastoma
Normoxia
Hypoxia
Relative BrdU Incorporation
1
C1.1
C1.2
C1.3
1.1
1.2
1.3
2.1
2.2
2.3
C1.1
C1.2
C1.3
1.1
1.2
1.3
2.1
2.2
2.3
B
Glioblastoma
Normoxia
Hypoxia
Relative BrdU Incorporation
1
2.1Res1
2.1EV1
2.3ResY
2.3EV1
2.1Res1
2.1EV1
2.3Res1
2.3EV1
2.1Res2
2.1EV2
2.3Res2
2.3EV2
2.1Res2
2.1EV2
2.3Res2
2.3EV2

Figure 18. Cells lacking eIF4E2 protein have reduced cell numbers in hypoxia.

Basic cell counts after 48 h incubation in normoxic and hypoxic conditions for [A] 786-0 [B] HCT116 [D-E] and U87MG cells. Data bars depict the mean of three experiments. Error bars indicate S.E.M. Significance was determined using student's t-test. (*, $P \leq 0.05$, **, $P \leq 0.01$, ***, $P \leq 0.001$).


A
Renal
Normoxia
Hypoxia
Relative cell number
1
C1.1 C1.2 C1.3 1.1 1.2 1.3 2.1 2.2 2.3 C1.1 C1.2 C1.3 1.1 1.2 1.3 2.1 2.2 2.3
B
Colorectal
Normoxia
Hypoxia
Relative cell number
1
C1.1 C1.2 2.1 2.2 2.3 C1.1 C1.2 2.1 2.2 2.3
C
Glioblastoma
Normoxia
Hypoxia
Relative cell number
1
C1.1 C1.2 C1.3 1.1 1.2 1.3 2.1 2.2 2.3 C1.1 C1.2 C1.3 1.1 1.2 1.3 2.1 2.2 2.3
D
Glioblastoma
Normoxia
Hypoxia
Relative cell number
1
2.1Res1 2.1EV1 2.3Res1 2.3EV1 2.1Res1 2.1EV1 2.3Res1 2.3EV1 2.1Res2 2.1EV2 2.3Res2 2.3EV2 2.1Res1 2.1EV2 2.3Res2 2.3EV2

4.6 eIF4E2 depletion is enough to inhibit *in vitro* tumour growth

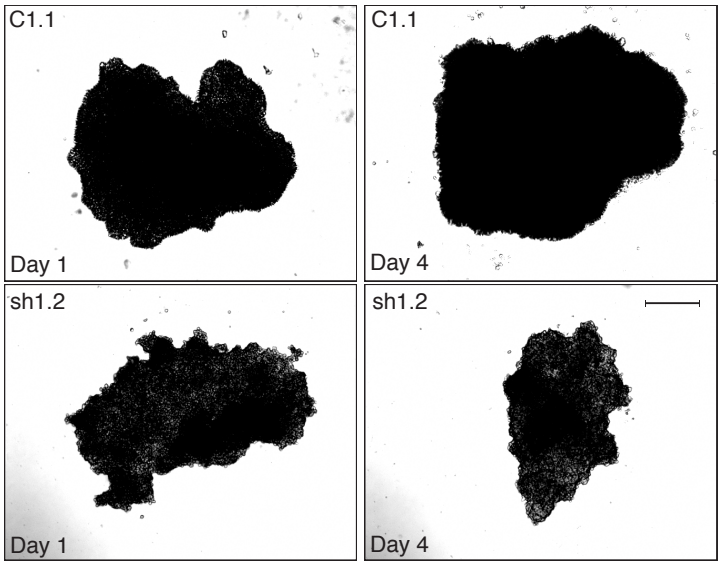
We suggest that eIF4E2 protein is translationally active in spheroids because eIF4E2 protein is associated with polysomes in hypoxic monolayers and spheroids (Figure 9, 12). As well, eIF4E2 is active in hypoxia, which is present in spheroids (Figures 10D, 11C). The effect of eIF4E2 shRNAs on proliferative capacity led us to investigate the growth capabilities of spheroid models. Cells lacking eIF4E2 protein made visibly smaller spheroids (U87MG; Figure 19A). U87MG, HCT116, and 786-0 cells lacking eIF4E2 protein made significantly smaller spheroids in comparison to scrambled shRNA-expressing cells (Figure 19B, 20). In the time points measured, cells expressing eIF4E2 specific shRNA decreased in size and remained at about 50% in HCT116 and U87MG cells, and 45% in 786-0 cells. Cell dependence on eIF4E2 for spheroid growth was confirmed by measuring spheroid growth in U87MG shRNA-2 cells that have eIF4E2 protein reintroduced. Compared to empty vector clones, rescued cell lines increased substantially whereas all four empty vector clones decreased by at least 50% (Figure 21).

Figure 19. Silencing eIF4E2 protein is sufficient to inhibit *in vitro* tumour growth of glioblastoma cells.

Scrambled (C1.1, C1.2, 1.3), eIF4E2 shRNA-1 (sh1.1, sh1.2, sh1.3) and shRNA-2 (sh2.1, sh2.2, sh2.3) expressing U87MG cells were plated over soft agarose to form *in vitro* spheres. [A] Representative images of control (C1.1) and eIF4E2-depleted (sh1.2) cells grown as spheroids for 1 or 4 days. Scale bar indicates 100 μm. [B] Area measurements were monitored daily. Data points represent means of three experiments. Error bars indicate S.E.M. Significance was determined at endpoint using student's t-test. (**, $P < 0.01$, ***, $P < 0.001$).

Glioblastoma Cell Line
U87MG

A



Glioblastoma Cell Line
U87MG

B

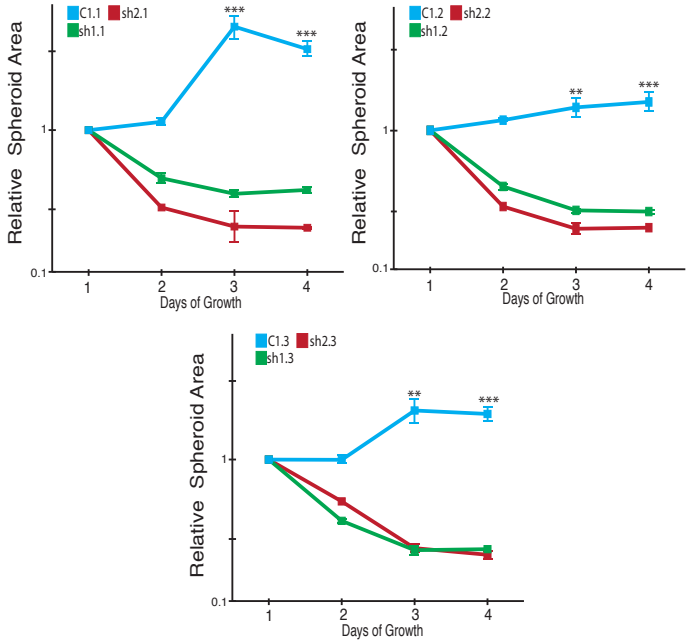


Figure 20. Silencing eIF4E2 is sufficient to inhibit *in vitro* tumour growth of renal adenocarcinoma and colorectal cancer.

Scrambled (C1.1, C1.2), eIF4E2 shRNA-1 (786-0: sh1.2, sh1.3) and shRNA-2 (786-0: sh2.2, sh2.3, HCT116: sh2.1, sh2.2, sh2.3) cells were grown over soft agarose to form *in vitro* tumours. [A-B] Area measurements were monitored daily. Data points represent means of three experiments. Error bars indicate S.E.M. Significance was determined at endpoint using student's t-test. HCT116 (***, $P < 0.001$, n=3) and 786-0 (***, $P < 0.001$, n=3).

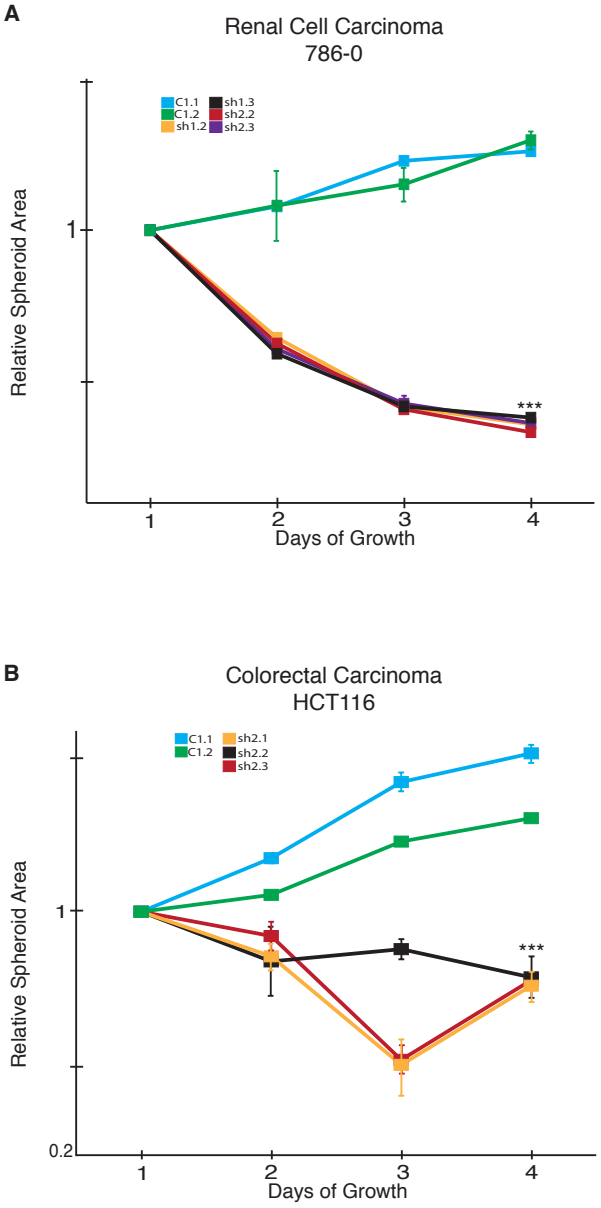
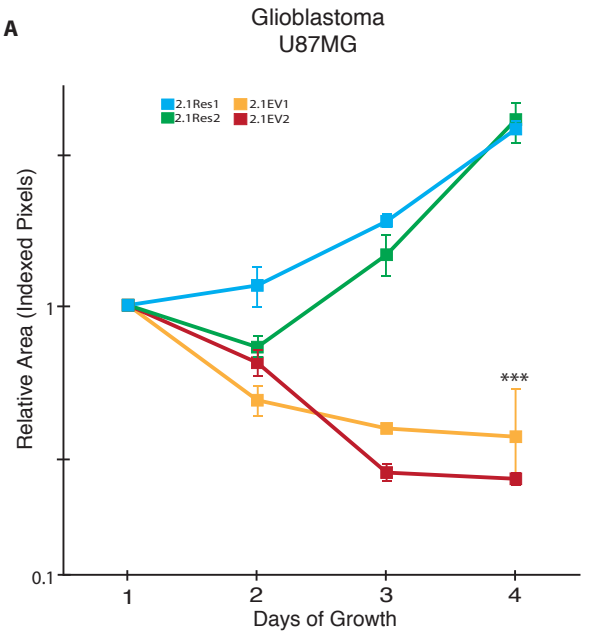
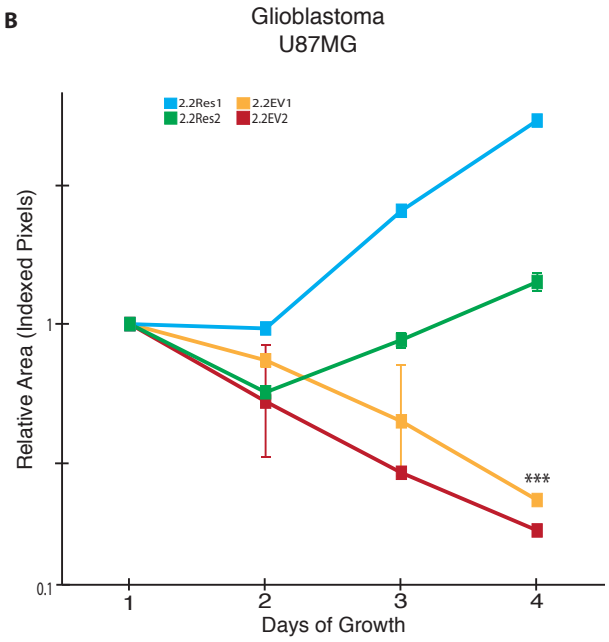

A
Renal Cell Carcinoma
786-0
C1.1
C1.2
sh1.2
sh1.3
sh2.2
sh2.3
Relative Spheroid Area
1
***
1
2
3
4
Days of Growth
B
Colorectal Carcinoma
HCT116
C1.1
C1.2
sh2.1
sh2.2
sh2.3
Relative Spheroid Area
1
0.2
***
1
2
3
4
Days of Growth

Figure 21. Loss of in vitro tumour growth is restored with reintroduction of eIF4E2. Spheroid formation was monitored in shRNA-2 cells that where transfected with either an empty vector plasmid (2.1EV1,1 and 2.2EV1,2) or a human eIF4E2-containing plasmid (2.1Res1,2 and 2.2Res1,2). [A-B] Area measurements were calculated daily. Data points represent means of three experiments. Error bars indicate S.E.M. Endpoints between controls and rescued cell lines were significantly different through student's t-test (***, $P < 0.001$, $n=3$).


A
Glioblastoma
U87MG
2.1Res1
2.1Res2
2.1EV1
2.1EV2
Relative Area (Indexed Pixels)
1
0.1
***
1
2
3
4
Days of Growth


B
Glioblastoma
U87MG
2.2Res1
2.2Res2
2.2EV1
2.2EV2
Relative Area (Indexed Pixels)
1
0.1
***
1
2
3
4
Days of Growth

4.7 Spheroids lacking eIF4E2 protein maintain cell division

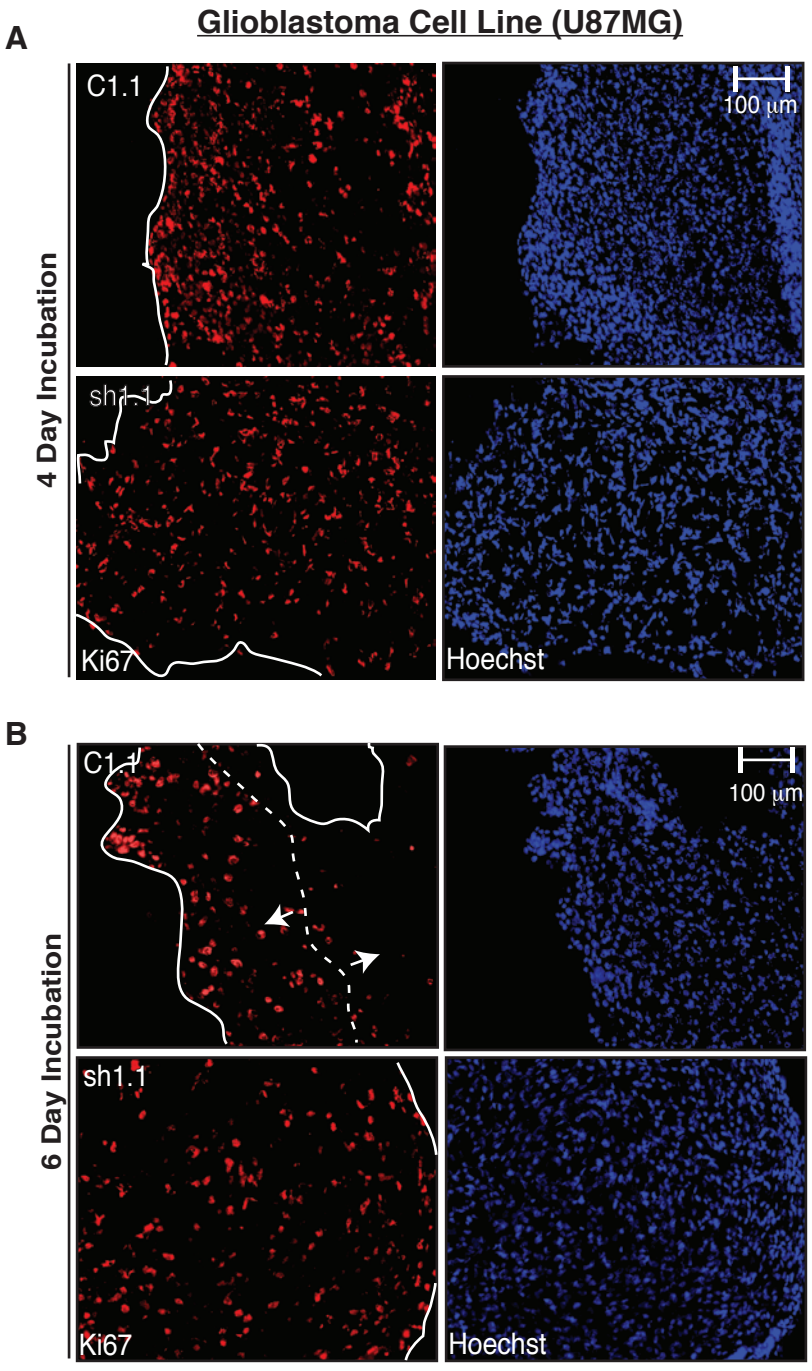
To confirm that eIF4E2 shRNA expression inhibited growth in spheroids, we proposed that growth would not be detectable in core regions. We stained for the mitotic protein Ki67, expressed in cells that are committed to cell division. To our surprise, in both day four and day six spheroids lacking eIF4E2, Ki67 was dispersed evenly regardless of location in the spheroid (Figure 22). After six days, scrambled shRNA-expressing cells formed spheroids that had less Ki67 staining in their cores and a prominent edge of proliferating cells (Figure 22B). These results concur with the physical observation that eIF4E2-depleted spheroids are more fragile and porous than scramble expressing spheroids.

4.8 Spheroids made from eIF4E2 shRNA expressing cells cannot become hypoxic

3-dimensional *in vitro* tumour spheroids mimic the tumour environment by containing regions of hypoxia, as illustrated in Figures 10-11. Unlike spheroids made from scrambled shRNA-expressing cells, we suggest that eIF4E2-silenced spheroids were unable to form hypoxic cores since P4EBP activity and phospho-S6 staining of day six spheroid cross sections appear throughout the spheroid (Figure 23A-B). Phosphorylated S6 is a product of mTORC1 activity. In hypoxia mTORC1 activity is inhibited resulting in less phospho-S6. eIF4E2-silenced spheroids had visibly more phospho-S6 protein expression. As well, these spheroids were Hypoxyprobe™ negative, suggesting that the spheroids were not hypoxic (Figure 23C). Measurement of HIF-2α protein levels was also completed in 2D (monolayer) and 3D

Figure 22. Loss of eIF4E2 protein does not inhibit cell division in glioblastoma spheroids.

Glioblastoma cells, stably expressing scrambled control (C1.1) or eIF4E2 shRNA (sh1.1), were cultured as multicellular spheroids and stained for Ki67 expression using basic immunohistological techniques. Spheroids were grown for four [A] or six [B] days and representative images were visualized at magnification 20X. Scale bar indicates 100 μm. Solid line, section border. Dotted line, border between edge and core. Arrows are used to indicate regions little Ki67 staining adjacent to high Ki67 staining.


Glioblastoma Cell Line (U87MG)
A
C1.1
100 µm
4 Day Incubation
sh1.1
Ki67
Hoechst
B
C1.1
100 µm
6 Day Incubation
sh1.1
Ki67
Hoechst

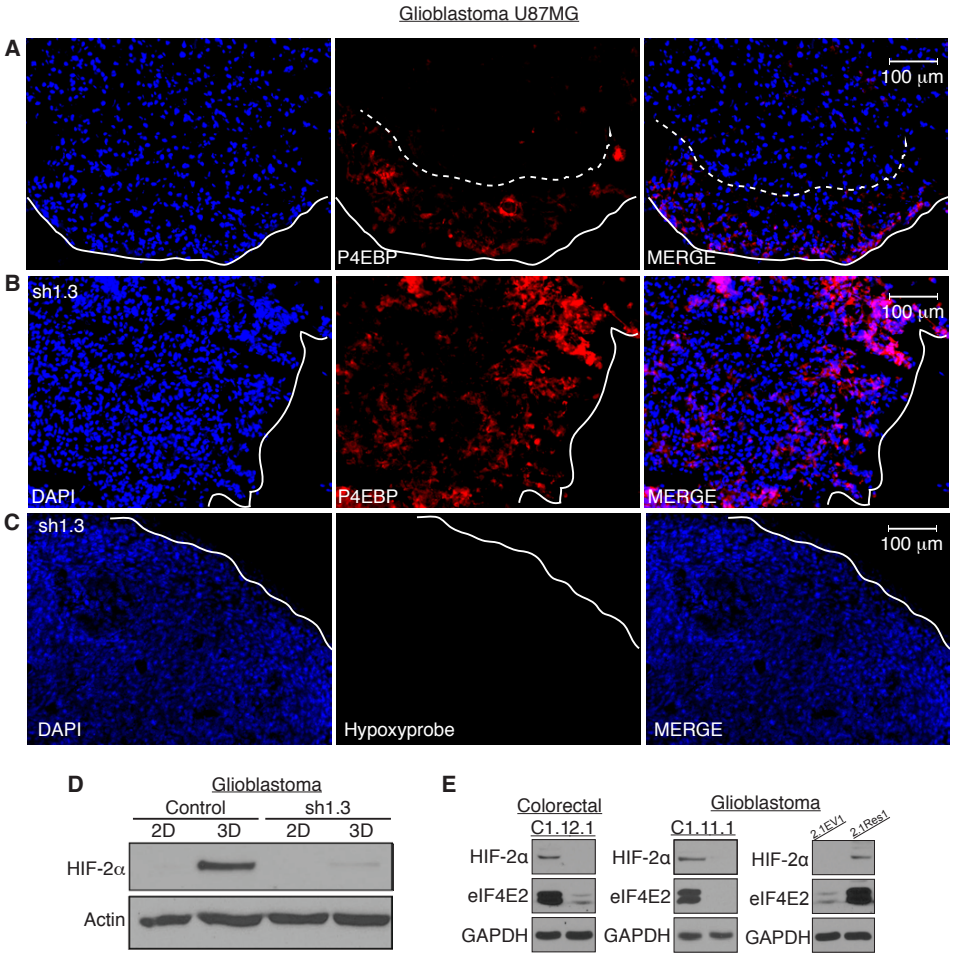
(spheroid) protein samples. HIF-2α is active under low oxygen conditions and can be used as an appropriate marker of hypoxia. Control and eIF4E2-silenced monolayers did not express HIF-2α (Figure 23D). When hypoxic however, all monolayers had an upregulation of HIF-2α protein (Figure 23). Control spheroids had an upregulation of HIF-2α protein, however, we did not see this same increase in spheroids where eIF4E2 expression was inhibited (Figure 23D). Following growth analysis of colorectal, and glioblastoma, spheroids were lysed and probed for HIF-2α protein. Cells lacking eIF4E2 protein did not become hypoxic, however eIF4E2 rescued cells could become hypoxic (Figure 23E). Taken together, these experiments suggest that the cores of spheroids made from eIF4E2-silenced cells are not hypoxic or small portions become transiently hypoxic and avoid detection through immunohistochemistry and western blot. This may be the result of cells being unable to cope through periods of hypoxia due to a lack of their ability to synthesize proteins resulting in cell death.

4.9 eIF4E2 is required for hypoxic survival of genetically unique cancers

Although hypoxia inhibits proliferation in cells lacking eIF4E2, spheroid analysis illustrates non-hypoxic tumour cores. Area measurements, however, indicated that without eIF4E2, spheroids cannot grow and in fact decreased in size. To answer these opposite results we hypothesized that the spheroids were undergoing massive cell death, which balanced the level of growth observed. Our first objective was to test hypoxic survival of monolayers. We predicted that cells stably expressing eIF4E2 shRNA would not survive in monolayer cultures grown at 1% O_2 either through apoptosis or necrosis.

Figure 23. eIF4E2-silenced human glioblastoma cells cannot form hypoxic *in vitro* tumours.

Spheroids made from cells expressing either scrambled (C1.2) or eIF4E2 shRNA-1 (sh1.3), were cultured for six days and stained through basic immunohistological techniques. [A-B] phosphorylated 4E-BP (P4E-BP) staining in spheroids made from C1.2 and sh1.3 cells. [C] Six-day-old spheroids made from sh1.3 expressing cells were incubated in Hypoxyprobe™ for 1 hr and stained using basic techniques. Solid line, section border. Dotted line, border between edge and core. [D] Western blot of hypoxic HIF-2α upregulation, in spheroid control cells (3D) and monolayers (2D). Actin is used as a loading control. [E] Western blot of HIF-2α using spheroids made from colorectal and glioblastoma cells expressing scrambled (C1.1), eIF4E2-targeting shRNA-1 (1.1), shRNA-2 or empty vector (2.1EV1) and shRNA-2 with eIF4E2 reintroduced (2.1Res1).


Glioblastoma U87MG
A
100 µm
P4EBP
MERGE
B
sh1.3
100 µm
DAPI
P4EBP
MERGE
C
sh1.3
100 µm
DAPI
Hypoxyprobe
MERGE
D
Glioblastoma
Control
sh1.3
2D
3D
2D
3D
HIF-2α
Actin
E
Colorectal
C1.12.1
Glioblastoma
C1.11.1
2.1EV1
2.1Res1
HIF-2α
eIF4E2
GAPDH
HIF-2α
eIF4E2
GAPDH
HIF-2α
eIF4E2
GAPDH

Our first experiments to answer this hypothesis were to culture cell monolayers in hypoxia for 48 h, followed by staining for the apoptotic marker active caspase-3. Cells that stain strongly for active caspase-3 protein are likely committed to apoptosis. Previous studies have indicated that cells treated with translation inhibitors, such as Cycloheximide, have upregulation of cleaved (active) caspase-3 protein (Blom et al., 1999; Chen et al., 2013). Cycloheximide is a drug that blocks the translocation of tRNA in the ribosomes when the complex is bound to mRNA, thereby inhibiting the elongation step of translation (Ennis and Lubin, 1964). In accord with these predictions, HCT116, 786-0, and U87MG cells had an increased expression of active caspase-3 after 48 h in hypoxia (Figure 24-25). This would suggest a significant increase in apoptosis of cells without eIF4E2 in hypoxia. Figure 24 illustrates a significant increase in active caspase-3 in HCT116 cell monolayers incubated hypoxia when eIF4E2 is depleted. Likewise, U87MG and 786-0 cells also have significantly more active caspase-3 staining in hypoxic cells that were depleted of eIF4E2 (Figures 25). There was approximately 4-5-fold increase in the percentage of cells expressing active caspase-3 in hypoxia, compared to control cells lines in hypoxia. Although there was an increase in active caspase-3 expression when control cells were cultured in hypoxia for 48 h, there was no significant difference between control and knockdown cells in normoxia (Figures 24-25). Reintroduction of eIF4E2 was sufficient to recover hypoxic survival (Figure 25C). Rescued cells lines proliferated similarly to control cells, even in hypoxia. Vehicle control cells were unable to survive in hypoxia, in a similar fashion to the eIF4E2 shRNA-expressing clones.

Figure 24. Programmed cell death is initiated in HCT116 cells lacking eIF4E2 during hypoxic incubation.

Levels of active caspase-3 were measured in colorectal cells expressing scrambled or eIF4E2-targeting shRNA-1 [A] Representative images of control cells (C1.1, C1.2) and eIF4E2-depeleted cells (1.1, 1.2) expressing active caspase-3 (red) in either hypoxic or normoxic conditions. The insert depicts Hoechst positive cells [B] The ratio of active caspase-3 positive to Hoechst positive cells is index to controls. Bars indicate mean of three experiments (**, $P < 0.01$,***, $P < 0.001$). Significance was determined using student's t-test. Error bars indicate SEM.

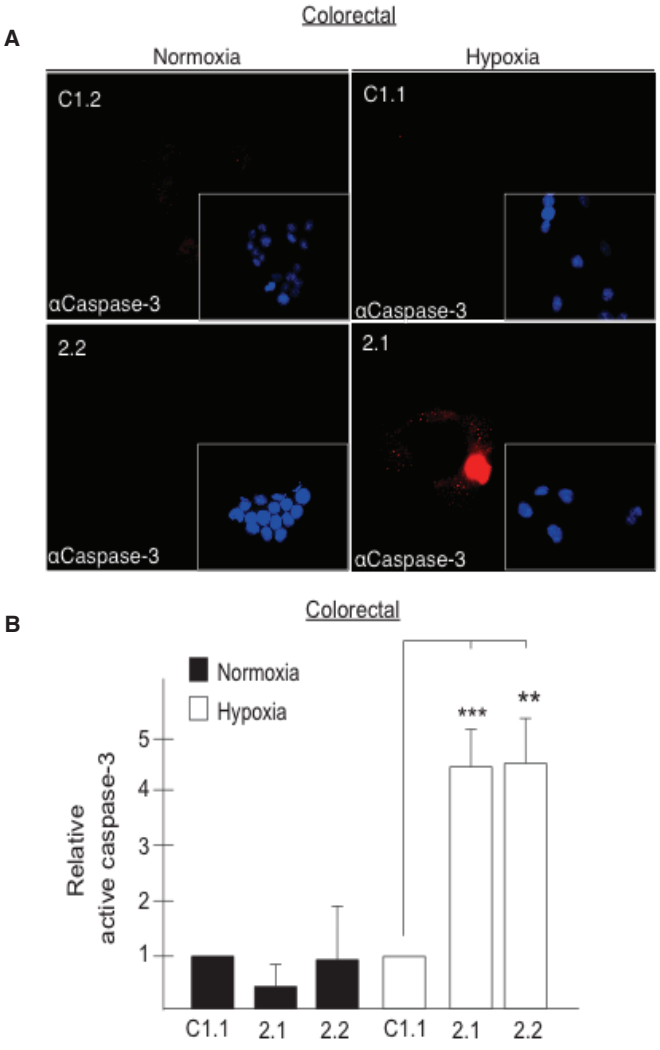
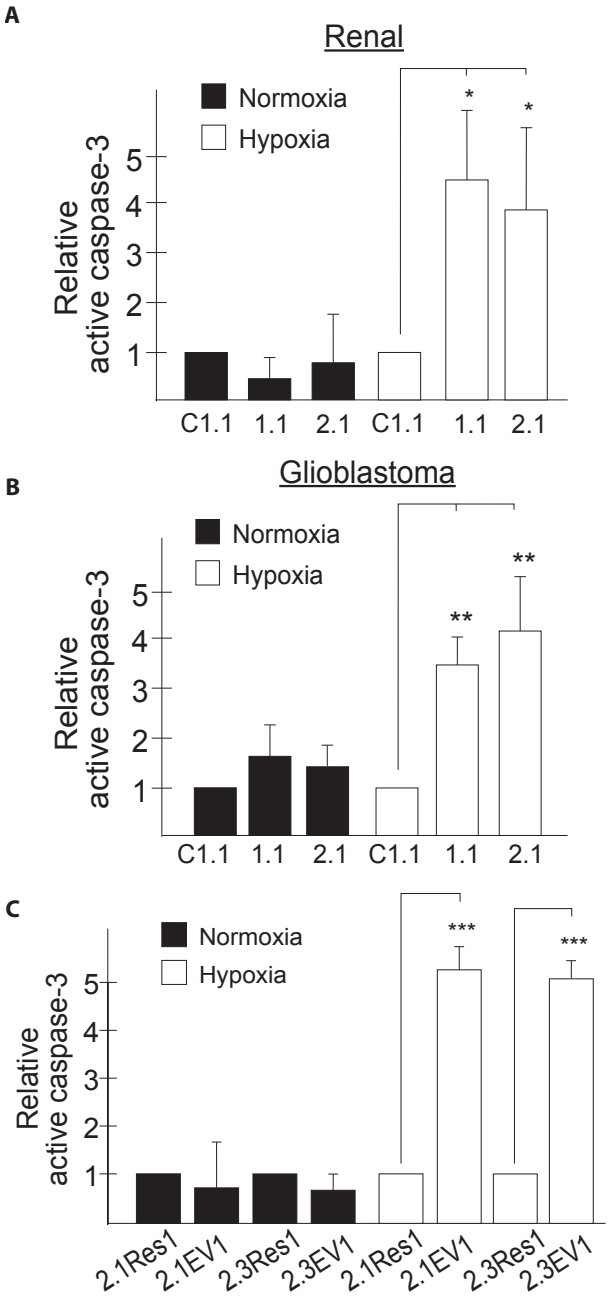

A
Colorectal
Normoxia
Hypoxia
C1.2
αCaspase-3
C1.1
αCaspase-3
2.2
αCaspase-3
2.1
αCaspase-3
B
Colorectal
Normoxia
Hypoxia
Relative active caspase-3
1
2
3
4
5
***
**
C1.1
2.1
2.2
C1.1
2.1
2.2

Figure 25. Human cancers require eIF4E2 to reduce programmed cell death in hypoxia.

Levels of active caspase-3 were measured in [A] renal adenocarcinoma [B] glioblastoma cells expressing scrambled (C1.1) or eIF4E2-targeting shRNA (1.1, 2.1). [C] eIF4E2-depleted empty vector controls (2.1EV1, 2 and 2.3EV1, 2) were compared to eIF4E2 rescued cells (2.1Res1,2 and 2.3Res1,2) for active caspase-3 positive staining. Cells were cultured in 5% FBS media at 21% O_2 or 1% O_2 for 48 hours. Significance was determined using student's t-test. Bars indicate mean of three experiments. Error bars indicate SEM. (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

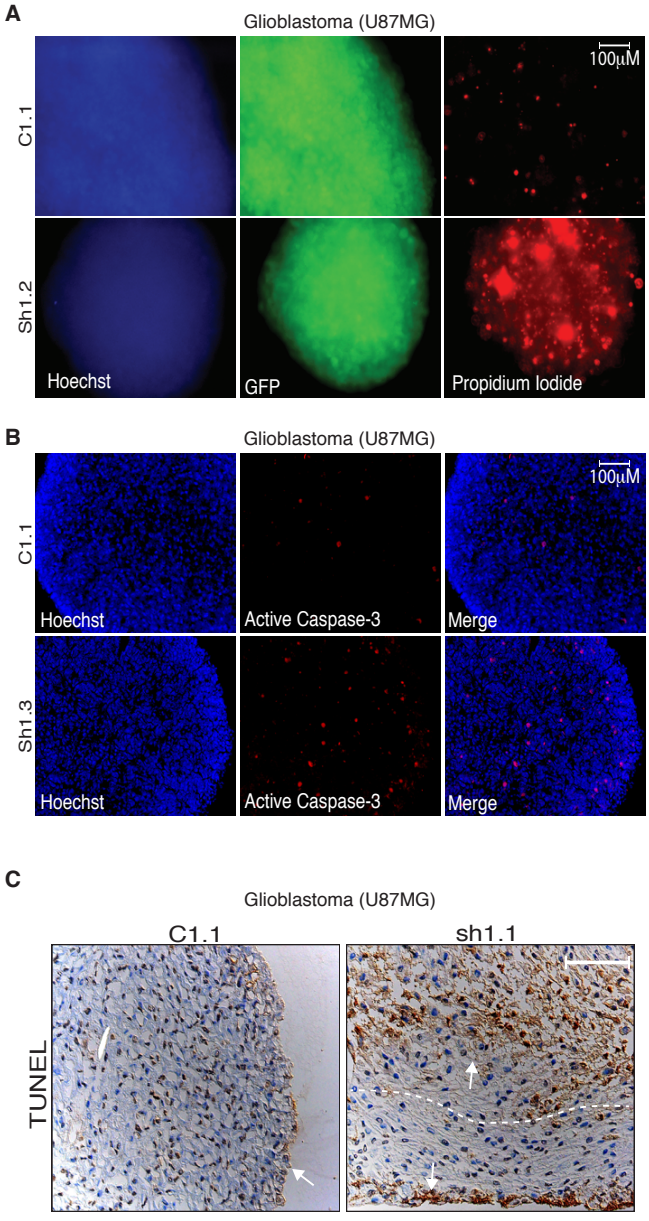

A
Renal
Normoxia
Hypoxia
Relative active caspase-3
1
2
3
4
5
*
*
C1.1
1.1
2.1
C1.1
1.1
2.1
B
Glioblastoma
Normoxia
Hypoxia
Relative active caspase-3
1
2
3
4
5
**
**
C1.1
1.1
2.1
C1.1
1.1
2.1
C
Normoxia
Hypoxia
Relative active caspase-3
1
2
3
4
5
***
***
2.1Res1
2.1EV1
2.3Res1
2.3EV1
2.1Res1
2.1EV1
2.3Res1
2.3EV1

4.10 Regions of massive cell death in spheroids compensate for regions of proliferation

Cell death in spheroids was measured with three separate markers: active caspase-3 staining, propidium iodide incorporation during live cell imaging and TUNEL immunohistochemistry. In Figure 26A it appeared that spheroids made from eIF4E2-silenced cells had more propidium iodide, compared to control spheroids. This would suggest that those specific regions were apoptotic or necrotic. In hypoxic monolayers, cells expressing eIF4E2 shRNA showed an elevated number of caspase-3 positive cells in comparison to scrambled shRNA-expressing cells (Figures 24-25). Spheroids were also stained for active caspase-3, which illustrates that knockdown spheroids have more death than their control counterparts (Figure 26B). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) uses an enzyme to mark broken DNA caused by apoptosis or necrosis. Spheroids lacking eIF4E2 experienced severe cell death in their core regions after only five days in culture. In comparison, control spheroids showed very little TUNEL staining (Figure 26C). This could suggest that cells lacking eIF4E2 were significantly less able to survive hypoxia, and may be the reason for an increased propidium iodide incorporation in spheroids grown with eIF4E2-silenced cells. This could be the result of inhibiting hypoxic translation causing cell death.

Figure 26. eIF4E2-depleted spheroids experience hypoxia-induced cell death

[A] Live cell imaging of four day old U87MG spheroids of scrambled expressing (C1.1) or shRNA-expressing (sh1.1) cells stained for Hoechst (blue), expressing GFP (green), and incubated in propidium iodide (red) [B] Immunolabeling with anti-active caspase-3 of four day old spheroid sections derived from U87MG glioblastoma cells stably expressing non-targeting shRNA (C1.1) or shRNA targeting eIF4E2 (sh1.1) [C] TUNEL staining of four day old spheroid sections derived from U87MG glioblastoma cells stably expressing non-targeting shRNA (C1.1) or shRNA targeting eIF4E2 (sh1.1). Scale bar indicates 100 μm. Dotted line, border between edge and core.


A
Glioblastoma (U87MG)
C1.1
Sh1.2
100μM
Hoechst
GFP
Propidium Iodide
B
Glioblastoma (U87MG)
C1.1
Sh1.3
100μM
Hoechst
Active Caspase-3
Merge
Hoechst
Active Caspase-3
Merge
C
Glioblastoma (U87MG)
C1.1
sh1.1
TUNEL

4.11 eIF4E2 depletion prevents the tumourigenesis of genetically diverse human cancers

Based on our *in vitro* observations, we proposed that eIF4E2 was a requirement for *in vivo* tumourigenesis. To explore the effects of silencing eIF4E2 *in vivo*, nude mice were injected subcutaneously in their flanks with U87MG, 786-O, or HCT116 cells stably expressing either control shRNA, shRNA targeting eIF4E2, rescued knockdown cells and knockdown empty vector controls. Four weeks after injection, U87MG cells stably expressing control shRNA produced large tumours over 200-300 mm^3 in volume (Figure 27A). Conversely, U87MG cells stably expressing shRNA-targeting eIF4E2 produced no noticeable tumours throughout the study (Figure 27A). Similar results were obtained with the 786-O (Figure 27B) and HCT116 (Figure 27C) stable cell lines whereby significantly larger tumours were observed in controls relative to eIF4E2-depleted xenografts.

Requirement of eIF4E2 protein was confirmed through *in vivo* injection of knockdown clones with eIF4E2 reintroduced or transfected with an empty vector. eIF4E2-rescued clones formed large tumours in the same size range as scramble expressing cells (200-300 mm^3) whereas empty vector controls failed to grow (Figure 28A-B). Once tumours reached an ethical endpoint, tumours were biopsied and their volumes were determined to confirm the accuracy of our measurements during the study. We were unable to excise and study tumours from the eIF4E2-depleted cells because of their absence. These results clearly establish a critical role for eIF4E2 and hypoxic protein synthesis in *in vivo* tumour cell proliferation in genetically diverse cancers.

Figure 27. eIF4E2-depleted cells cannot form *in vivo* tumours.

Silencing of eIF4E2 prevents the tumourigenesis of genetically diverse human cancers. [A-C] Weekly tumour volume measurements in nude mouse xenograft assays performed with [A] U87MG glioblastoma, [B] 786-O renal cell carcinoma and [C] HCT116 colorectal carcinoma cells stably expressing non-targeting shRNA (C1.1 and C1.2) or shRNA targeting eIF4E2 (1.1, 1.2, 2.1 and 2.2). Representative mice that were injected subcutaneously with control cells on the left flank and eIF4E2-depleted cells on the right flank were photographed at the ethical endpoint. Data are mean of at least three independent experiments. Error bars indicate SEM. Significance measured by student's t test (* $p < 0.05$, ** $p < 0.01$). (Contributors: J.K.P., J.U., and C.F.)

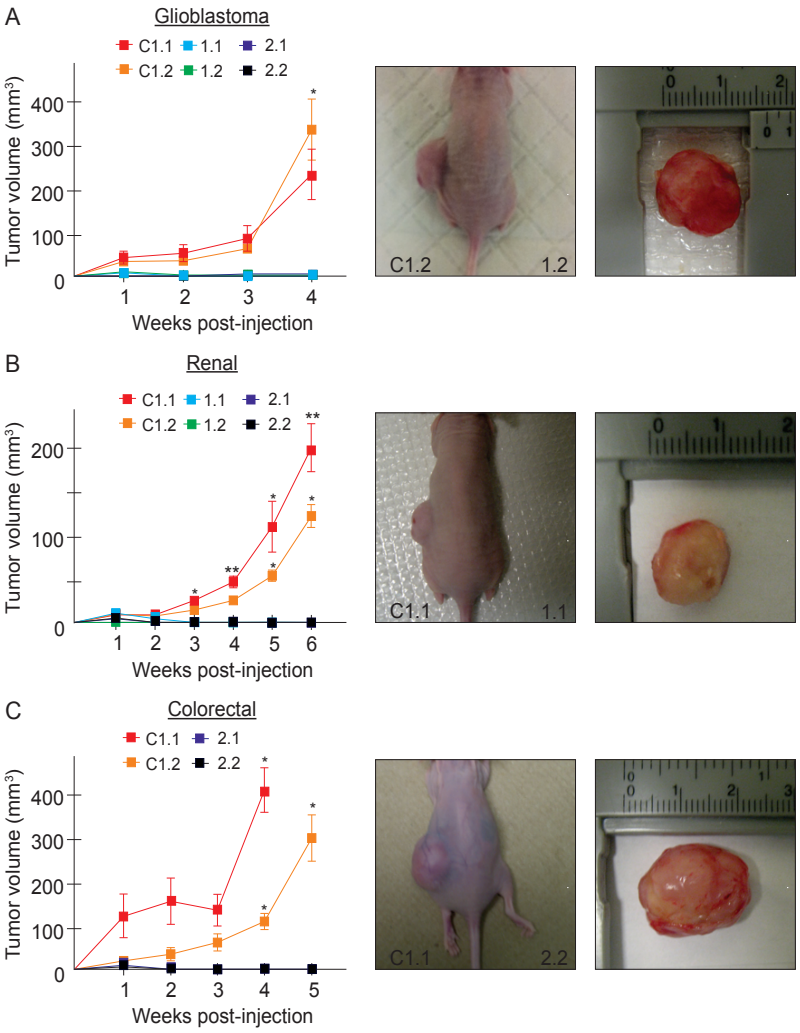
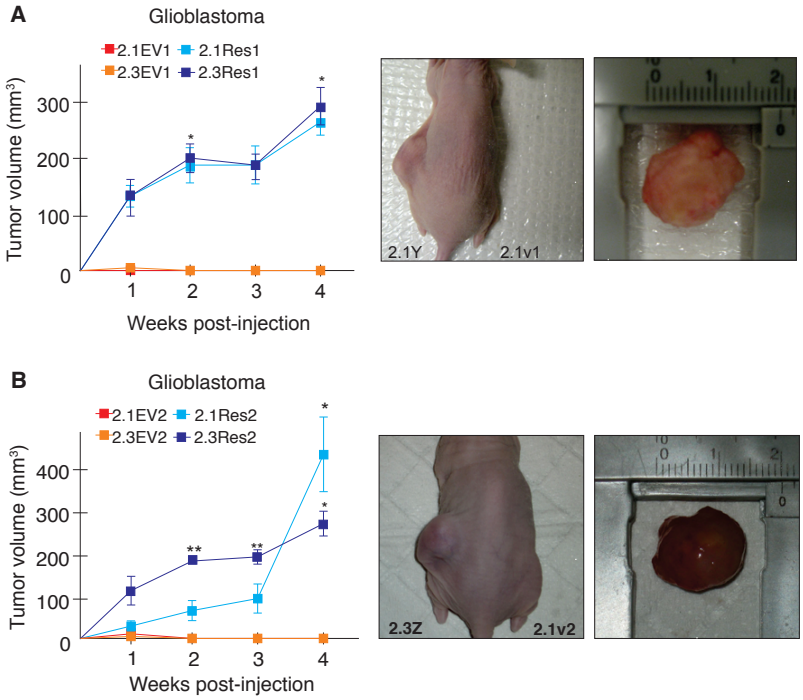

A
Glioblastoma
C1.1
1.1
2.1
C1.2
1.2
2.2
Tumor volume (mm3)
400
300
200
100
0
1
2
3
4
Weeks post-injection
C1.2
1.2
B
Renal
C1.1
1.1
2.1
C1.2
1.2
2.2
Tumor volume (mm3)
200
100
0
1
2
3
4
5
6
Weeks post-injection
C1.1
1.1
C
Colorectal
C1.1
2.1
C1.2
2.2
Tumor volume (mm3)
400
300
200
100
0
1
2
3
4
5
Weeks post-injection
C1.1
2.2

Figure 28. Reintroduction of eIF4E2 protein in previously silenced cells rescues the tumour phenotype.

Reintroduction of eIF4E2 protein is sufficient to return tumourigenic ability in previously eIF4E2-depleted U87MG cells. [A-B] Weekly tumour volume measurements in nude mouse xenograft assays were performed with empty vector control eIF4E2 knockdown cells (2.1EV1, 2.1EV2 and 2.3EV1, 2.3EV2) and eIF4E2 rescues (2.1Res1, 2 and 2.3Res1, 2). Representative mice that were injected subcutaneously with eIF4E2 rescues on the left flank and vehicle control cells on the right flank were photographed at the ethical endpoint. Data points represent of at least three independent experiments. Error bars show S.E.M. Significance measured by student's t test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). All experiments were performed in U87MG glioblastoma. (Contributors: J.K.P., J.U., and C.F.)


A
Glioblastoma
2.1EV1
2.1Res1
2.3EV1
2.3Res1
Tumor volume (mm3)
300
200
100
0
1
2
3
4
Weeks post-injection
*
2.1Y
2.1v1
B
Glioblastoma
2.1EV2
2.1Res2
2.3EV2
2.3Res2
Tumor volume (mm3)
400
300
200
100
0
1
2
3
4
Weeks post-injection
**
*
2.3Z
2.1v2

5.0 Discussion

5.1 Summary of findings

Cancers result from genetic mutations that lead to abnormal cell growth. Several different genetic mutations have been identified in the development of cancer cells; however, in this complexity there are also some shared traits between cancers. Tumours share a unique microenvironment that includes acidosis, nutrient starvation, and hypoxia. In this thesis we propose that tumours hijack eIF4E2-mediated hypoxic protein synthesis in order to develop into life-threatening tumours. We provide evidence for eIF4E2 activity in both *in vivo* and *in vitro* tumours (Figures 12-13), which contain regions of hypoxia and mTORC1 inhibition (Figures 10-11, 13). Cancer cells depleted of eIF4E2 have reduced survival in hypoxia, and show elevated levels of apoptosis in these conditions (Figures 24-25). Moreover, eIF4E2 is required to maintain the proliferative potential of cancer cells in hypoxia, but not normoxia (Figures 16-18). Early tumourigenic analysis shows that eIF4E2-depleted cells from three genetically distinct cancers cannot form hypoxic spheres (Figure 23). Although they maintain consistent cell proliferation, a large degree of death results (Figure 26). The data suggests that tumours will grow to a critical mass that is governed by the oxygen diffusion distance. After this point hypoxia will lead to necrosis and apoptosis in the absence of eIF4E2-mediated protein synthesis, which compensates for cell division and maintains spheroids to a small size (Figures 19-21). Reintroduction of human eIF4E2 rescues the cells ability to divide, survive, and form *in vitro* tumours. Nude mouse xenografts show that without eIF4E2 tumours cannot be observed, whereas with this cap-binding protein large tumours persist (Figures 27-28). Our results suggest that eIF4E2 inhibitors may be suitable as chemotherapeutics because

a switch in protein synthesis machinery occurs in physiologically irrelevant hypoxia. Therefore, eIF4E2 may be a method of specifically targeting the hypoxic tumour cells that are the most detrimental to prognosis.

5.2 A switch in the protein synthesis machinery occurs in regions of *in vitro* and *in vivo* tumours

The switch in cap-binding protein (Figure 12, 13) appears to be necessary for tumourigenesis (Figure 27-28). *In vivo* analysis indicates that cells lacking eIF4E2 protein form barely measurable tumours at any point (Figure 27). eIF4E2 must, therefore, be required for early tumourigenesis. We explore early tumourigenesis *in vitro,* through the use of spheroid models. Spheroids are less than 1000 μm in diameter and can survive for days *in vitro* while developing a hypoxic core (Figures 10-11). It has been well documented that in spheroids and xenografts the oxygen diffusion distance is 100-150 μm (Groebe and Vaupel, 1988; Rijken et al., 2000). It is not just due to the ability of oxygen to penetrate tissues, since some large molecules can penetrate up to 400 μm, but because the outer regions of cells are using up oxygen for energy production (Kwasiborski et al., 2012). In regions of oxygen availability eIF4E is the predominant cap-binding protein, responsible for initiating protein synthesis. With 100 times more affinity for the cap and 10 times more abundance than eIF4E2 in the cell, it is thought that eIF4E greatly outcompetes its homologue for cap-binding responsibility. This is observed in polysome profiles of monolayers at 21% O_2 (Figure 9A). Uniacke et al. (2012) clearly describe that a switch in protein synthesis machinery occurs when cells are exposed to low oxygen. We suspect that the tumour regions exceeding the oxygen

diffusion distance are producing proteins in an eIF4E2-mediated manner. For this to occur, eIF4E must be inhibited, thereby allowing for eIF4E2 to compete for the cap structure. In spheroids made from U87MG and HCT116 cells, the potent eIF4E inhibitor 4E-BP is active in regions approximately 100-150 μm away from the edge (Figures 10-11). This result is in accord with regions of hypoxia, which we suspect is the cause of eIF4E inhibition (Figures 10A-B, 11A-B). These regions are thus potential sites of eIF4E2 activity. Polysome analysis confirms that eIF4E2 is active in both xenografts and spheroid models (Figures 12, 13C). The hypoxic regions account for about 35% of spheroid volumes. Xenografts are a more complex model because they contain vasculature, which reduces regions of hypoxia (Figure 13B). Continued eIF4E activity in polysome profiles of spheroids may result from regions of oxygen availability. We suggest that the greater the hypoxic to normoxic ratio in a tumour, the closer the polysome profiles would be to that of Figure 9A. These results would suggest that certain regions of early tumours have switched from eIF4E to eIF4E2 mediated protein synthesis. If our analysis is correct then current drugs targeting eIF4E-mediated translation may be ineffective in targeting the hypoxic tumour cores that are known to result in a detrimental prognosis. Conversely, eIF4E2 inhibitors may be suitable to selectively target the hypoxic core of tumours.

5.3 eIF4E2 requirement is specific to regions of hypoxia

We silenced eIF4E2 in three genetically unique cell lines without detrimental consequences under normal oxygen conditions. There were no apparent morphological differences between cells expressing non-targeting scrambled shRNA or shRNA

targeting eIF4E2. Cell division was also unaltered under normal conditions (Figures 16, 17A). These results imply that eIF4E2 inhibition in normoxic conditions is not detrimental to the cell. To our knowledge, eIF4E2 is not required outside of hypoxic translation. One might predict a higher rate of tumourigenesis in eIF4E2-depleted cells because eIF4E is not challenged for cap responsibility, removing a possible area of regulation (Morita et al., 2012). Although we have not studied the response of non-cancer cells to eIF4E2 inhibition, studies in normoxic conditions suggest that depletion of eIF4E2 does not make cells more proliferative. We know that in hypoxic monolayers of eIF4E2-depleted cells, a drastic, five fold, increase in programmed cell death occurs (Figures 24, 25A-B) and that reintroduction of eIF4E2 is enough to restore hypoxic survival (Figure 25C). Interestingly, even eIF4E2-depleted cells did not have a statistically significant amount of cell death when cultured in normoxia. Inhibition of normoxic protein synthesis causes an elevation of apoptosis (Babu et al., 2012). Thus, a rise in active Caspase-3 protein levels may have resulted from inhibition of the dominant mRNA translation machinery in these cells. Since eIF4E can function in normoxia, a significant difference in apoptosis is not found between scramble, rescue, and eIF4E2 shRNA expressing cells. Restoration of eIF4E2 protein in shRNA-2 cells was not detrimental to cell growth suggesting that we did not overexpress the protein enough to inhibit eIF4E mediated translation. We have shown that in hypoxia, eIF4E2 is required for proliferation (Figures 16, 17). We predict that the drop in protein synthesis capacity is inhibiting the growth phases of the cell cycle. A decrease in cell division coupled with reduced survival accounts for the reduced cell numbers that were observed. These experiments clearly describe that hypoxia is more detrimental to cells lacking eIF4E2

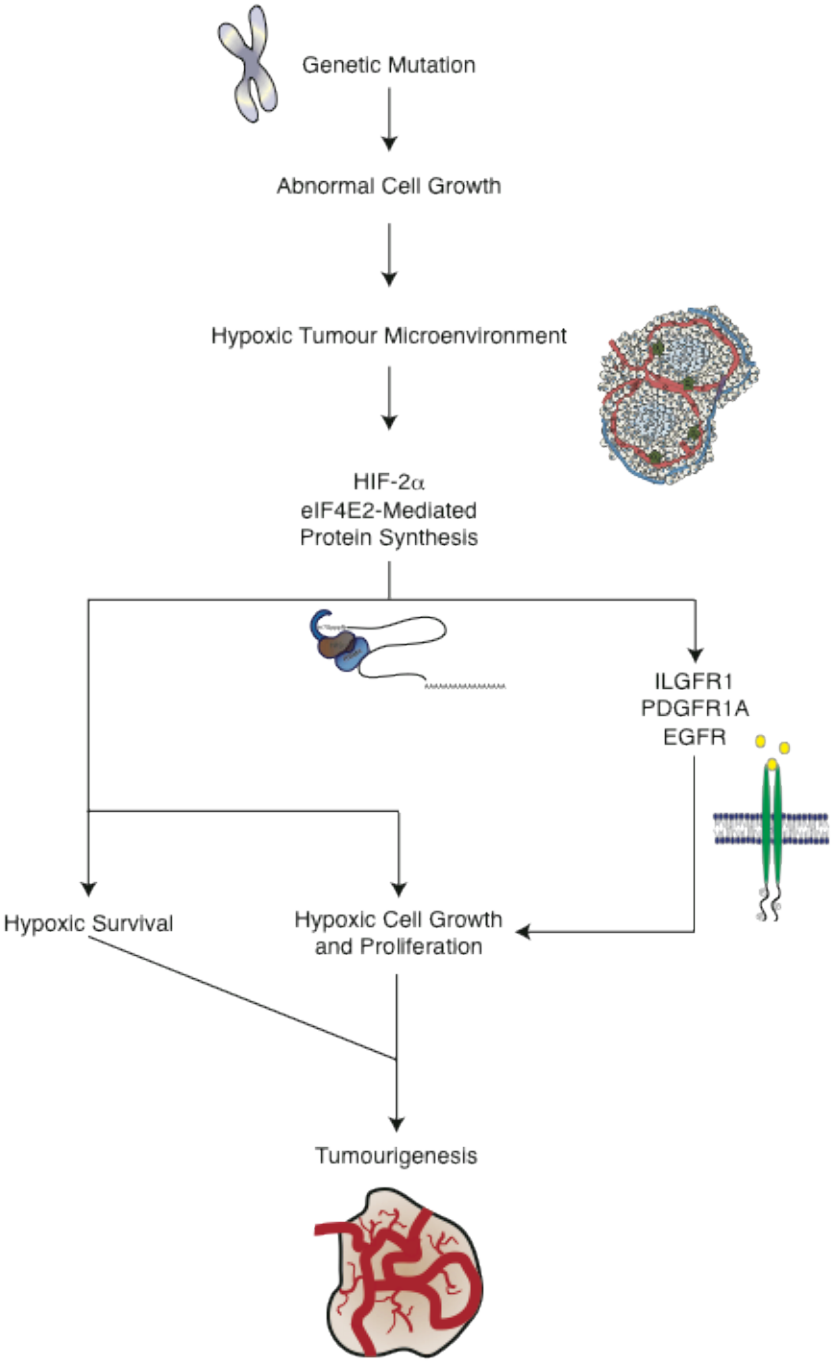
than their control counterparts. The requirement of eIF4E2 is summarized in Figure 29. Even more interesting is that eIF4E2 shRNA expressing cells are unaffected in normal oxygen conditions. This suggests that we can selectively terminate hypoxic cells by inhibiting eIF4E2. As well, it seems that hypoxic survival is dependent on eIF4E2 and its function cannot be compensated for by another protein in the cell. We predict that eIF4E2 requirement at early tumourigenesis is the reason eIF4E2-depleted *in vivo* tumours could not grow, limiting growth to the oxygen diffusion distance.

5.4 Inhibition of hypoxic protein synthesis machinery leads to cell death

Spheroids act as an avascular tumour model. Thus their growth is indicative that these cells can form a tumour and survive a simulated tumour microenvironment. Growth was inhibited in three sets of genetically unique spheroids lacking eIF4E2 protein (Figures 19-20). Spheroids decrease in size within a few days. It appears that these spheroids are plateauing at approximately half of their starting size (approximately 300 μm in radius). Colorectal and glioblastomal cells expressing non-targeting shRNA were able to form dense spheroids with hypoxic regions (Figures 10-11). Spheroids lacking eIF4E2 were fragile and more porous. They were unable to develop hypoxia, as tested through immunohistochemistry, western blot, and Hypoxyprobe staining (Figure 23). This distinct physical identity may result from the cell's inability to survive in hypoxia, resulting in regions of cellular debris and poor support (Figure 30). The spheroid cores act as a scaffold for growth, which is clear in Ki67 staining of late day spheroids (Figure 22). Although cell division occurs in all regions of eIF4E2-depleted spheroids, physical

Figure 29. eIF4E2 is allows for abnormal growth into tumours

Genetic mutations that lead to abnormal cell growth and cancer often cause abnormal vasculature development and hypoxia in tumour cores. During these hypoxic episodes eIF4E2 is required for cell division and survival, which in turn promotes tumourigenesis.


Genetic Mutation
Abnormal Cell Growth
Hypoxic Tumour Microenvironment
HIF-2α
eIF4E2-Mediated
Protein Synthesis
ILGFR1
PDGFR1A
EGFR
Hypoxic Survival
Hypoxic Cell Growth
and Proliferation
Tumourigenesis

growth is not seen. This would suggest that a critical mass is reached in spheroids, which once passed would lead to hypoxia, inhibition of growth, and subsequently, cell death.

Chronic hypoxia typically reduces cell division in control spheroids as seen in Figure 22B. At this stage we typically see a dense proliferative rim of cells. In eIF4E2-depleted spheroids, proliferation is observed uniformly throughout the spheroid, which correlates with a lack of hypoxia, but is not consistent with a reduction in spheroid size. In late day spheroids of control cells, these regions eventually have reduced cell proliferation, but the stress on cells in this microenvironment may have selective pressure leading to an aggressive phenotype. As well, hypoxic survival for days may help promote angiogenesis alleviating the stress from growing tumours. If eIF4E2 could be targeted in early stage tumours, the scaffold from which tumours can grow and evolve would be compromised, leading to tumours that remain within the oxygen diffusion distance.

TUNEL staining indicated a large region of necrosis in spheroids lacking eIF4E2 protein (Figure 26C). TUNEL positive regions encompass many more cells than active Caspase-3 staining, suggesting that more cells die from necrosis (Figure 26B-C). Our leading hypothesis is that cells lacking eIF4E2 can produce tumours until they develop a hypoxic microenvironment. Once hypoxia permits, extensive cell death and reduced proliferation occur collapsing regions of the tumour (Figure 30). We propose that this critical size is fairly small, since we achieved it with only 2×10^4 cells. With such a small mass of cells we were able to develop hypoxia within days. It is possible that smaller regions of hypoxia exist throughout the development of the spheroids contributing to the loss of size in eIF4E2-lacking spheroids.

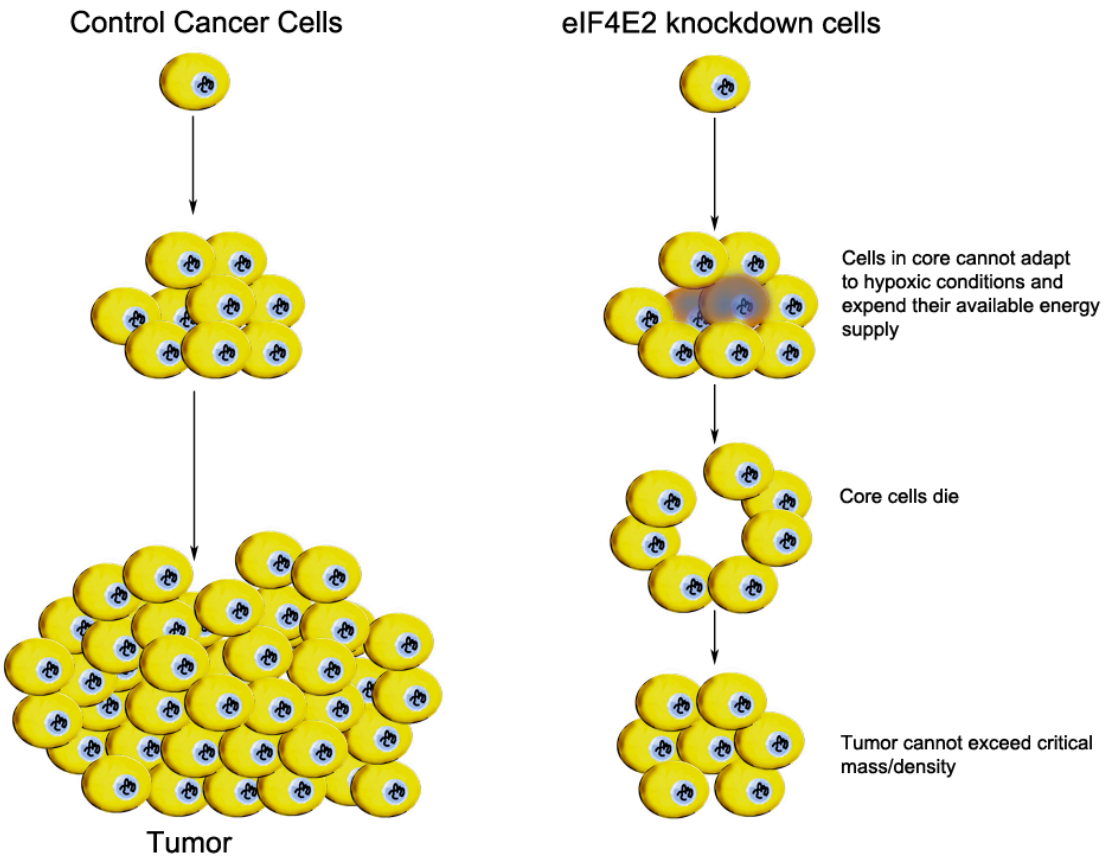
5.5 Inhibiting eIF4E2 may selectively target hypoxic tumour cells

By the 1950s scientists were exploiting differences between bacterial and mammalian cells as antibiotic treatments. Several antibiotic drugs target bacterial metabolism and protein synthesis. Specifically, these drugs target differences in metabolic pathways as to not harm the host organism. Likewise, cancers have unique characteristics that we believe can be exploited to develop anti-cancer drugs. Our results suggest that targeting eIF4E2 is an appropriate way of reducing tumour size. More importantly, eIF4E2 is active in regions of hypoxia below 1% O_2. These conditions are physiologically unique, and we may thus impede eIF4E2 to selectively target hypoxic tumour cells. Currently, a few translation inhibitors are in phase III clinical trials that target eIF4E2's normoxic counterpart. eIF4E is overexpressed in several cancers such as in neck and head carcinomas (Nathan et al., 1997), breast cancer (Kerekatte et al., 1995), colon cancer (Rosenwald et al., 1999), myelogenous leukemia (Topisirovic et al., 2003), and non-Hodgkin's Lymphoma (Wang et al., 1999). This has lead to various drugs that target eIF4E as a cancer therapeutics (Graff et al., 2008). However, conflicting studies suggest that eIF4E inhibition leads to IRES-mediated translation which causes tumourigenesis though angiogenic proteins; vascular endothelial growth factor – A (VEGF-A), HIF-1α (Lang et al., 2002), and inhibitor of apoptosis; Bcl2 (Braunstein et al., 2007; Sherrill et al., 2004).

The inhibitor of mTOR, rapamycin, has shown modest results since it does not bind to the catalytic site of mTOR. This method is partially ineffective because 4E-BP activity returns after a few hours during rapamycin treatment, even though S6K is still

Figure 30. eIF4E2 confers hypoxic survival and tumourigenesis.

eIF4E2-depleted cells are limited in their growth potential since the development of hypoxia leads to cell death. Consequently, these masses remain at a critical mass.


Control Cancer Cells
eIF4E2 knockdown cells
Cells in core cannot adapt to hypoxic conditions and expend their available energy supply
Core cells die
Tumor cannot exceed critical mass/density
Tumor

unphosphorylated (Choo et al., 2008). Although some normoxic translation inhibitors have shown success (Yao et al., 2011) clinical trials show no change in the metastatic phenotype (Yao et al., 2013). Novel attempts have targeted the 5' m7GpppN cap of mRNA (Kentsis et al., 2004) and the eIF4E-eIF4G interaction (Moerke et al., 2007) for eIF4E inhibition. Although relatively tolerated, mTOR inhibitors result in common side effects that include diarrhea, vomiting, and nausea. Second generation rapamycins are more toxic than first generation drugs, and seem to be ineffective in cancer lines containing KRAS mutations (Zaytseva et al., 2012). Other side effects of these drugs include hyperglycemia and high cholesterol and triglyceride levels (Morrisett et al., 2002). The eIF4G inhibitors cause cell death that is most likely not selective to cancer cells (Fan et al., 2010). eIF4E may not be an appropriate target because it is required by all of the cells in your body and would cause collateral damage. eIF4E2-mediated translation would be a more appropriate target. In our hands, inhibiting normoxic levels of eIF4E was detrimental to U87MG cells leading to cell death, and the inability to select viable stable clones. 786-0 cells were malleable with eIF4E, however, it appeared that the eIF4E knockdown was lost over time, and cells could not divide. Cell lines obtained from the Sonenberg lab (McGill University) were tested by colleagues in our lab and appeared to have a reduction in eIF4E, however, catalytic amounts might have been enough to support cell survival in normoxia. One can imagine the detrimental side effects of inhibiting eIF4E in mammals, as explained above.

5.6 An alternative cap-dependent protein synthesis complex as a chemotherapeutic target

Despite genetic differences between renal adenocarcinomas, glioblastoma, and colorectal cancers, each cell type requires eIF4E2 for upregulation of EGFR protein. Thus we suspect that each genetically unique line is using eIF4E2 as a translation initiator in the presence of HIF-2α. As indicated in the literature, the eIF4E2-dependent mRNA translation complex is responsible for synthesizing EGFR protein (Uniacke et al., 2012). Targeting the cap-binding protein eIF4E2 was sufficient to inhibit the protein synthesis complex, as measured through hypoxic EGFR protein levels (Figure 15). Currently we know that HIF-2α, RBM4, and eIF4A are involved with this complex. We suspect that inhibition of tumourigenesis in our study is a result of eIF4E2's role in protein synthesis. If this is true then we can target other members of the protein synthesis complex and achieve the same result. Prior to the identification of HIF-2α involvement with the eIF4E2 cap-binding structure, Franovic et al. (2009) noted its importance in tumour growth. Two of the proteins require stress-mediated stability for their activity. HIF-2α is only stabilized in low oxygen and eIF4E2 can only compete for the cap structure when eIF4E's activity is reduced. 786-0 cells have a *VHL*-null mutation, which results in the overabundance of HIF-2α protein in normoxic conditions. Interestingly, removing eIF4E2 actually inhibits EGFR production in normoxia in these renal adenocarcinoma cells. Previously, this result was observed in cells that were depleted of HIF-2α (Franovic, 2009). Since EGFR has been linked to cancer (Nicholson et al., 2001), this result suggests that 786-0 cells partially attain their tumourigenic phenotype from eIF4E2-mediated translation. This observation has implications on the binding partners

of eIF4E2. HIF-2α acts as the oxygen sensor since it is usually inactive in normoxic conditions. There must not be another oxygen-regulated molecule in this complex because knockdown of HIF-2α or eIF4E2 is sufficient to inhibit EGFR upregulation (Franovic, 2009). If another oxygen regulated protein was involved then EGFR would not be overexpressed in normoxia through the eIF4E2 pathway. Since HIF-2α is also a transcription factor, we cannot confirm whether tumours could not grow because of lack of hypoxic protein synthesis inhibition or inhibition of transcriptional upregulation. Thus, unraveling the molecular components that contribute to cap-dependent protein synthesis in hypoxia could lead to various chemotherapeutic targets.

5.7 eIF4E2 and the cancer phenotype

In patients with lung cancer treated with Paclitaxel and Carboplatin, eIF4E2 small nucleotide polymorphisms (SNPs) had a large effect on patient survival (Sato et al., 2011). Three nucleotide alterations were identified; AA, AG, and GG. Each phenotype has a different survival time. The mean survival time for this cancer was 17.4 months. With an AA SNP patient survival time decreased to 15.6 months and patients who had a GG SNP were even more affected only surviving 7.69 months. AG SNPs seemed beneficial, with patients surviving on average 24 months (Sato et al., 2011). The distribution of these alleles varies in the population. Unusually, western and northern European descendants have a large incidence of the detrimental GG genotype (rs1656402). These detrimental SNPs may result in a gain-of-function mutation, or increase eIF4E2's competitiveness to bind the cap structure, bracing cells for hypoxic survival. Cancers are caused by numerous genetic and environmental factors; however,

Europeans seem to have a greater chance of cancer compared to non-Europeans. In a study of non-western European immigrants to Western Europe it was found that these immigrants were only more likely to have, and die, from cancers that could be traced back to an environmental cause. Overall, non-western European immigrants, living a western European lifestyle, were less likely to get cancer (Arnold et al., 2010).

In 2003, a *Nature Genetics* paper illustrated that metastatic cells might not evolve from somatic mutations that result in naturally metastatic cells (Ramaswamy et al., 2003). Instead they suggest that metastasis is the result of a unique mRNA expression profile. This group compared primary tumours to metastatic cancer cell lines, and identified a set of upregulated, and down-regulated mRNAs unique to the metastatic cells, termed the metastatic signature. Interestingly, eIF4E2 (named eIF4EL3) was one of the upregulated mRNAs. This metastatic signature was used to correctly predict the metastatic potential of several primary tumours. In our hands, eIF4E2 protein levels do not seem to change, and appear to be similar even between separate cell lines. We, however, have not compared cancer cells to non-cancerous cell lines.

There is some evidence that eIF4E2 regulation does occur in the cell. Two bands appear on western blots approximately 2 kDA apart, thought to be splice isoforms (Nygard et al., 2010). Interestingly, when using our 3'UTR targeting shRNA only the top band was removed (Figure 14B). The coding sequence targeting shRNA was sufficient to knock out both bands observed via western blot. Both sets of clones behaved similarly suggesting that removal of the heavier isoform is enough to inhibit tumourigenesis. In our 3'UTR lines, the smaller isoform seemed to increase in expression to compensate for the loss of the heavy isoform. When eIF4E2 protein was reintroduced, this smaller isoform

decreased in expression, possibly due to the over expression of the heavy isoform. This information could suggest that eIF4E2 levels are detected through some mechanism that attempts to balance this loss. We have not identified a role for the lighter isoform. eIF4E2 is thought to translate mRNAs that have a unique sequence of nucleotides known as the rHRE. A PAR-CLIP analysis indicates that several mRNAs thought to be important in tumourigenesis contain this sequence. Amongst these mRNAs are proliferative receptors EGFR, PDGFRA, IGF1R, and the angiogenic promoter VEGF1. Thus, the data suggests that eIF4E2-mediated translation aids in the development of the cancer phenotype. This would partially explain why hypoxic tumour cells are more aggressive and metastatic. Cells that naturally have an increase in eIF4E2 mRNA would be better equipped to cope in hypoxia and promote cell proliferation and angiogenesis.

6.0 Conclusion

Although protein synthesis has been studied intensively, the alternative molecular components that compensate for inhibition of the eIF4F complex were poorly understood until recently. An oxygen-regulated switch in the mRNA translation machinery permits an alternative translation complex that synthesizes the bulk of proteins in hypoxia. It appears that tumours depend on this alternative translation complex for growth and proliferation. Here we have illustrated a potential target that, if inhibited, can selectively target hypoxic tumours cells. Targeting the cap-binding protein eIF4E2 can inhibit this alternative cap-dependent mRNA translation complex. This eIF4E homologue is weakly inhibited during stress, and is clearly activated when cells are exposed to low oxygen (Figures 12, 13C). Consequently it mediates the bulk of protein synthesis when eIF4E is inhibited. Removal of eIF4E2 protein does not seem to be detrimental to the cells in normoxia (Figures 16-18, 24-25), unlike removal of eIF4E. However, when cells are cultured in hypoxic conditions, eIF4E2 is essential for growth and survival (Figures 16-18, 24-25). As result early tumour models show inability of cells to form large spheroids without eIF4E2 (Figures 19-21). Although growth occurs throughout these spheroids, hypoxic regions cause excess cell death, preventing tumour spheroids from exceeding a critical limit (Figures 23, 26). We propose that this limit is very small because *in vivo* tumours are not detectable without eIF4E2. eIF4E2 is active in regions of low oxygen that are physiologically unique (Carreau et al., 2011). We suggest that eIF4E2 inhibitors would act similarly to antibiotics because they would exploit a translation machinery difference between tumour cells and mammalian tissue. Although we are the first to show a direct link between eIF4E2 and tumour progression, eIF4E2 has been linked to the

metastatic phenotype, and patient survival (Ramaswamy et al., 2003; Sato et al., 2011). Cancer cells exploit a distinctive protein synthesis machinery for tumourigenesis which identifies a method of selectively killing tumour cells.

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Appendix

Table 1. Contributors to Results Section

Results Figure	Contributors
Figure 9	[A] J.U. [B] J.K.P.
Figure 10	J.K.P.
Figure 11	J.K.P.
Figure 12	J.U.
Figure 13	[A] J.K.P. [B] J.K.P. [C] J.U.
Figure 14	[A-E] J.K.P.
Figure 15	[A-D] J.K.P. grew cell lines and treated cells, final western blots were completed by J.U.
Figure 16	J.K.P.
Figure 17	J.K.P.
Figure 18	J.K.P.
Figure 19	J.K.P.
Figure 20	J.K.P.
Figure 21	J.K.P.
Figure 22	J.K.P.
Figure 23	J.K.P.
Figure 24	J.K.P.
Figure 25	J.K.P.
Figure 26	J.K.P.
Figure 27	J.K.P. made stable cell lines. J.K.P. and J.U. grew cells for injections. J.K.P, J.U., and C.F. prepped cells for injection into mice. J.K.P. and C.F. measured tumours over time. J.K.P. biopsied tumours post study.
Figure 28	J.K.P. made stable cell lines. J.K.P. and J.U. grew cells for injections. J.K.P, J.U., and C.F. prepped cells for injection into mice. J.K.P. and C.F. measured tumours over time. J.K.P. biopsied tumours post study.

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