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Subject Review

"Translating" Tumor Hypoxia: Unfolded Protein Response (UPR)–Dependent and UPR-Independent Pathways

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Abstract

Poor oxygenation (hypoxia) is present in the majority of human tumors and is associated with poor prognosis due to the protection it affords to radiotherapy and chemotherapy. Hypoxia also elicits multiple cellular response pathways that alter gene expression and affect tumor progression, including two recently identified separate pathways that strongly suppress the rates of mRNA translation during hypoxia. The first pathway is activated extremely rapidly and is mediated by phosphorylation and inhibition of the eukaryotic initiation factor 2α. Phosphorylation of this factor occurs as part of a coordinated endoplasmic reticulum stress response program known as the unfolded protein response and activation of this program is required for hypoxic cell survival and tumor growth. Translation during hypoxia is also inhibited through the inactivation of a second eukaryotic initiation complex, eukaryotic initiation factor 4F. At least part of this inhibition is mediated through a Redd1 and tuberous sclerosis complex 1/2–dependent inhibition of the mammalian target of rapamycin kinase. Inhibition of mRNA translation is hypothesized to affect the cellular tolerance to hypoxia in part by promoting energy homeostasis. However, regulation of translation also results in a specific increase in the synthesis of a subset of hypoxia-induced proteins. Consequently, both arms of translational control during hypoxia influence gene expression and phenotype. These hypoxic response pathways show differential activation requirements that are dependent on the level of oxygenation and duration of hypoxia and are themselves highly dynamic. Thus, the severity and duration of hypoxia can lead to different biological and therapeutic consequences. (Mol Cancer Res 2006;4(7):423–36)

Introduction

Dynamics, Severity, and Clinical Importance of Tumor Hypoxia

Over the past 10 to 15 years, it has been convincingly shown that the majority of solid human tumors contain regions that are poorly oxygenated (1). Clinical studies with oxygen electrodes and molecular markers of hypoxia have shown not only that tumors are poorly oxygenated but also that oxygenation is extremely heterogeneous. This heterogeneity occurs both within individual tumors at the microregional level and between tumors from different patients with the hypoxic fraction ranging from 0 to ~100%. Because hypoxia alters cellular behavior and causes resistance to both radiotherapy and chemotherapy, this heterogeneity may be a key determinant of prognosis. Indeed, hypoxia has been shown to predict for worse outcome in the treatment of cancer of the head and neck, uterine cervix, and soft tissue sarcomas (for recent reviews, see refs. 2, 3).

The exposure of tumor cells to both chronic and acute hypoxia has profound consequences for tumor growth characteristics and tumor response to radiotherapy and chemotherapy. Hypoxic tumor cells are more radioresistant than their well-oxygenated counterparts due to the requirement of oxygen for the induction of DNA damage by radiation. Cells in hypoxic tumor areas are also chemoresistant due to limited diffusion of drugs to these areas and due to the general slowdown of the cell cycle and metabolism in hypoxic tumor cells (4, 5). Importantly, the prognostic value of hypoxia has been observed independent of treatment modality, even for patients treated with surgery where no "intrinsic" resistance is expected (6). It has therefore been proposed that hypoxia not only modifies therapy resistance but also may affect malignancy. Hypoxia has been shown to promote lymphovascular space involvement and parametrial infiltration in cervical cancer (6). Furthermore, several clinical studies have found an association between hypoxia and metastases (6, 7). These findings strongly suggest that hypoxia fundamentally alters the physiology of the tumor toward a more aggressive phenotype (8). In all of these studies, arbitrary threshold levels for hypoxia were defined.

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Two crucial physiologic and biological aspects of tumor oxygenation have been largely overlooked in many biological and clinical studies. First, the severity of hypoxia (the absolute level of oxygen) within tumors is highly variable, ranging from normoxic to completely anoxic (see Fig. 1). Second, oxygenation is highly dynamic, characterized by periodic cycling of cells between various levels of oxygenation. These patterns of oxygenation are expected to have very different biological and clinical implications. The biological importance of these factors derives both from the nature of the mechanisms that cause hypoxia and to the differential activation of hypoxic response pathways. Multiple mechanisms are known to contribute to the development of regions of low oxygen within a tumor and the list seems to be ever growing. These include (*a*) the outpacing of new blood supply by the rate of tumor growth-creating areas of "diffusion limited hypoxia," (*b*) the abnormal architecture and blood flow dynamics of newly formed blood vessels, and (*c*) the spontaneous vasoconstriction and vasodilation of tumor blood vessels (9-11). Recent data from spontaneous and experimental animal tumors indicate that large fluctuations in tumor pO_2 levels can occur due to transient changes in blood flow and are independent of treatment or overall tumor oxygenation status. As flow changes from high to low, the proportion and severity of hypoxia increases. These fluctuations are readily seen in animal tumor models, lasting on average between 30 minutes and 2 hours (12), and are implicated in human tumors (13). Perfusion changes in tumor vasculature occur because it is often immature, lacking smooth muscle cells and other structural components. Combined with high interstitial pressures, this can lead to rapid and dramatic changes in perfusion, including complete temporary shutdown of vessels. This so-called acute hypoxia can vary significantly among different tumors and in some cases account for a large proportion of the hypoxic cells at any given time (14, 15). It can also result in small tumor regions that experience transient exposure to very low (anoxic) oxygen levels. Although tumor cells cannot survive indefinitely under complete anoxia, they are well adapted to survive in extremely low oxygen concentrations for prolonged periods of time.

Biological Responses to Hypoxia

To survive the hypoxic environment, tumor cells must adapt to the reduced availability of oxygen. It should be emphasized that, during tumorigenesis and perhaps during tumor progression, hypoxia can in fact act as a selective pressure to eliminate apoptotically sensitive cells via pathways largely mediated through activation of the tumor suppressor p53 (16, 17). However, hypoxia still develops in growing tumors that have lost their p53 status and other pathways must contribute to the resistance of tumor cells to this stress. The adaptive biochemical pathways that are activated in response to hypoxia/anoxia can be broadly divided into two main categories. The first is manifested as an attempt by the tumor cells both to increase tumor oxygenation by stimulating angiogenesis and to switch the metabolic profile of the hypoxic cell. These processes are governed in large part by the hypoxia-inducible factor (HIF) family of transcription factors. These transcription factors become activated primarily via post-transcriptional mechanisms under conditions of hypoxia and subsequently promote the expression of a large number of

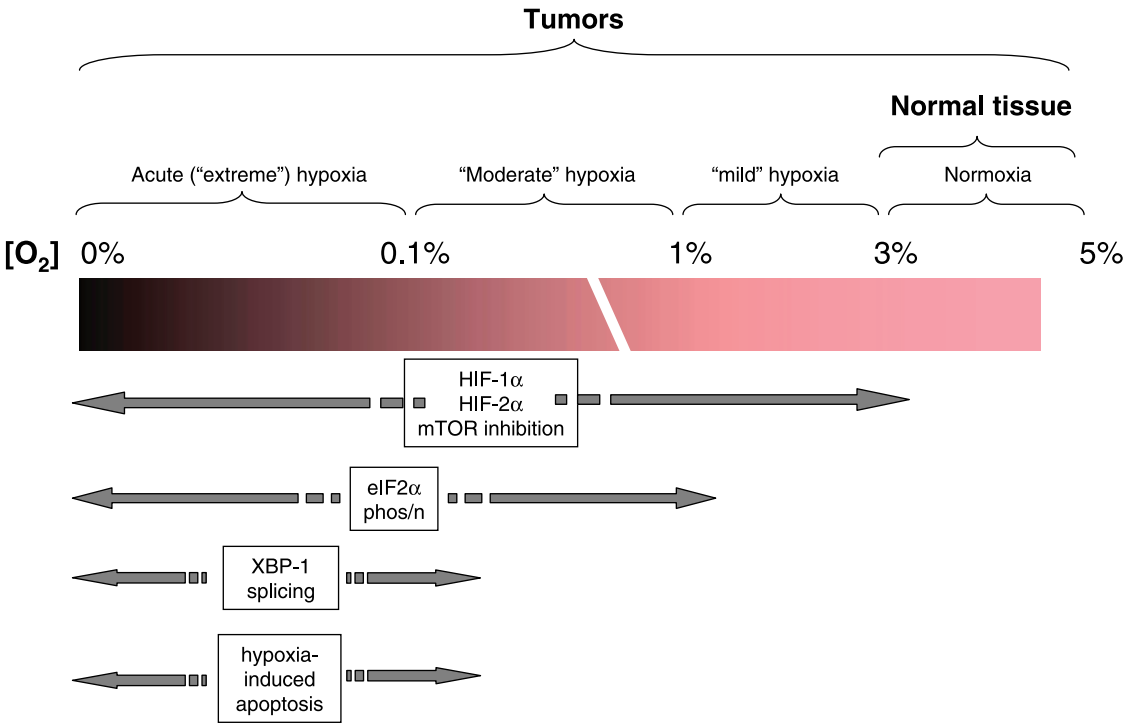

Figure 1. Arbitrary definitions for different levels of hypoxia and approximate ranges for biological responses mentioned in this review. Oxygen levels in tissues are also expressed in mm Hg. For comparison, 1% O_2 equals 7.6 mm Hg.

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genes whose products are involved in neoangiogenesis, metabolic transition to anaerobic glycolysis, and cell motility and invasion. HIF-1 and HIF-2 levels are up-regulated at oxygen levels as high as 1% to 3%. This "moderate" level of hypoxia is found in many normal physiologic situations, consistent with the requirements of HIF in many nonpathologic conditions. Although this level of moderate hypoxia is undoubtedly sufficient to induce HIF-mediated responses, numerous measurements using polarographic electrodes, hypoxia-sensitive dyes, and other means clearly indicate that the majority of experimental and spontaneous tumors also contain regions that are severely hypoxic and or anoxic. These observations are also supported by the fact that tumor cells do not become fully radioresistant until oxygen levels are below ~0.1%.

The stability and activity of HIF-1α is regulated not only by the absolute level of oxygenation but also by the length of hypoxic exposure. The selective degradation of HIF-1α under aerobic conditions occurs due to modification of two proline residues in its oxygen-dependent degradation domain, by one of a family of prolyl hydroxylase enzymes 1 to 3 (18). The prolyl hydroxylase enzymes are themselves targets of HIF, leading to the establishment of a negative feedback loop and an increased rate of HIF degradation during continued hypoxia (see Fig. 2). Thus, HIF-dependent transcription may be highly temporal, especially at moderate oxygen concentrations (~1%).

The second category of hypoxic adaptation involves the general slowdown of processes that involve oxygen and energy consumption, including the reduction of cell proliferation, DNA replication, and a decrease in the overall rate of protein synthesis. Together, these adaptive strategies result in an overall increase in tolerance to hypoxia by aiding in the maintenance of energy levels. Hypoxic stress has been shown to cause a pronounced depression in the rate of oxygen consumption and of energy turnover. It has been estimated that, under severe hypoxia, the ATP demand for protein synthesis drops to ~7% of that of normoxic cells. This drop correlates with a substantial and rapid drop in the rate of protein synthesis, which occurs initially at the levels of translation and later extends at the level of transcription as well. Because protein synthesis is the second costliest cellular process in terms of ATP demand besides the Na^+ pump, a decrease in the rate of translation may be crucial for cellular adaptation to the new environment of low oxygen and of energy deficiency. Over the last several years, the molecular basis of this decrease in protein synthesis has been largely explained. The inhibition in protein synthesis in hypoxic cells is tightly regulated in a two-step choreographed process: one mediated by events occurring in the endoplasmic reticulum (ER) and the other in the cytosol. These processes are dependent on the severity of hypoxia, hypoxic exposure time, and other cell signaling pathways. These two pathways ensure a rapid and pronounced repression of translation of the majority of cellular mRNAs on the one hand and simultaneously promote the synthesis of specific mRNAs that may be important for hypoxic tolerance and tumor growth. Here, we have attempted to summarize several recent reports that have delineated the mechanistic basis for control of mRNA translation during hypoxia and its importance for gene expression, hypoxia tolerance, and tumor growth. The data suggest that these adaptive processes are essential for cell survival under hypoxic stress and that they may be interesting targets for hypoxia-specific treatment approaches.

ER Stress, Unfolded Protein Response, and Hypoxia

PERK-Eukaryotic Initiation Factor 2α Control of Translation during Hypoxia

Several recent reports from both our laboratories and others indicate that changes in protein synthesis under hypoxia can be accounted for in large part by changes in the rate of mRNA translation. Initiation of eukaryotic translation is a complex and highly regulated process, carried out by a family of at least nine eukaryotic initiation factors (eIF; refs. 19-22). One of the most important control points in this process is mediated by eIF2, which forms a ternary complex with GTP and the initiator (Met)-tRNA. The ternary complex associates with the 40S ribosomal subunit and eIF1, eIF1A, and eIF3 to form the 43S preinitiation complex, which scans the 5′ end of the mRNA for the AUG initiation codon. The eIF5 protein then stimulates hydrolysis of the eIF2-bound GTP into GDP, resulting in release of the initiation factors and initiation of the elongation phase of translation. Creation of a new ternary complex requires exchange of GDP for GTP on eIF2 by eIF2B, which can be inhibited when one of the two subunits of eIF2, eIF2α, becomes phosphorylated at Ser^{51}. Because the eIF2B subunit exists in significantly smaller amounts than eIF2α (~20-30% of eIF2α), a less than complete phosphorylation of eIF2α molecules is sufficient to inhibit the exchange activity of eIF2B. Several years ago, we found that exposure of both normal and transformed cells to severe hypoxia (<0.05%) is sufficient to cause rapid phosphorylation of eIF2α and a reduction in protein synthesis (23). Phosphorylation of eIF2α during severe hypoxia occurs in standard culture conditions (serum replete) within 30 minutes of hypoxic exposure and is fully reversible on return to oxygenation. Under severe hypoxia, eIF2α phosphorylation levels are highest at short times after hypoxia and show a partial recovery after 4 to 8 hours (23, 24). This recovery is consistent with a well-established feedback loop that is activated following inhibition of mRNA translation through this pathway (see below). A causal link between eIF2α phosphorylation and inhibition of translation under hypoxia was shown by the ability of a transdominant mutant allele of eIF2α (25, 26), which encodes a protein with a single amino acid substitution at position 51 (Ser-to-Ala; S51A), to inhibit or delay the phosphorylation of endogenous eIF2α in response to stress or treatment with agents that induce its phosphorylation. We found that phosphorylation of eIF2α also occurs in response to more moderate hypoxia (0.2% and 1% oxygen) but requires significantly longer hypoxic exposures (>8 hours). Several additional reports have confirmed the increase in eIF2α phosphorylation during both severe and moderate hypoxic exposures (27-29).

The consequences of the extremely rapid phosphorylation of eIF2α on the kinetics of mRNA translation inhibition during hypoxia was studied in more detail by analyzing the overall levels of ribosome association with mRNA (polysomes) as a

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function of hypoxic exposure time. We found that severe hypoxia caused a dramatic loss in polysomal mRNA with kinetics that reflected eIF2α phosphorylation (24). The percentage of ribosomes found in the polysomes dropped from >60% under normal conditions to ~20% within 1 hour of severe hypoxic exposure. Interestingly, cells exposed to longer hypoxic exposures exhibited a partial recovery in both eIF2α phosphorylation and translation after 4 to 8 hours, although overall levels of translation remained much lower than normal. The rapid inhibition in translation did not occur in cells derived from knock-in mice containing a nonphosphorylatable allele of eIF2α (S51A), indicating that this rapid effect requires phosphorylation of this protein. However, after long times of severe hypoxia (16 hours), translation inhibition in the S51A cells was identical to that of the wild-type (WT) cells. Together, these data imply that eIF2α phosphorylation is responsible and required for the rapid inhibition of translation during severe hypoxia but that a second mechanism of translational control causes reduced protein synthesis after longer hypoxic exposures. This finding may also explain a recent publication from Connolly et al., which showed that 16 hours of moderate hypoxia (0.5%) caused no change in eIF2α phosphorylation. Similar to the results with eIF2α S51A knock-in cells, these authors also found no requirement for eIF2α phosphorylation for inhibition of protein synthesis at this time point (30).

At least four distinct kinases have been shown to phosphorylate eIF-2α at Ser^{51} and to regulate translation initiation in response to stress (reviewed in refs. 19, 31). These are (*a*) PKR, an IFN-induced, dsRNA-activated protein kinase; (*b*) HRI, a heme-regulated inhibitor of protein translation found predominantly in erythroid cells; (*c*) GCN2, a kinase originally identified in yeast, which is activated on nutrient (amino acid) deprivation; and (*d*) PERK, an ER resident kinase, which is proposed to participate in the unfolded protein response (UPR) to inhibit protein translation and which itself becomes phosphorylated during this process. We found evidence that hypoxia can activate PERK and furthermore that PERK is required for hypoxia-induced phosphorylation of eIF2α. Cells derived from $PERK^{-/-}$ mice and tumor cells stably expressing a dominant-negative PERK allele (32) are defective in eIF2α phosphorylation during hypoxia. Notably, cells with inactivated PERK activity also exhibit attenuated inhibition of protein synthesis in response to hypoxia. These data indicate that PERK is the primary kinase that phosphorylates eIF2α leading to down-regulation of protein synthesis in response to hypoxic stress (see Fig. 3).

PERK resides in the lumen of the ER with its kinase domain in the cytoplasmic side and is activated by stresses that result in an increase in unfolded proteins (33). The lumenal domain of PERK is bound to BiP/GRP78 and perturbation of protein folding leads to a dissociation of these two proteins and activation of PERK (34). Therefore, a model has emerged, in which BiP acts as a repressor of PERK activation, and on ER stress and an increase in protein misfolding, a relief of this repression leads to PERK activation, eIF2α activation, and inhibition of protein synthesis. These data are consistent with previous work showing that BiP is induced by hypoxia (35).

UPR during Hypoxia

The activation of PERK and phosphorylation of eIF2α serve two major functions in a cell experiencing ER stress. The first is to rapidly down-regulate protein synthesis, which in turn reduces the load of misfolded proteins in the ER and leads to lower energy expenditure, because both protein synthesis and protein folding are ATP-requiring processes (36-38). The second function is to up-regulate genes that promote protein folding capacity, redox homeostasis, and amino acid sufficiency (39), thereby further promoting cell survival. PERK-dependent signaling is mediated by at least two downstream pathways. The first is mediated by the activating transcription factor (ATF) 4, which is translationally up-regulated in an eIF2α phosphorylation-dependent manner (39, 40). ATF4 activates the induction of downstream UPR genes but has also been

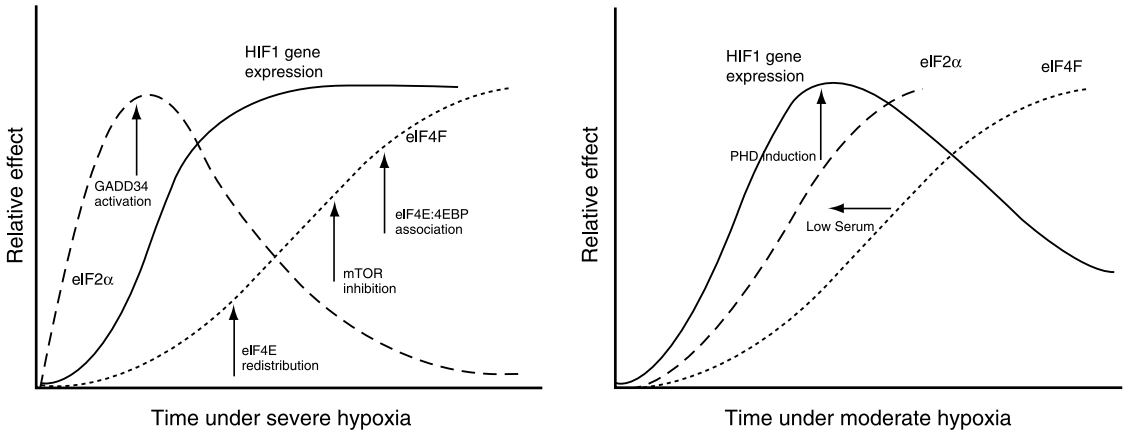

Figure 2. Cellular responses under chronic and acute hypoxia are highly dynamic and dependent on the severity of hypoxia. Under severe hypoxia (<0.05%), phosphorylation of eIF2α occurs rapidly but is transient (see PERK-Eukaryotic Initiation Factor 2α Control of Translation during Hypoxia). During moderate hypoxia (0.5-1%), phosphorylation of eIF2α requires much longer hypoxic exposures. Inhibition of eIF4F occurs under both severe and moderate hypoxia and requires 6 to 24 hours to show maximum effect, although inactivation of mTOR during moderate hypoxia can occur much faster in the absence of growth factors. For reference, the expected responses for HIF-1-dependent genes are also shown. Under moderate hypoxia, HIF-1 activates a negative feedback loop by inducing the expression of its negative regulators, the prolyl hydroxylase enzymes. The prolyl hydroxylase enzymes are inactive under severe hypoxic conditions.

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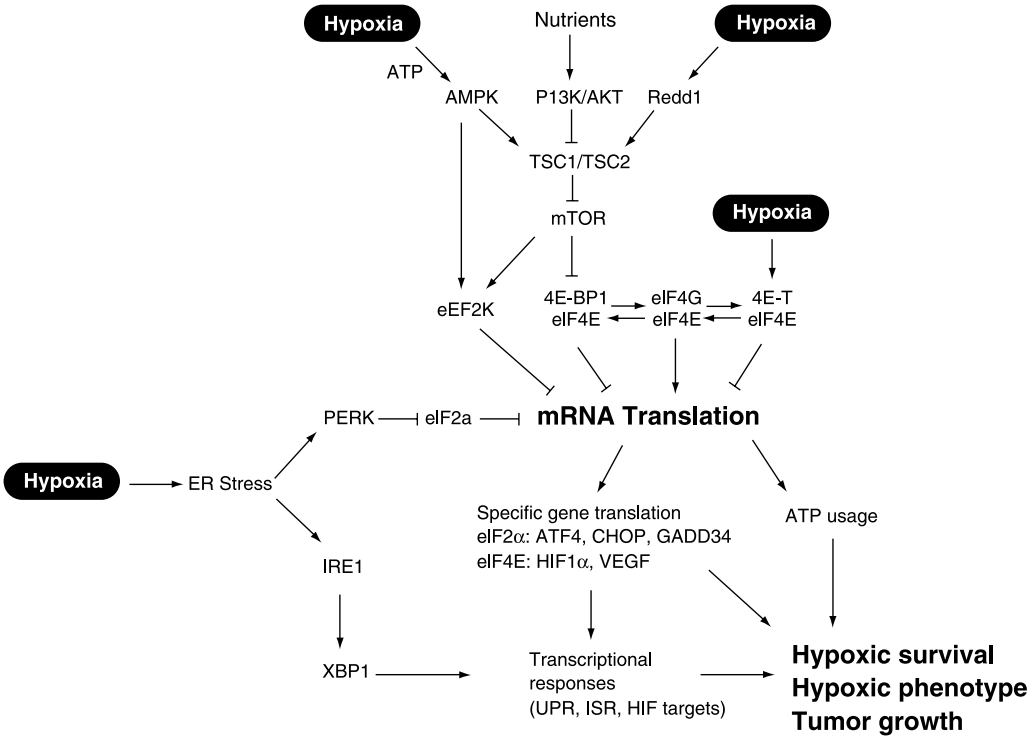

Figure 3. Schematic depiction of translational control by hypoxia through both eIF4F- and UPR-dependent pathways. See text for details.

implicated in antioxidant cellular defense processes (39, 41, 42). This pathway, known as the integrated stress response (43), constitutes one arm of a larger coordinated program called the UPR, which promotes cellular adaptation to conditions of ER stress. The second PERK-dependent pathway involves nuclear import of the Nrf2 transcription factor following its dissociation from the cytoplasmic protein Keap1 (44). Cells harboring a targeted deletion of Nrf2 exhibit increased cell death following exposure to ER stress compared with WT counterparts.

The other two UPR pathways are initiated by the ER transmembrane proteins IRE1 and ATF6 (45). Interestingly, in unstressed cells, IRE1 and ATF6 are held inactive by being bound to BiP, in a manner similar to PERK. However, recently, the proapoptotic proteins Bax and Bak were also implicated in the activation of IRE1 signaling by direct binding to its cytoplasmic domain (46). Mammalian IRE1 acts as a site-specific endonuclease to cleave and remove a small intron in the mRNA of the bZIP transcription factor XBP-1. This is followed by religation of the 5′ and 3′ fragments to produce a processed mRNA that is translated more efficiently and encodes a more stable XBP-1 protein (47, 48). XBP-1 migrates to the nucleus and is able to bind response elements in the promoters upstream of both UPR and ER stress genes, such as proteins involved in folding (BiP), disulfide bond formation (protein disulfide isomerase), and protein degradation (EDEM; refs. 48, 49). Interestingly, it has been shown that XBP-1 is also important for resistance to hypoxic stress *in vitro* and, more importantly, for optimal tumor growth *in vivo* (50), in a manner that is similar to PERK. ATF6 translocates to the Golgi in response to ER stress, where it is cleaved, and the NH_2 terminus translocates to the nucleus (51). Both ATF6 and XBP-1 regulate the expression of downstream genes by binding to the ER stress element of downstream gene promoters, including that of the proapoptotic protein cyclophosphamide-Adriamycin-vincristine-prednisone (CHOP; ref. 41). Preliminary data suggest that ATF6 is also activated and translocates to the nucleus under prolonged acute hypoxia.[3] Collectively, these results strongly implicate the whole UPR in the hypoxic adaptation of tumor cells.

Cells experiencing prolonged or acute ER stress undergo apoptosis, which is thought to be primarily initiated from the ER. Conversely, due to inability to down-regulate protein synthesis, cells with a compromised UPR, such as cells with abrogated PERK and eIF2α signaling, are substantially more sensitive to ER-induced cell death than WT cells presumably due to the continuous accumulation of misfolded proteins in the ER, a process that is termed "proteotoxicity" (26, 38). The mechanisms for ER-induced apoptosis are not completely understood, but there is strong evidence that Ca^{2+} release from ER stores and subsequent caspase activation mediates programmed cell death under these conditions (52-54). Other crucial activators of ER stress-induced apoptosis seem to be Bcl-2 family proteins. In addition to their contribution to the intrinsic apoptotic pathway through the mitochondria, Bcl-2 family members are also localized to the ER. Antiapoptotic Bcl-2 was shown to be present in the ER membrane and may contribute to ER membrane permeability by maintaining the prodeath Bak and Bax in their inactive conformations (55-57). ER stress induces oligomerizaiton of Bax and Bak to their

[3] Diane Fels and Constantinos Koumenis, unpublished observations.

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active states, which can then induce an ER Ca^{2+} leak that triggers apoptosis. ER release of calcium is known to activate calpains, cytosolic calcium-dependent proteases that activate caspase signaling cascades (53, 57-59), including cleavage of Bid. Cleavage of Bid enhances its capacity to induce mitochondria membrane permeabilization, leading to cytochrome *c* release and activation of downstream caspases. We have shown that cells unable to elicit a UPR or integrated stress response undergo apoptosis much more readily and exhibit higher levels of cleavage of caspase-3 and poly(ADP-ribose) polymerase in response to hypoxia compared with cells with an intact UPR. Preliminary data also suggest that expression of an ER-targeted bcl-2 variant can protect PERK-deficient cells from hypoxia-induced apoptosis.[3]

Regulation of mRNA Translation through Tuberous Sclerosis Complex 2/Mammalian Target of Rapamycin and eIF4F

Disruption of eIF4F during Hypoxia

A flurry of recent reports have implicated control of eIF4F, a complex that binds to the 5′ cap structure of mRNA to facilitate recruitment of the 43S preinitiation complex, as a second important regulator of mRNA translation during hypoxia. As described above, we have shown recently the existence of a pathway capable of strongly inhibiting translation during severe hypoxia that is independent of eIF2α phosphorylation. This was most clearly evident by the observation that translation inhibition following 16 hours of severe hypoxia is as strong in cells expressing the nonphosphorylatable eIF2α S51A allele as in WT cells. Instead, translation inhibition at these longer time points is associated with disruption of eIF4F, a complex consisting of three proteins: the m^7GpppN cap-binding protein eIF4E, a scaffolding protein eIF4G, and an ATP-dependent helicase eIF4A. Control over the formation of this complex is influenced in large part by the availability of eIF4E, levels of which are considered rate-limiting in overall protein synthesis (see Fig. 3). The availability of eIF4E is repressed by a family of eIF4E-binding proteins (4E-BP1, 4E-BP2, and 4E-BP3; refs. 60, 61), which compete with eIF4G for an overlapping binding site. In their underphosphorylated forms, the 4E-BPs bind eIF4E with high affinity and thus prevent eIF4F formation. A role for this pathway in controlling translation during hypoxia is supported by reports that both moderate (28, 30, 62, 63) and severe (24) hypoxia cause dephosphorylation of 4E-BP1 and a corresponding increase in the association of this protein with eIF4E. Recent data from Connolly et al. have functionally linked 4E-BP1 to the regulation of translation during moderate hypoxia. Using small interfering RNA against 4E-BP1, they showed that this protein is necessary for the down-regulation of protein synthesis following 24 hours of 0.5% oxygen. Interestingly, they found that the ability of hypoxia to control 4E-BP1 was also cell type dependent and may be altered in cancer (see below).

Control of Translation during Moderate Hypoxia via Mammalian Target of Rapamycin

Phosphorylation of 4E-BP1 is controlled in large part through the activity of the mammalian target of rapamycin (mTOR) kinase (64). On activation, mTOR directly phosphorylates at least two sites in 4E-BP1, Thr^{37} and Thr^{46}, and may be involved in the phosphorylation of several others. mTOR is thought to integrate signals from several upstream pathways that respond to growth factors, nutrients, and energy to regulate metabolism, cell growth (mass), and cell division (65). Arsham et al. provided the first evidence that mTOR signaling may be a target of hypoxia. They showed that moderate hypoxia (1.5%) for as little as 15 minutes was sufficient to block the activation of mTOR (as assessed by its autophosphorylation on Ser^{2481} and phosphorylation of its downstream targets 4E-BP1 and p70 S6 kinase) when serum-starved cells were stimulated with insulin, a known activator of mTOR (63). In this study, the effect of hypoxia-induced mTOR inhibition on overall protein synthesis was not assessed. More recently, this same group showed that moderate hypoxia (1.5%) can also effectively inhibit mTOR activity in serum-replete conditions, although this requires hypoxic exposures of at least 6 hours with a maximum inhibition occurring only after 20 hours (28). Thus, it seems that the ability of hypoxia to inhibit mTOR is influenced by other signaling pathways that are active when cells are cultured in the presence of serum (see Fig. 3).

The study by Liu et al. also illustrates that mTOR inhibition during hypoxia is not sufficient to assume a significant effect on overall protein synthesis. Despite the clear inhibition of mTOR and dephosphorylation of 4E-BP1, no effect on overall protein synthesis is observed within the first 24 hours of moderate hypoxic exposure. Serum-deprived cells showed ~30% inhibition in protein synthesis after 16 hours of hypoxia and cells exposed to more severe hypoxia (0.3%) showed a similar inhibition in serum-replete medium within 6 hours. However, the direct contribution of mTOR to overall translation inhibition at these time points was not investigated and is difficult to predict because eIF2α is also activated by these conditions. The lack of a significant change in translation at time points that show clear inhibition of mTOR may be due to changes in the expression of either eIF4E or 4E-BP1. If levels of eIF4E are significantly higher than that of 4E-BP1, mTOR inhibition is less able to affect protein synthesis (66). Given that eIF4E is frequently overexpressed in cancer (67, 68), mTOR inhibition under hypoxia may have substantially different consequences among different tumor cells.

AMPK- and Redd1-Dependent Signaling to mTOR through Tuberous Sclerosis Complex 2

At least three upstream pathways are able to influence mTOR activity, two of which seem to be regulated by hypoxia (65). These three pathways signal to mTOR by altering the activity of the tuberous sclerosis complex (TSC), which functions as a negative regulator of mTOR activity by acting as a GTPase-activating protein toward the small G protein RheB. Regulation of mTOR by growth factor signaling has been extensively studied and is thought to occur primarily via activation of the phosphatidylinositol 3-kinase/AKT pathway and subsequent phosphorylation and inhibition of TSC2 (69). However, this molecular mechanism does not seem to be responsible for mTOR inhibition during hypoxia (63, 70). A second mTOR-regulating pathway involves a 25-kDa protein called Redd1, which is induced during hypoxia and energy

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stress (71) in a HIF-1-dependent manner (72). Redd1 activation was identified in a *Drosophila* screen for suppressors of insulin signaling and its overexpression is sufficient to inactivate mTOR (73). Brugarolas et al. showed that both Redd1 and the TSC1/TSC2 complex are required for the reduction in mTOR activity (as assessed by reduced phosphorylation of S6 kinase) following exposure to moderate hypoxia (1%) for as little as 3 hours (70). Because Redd1 is a direct target of HIF-1 and is hypoxia inducible, these data also suggest a mechanism for oxygen sensing in the activation of this pathway. These authors provided convincing data that Redd1 was acting upstream of TSC2 and cells deficient in TSC2 also showed a reduction in hypoxia-induced association of eIF4E with 4E-BP1. Thus, it seems that mTOR inhibition under moderate hypoxia is at least partially due to Redd1 activation of TSC2. Unfortunately, the investigators did not determine whether the inhibition of mTOR through this pathway was capable of significantly influencing overall levels of protein synthesis. As discussed above, changes in mTOR activity are not sufficient to assume a measurable change in overall translation.

The TSC1/TSC2 complex also regulates mTOR function in response to changes in energy status. Under low energy conditions (increases in AMP), LKB1 phosphorylates and activates AMPK. This leads to phosphorylation of TSC2 and, in contrast to AKT signaling, activation of the TSC1/TSC2 complex and inhibition of mTOR (74, 75). LKB1 and AMPK thus promote maintenance of cellular energy homeostasis through mTOR-dependent inhibition of mRNA translation. A reduction in ATP after long exposures to moderate hypoxia is expected because cells are less able to use oxidative phosphorylation; thus, the efficiency of ATP production from glucose is dramatically reduced. This may provide a possible explanation for the long times required to observe significant changes in protein synthesis (32-48 hours) in response to moderate hypoxia (28). Indeed, activation of AMPK during moderate hypoxia in serum-replete conditions occurred progressively over 20 hours of exposure. However, and somewhat surprisingly, it was also reported that, in serum-depleted conditions, AMPK activation can occur much more quickly, within 30 minutes of moderate hypoxia, and this correlates with severe inhibition of mTOR. Unlike the effects of prolonged hypoxia, the rapid inhibition of mTOR by moderate hypoxia in serum-deprived cells was strongly dependent on TSC2 (28). Activation of AMPK and subsequent inhibition of mTOR through TSC2 under these conditions correlated with a drop in ATP, which was only seen in hypoxic cells that were also serum deprived. Inhibition of mTOR via AMPK activation during short exposures to moderate hypoxia thus provides a mechanism to explain a previous observation that hypoxia can inhibit mTOR in a TSC2-dependent pathway that is independent of HIF-1 (and thus presumably Redd1; ref. 63). However, the consequences of AMPK signaling to TSC2 on overall protein synthesis are once again difficult to interpret because although mTOR was effectively inhibited after 30 minutes during these conditions, no decrease in protein synthesis was observed for >24 hours (28).

There is also some evidence that moderate hypoxia may affect protein synthesis as a result of an AMPK-dependent or mTOR-dependent phosphorylation of the eEF2 kinase. Both AMPK and mTOR have been shown to phosphorylate eEF2 kinase, which leads to subsequent phosphorylation of the elongation factor eEF2 and inhibition in translation elongation (76, 77). Liu et al. showed that activation of AMPK during hypoxia resulted in strong eEF2 phosphorylation. However, this did not seem to directly affect protein synthesis in a measurable way because strong phosphorylation of eEF2 was observed in serum-deprived cells after only 30 minutes of moderate hypoxia, but no change in the rate of protein synthesis was observed for >24 hours.

Thus, from all of these studies described thus far, there is no evidence that Redd1-TSC2, AMPK-TSC2, or AMPK-eEF2K signaling during moderate hypoxia can directly affect the overall rates of cellular protein synthesis. However, in a recent publication, Connolly et al. have provided evidence directly linking these pathways with the suppression of protein synthesis and interestingly suggest that the ability for this to occur may strongly depend on cell type (30). Concomitant with the increased association between eIF4E and 4E-BP1 mentioned earlier, 24 hours at 0.5% O_2 also resulted in activation of eEF2 kinase. In untransformed cells, this correlated with a decrease in protein synthesis of ~70%. Furthermore, small interfering RNA against both eEF2K and 4E-BP1 indicated that both pathways contributed functionally to this inhibition.

Cell Type–Specific Regulation of mTOR

As alluded to above, the recent publication by Connolly et al. raise the interesting possibility that translational control under hypoxia may be altered during carcinogenesis. They found that moderate hypoxia failed to inhibit mTOR or translation, which was interpreted as an indication that translational control during moderate hypoxia may be lost during cellular transformation. This finding is also supported by a recent publication by Kaper et al. who showed that inhibition of mTOR under severe hypoxia (<0.02%) may also be cell type specific (78). Mutations in the phosphatidylinositol 3-kinase/PTEN/TSC2 pathway seemed to affect the ability of severe hypoxia to inhibit mTOR. Inhibition of mTOR was less effective in cells that had increased levels of AKT signaling as a result of mutations in PTEN. Thus, although phosphatidylinositol 3-kinase signaling to mTOR does not seem to be directly modulated by hypoxia, it may influence the ability of Redd1 or AMPK to activate TSC2 and inhibit mTOR. Only after prolonged hypoxia (24 hours) was mTOR inhibition complete (as assessed by the inability of rapamycin to further reduce protein synthesis).

mTOR-Independent Regulation of eIF4F

The fact that mTOR inhibition is incomplete after short severe hypoxic exposures is also in agreement with our data that suggest existence of a mTOR/4E-BP1-independent translational control mechanism at these early time points. The association of eIF4E with eIF4G1 and eIF4G2 is dramatically reduced after both 4 and 16 hours of severe hypoxia, whereas increased binding between eIF4E and 4E-BP1 is observed only after 16 hours (24). Therefore, disruption of the eIF4F complex cannot be explained solely by a mTOR-stimulated increase in binding between eIF4E and 4E-BP1. We showed that after 4 hours of hypoxia the disruption of eIF4F may also occur as a result of redistribution of eIF4E and the eIF4E translocator

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protein (4E-T) into either the nucleus or the recently discovered cytoplasmic bodies of mRNA processing (P-bodies; refs. 79, 80). Translocation of eIF4E into either of these cellular locations makes it unavailable to participate in translation initiation. The function of eIF4E in these sites is not clearly understood, but 5% to 20% of total eIF4E has been reported to be present in the nucleus (81). The recent finding that eIF4E also colocalizes with 4E-T in P-bodies, where mRNA is degraded or stored (80) and where small interfering RNA–based silencing takes place (82), also suggests novel consequences for hypoxia on gene expression.

Implications for Tumor Growth

UPR in Hypoxia Tolerance and Tumorigenesis

The importance of hypoxia-induced, PERK-dependent phosphorylation of eIF2α on hypoxia tolerance suggests a possible role for this pathway in supporting the growth of tumors, which frequently contain regions of hypoxia. We found that tumor cells with abrogated PERK activity (e.g., $PERK^{-/-}$ mouse embryonic fibroblasts or HT29 colorectal carcinoma cells expressing dominant-negative PERKΔC) as well as cells with abrogated eIF2α phosphorylation or ATF4 activity display significantly reduced clonogenic survival compared with the corresponding WT cells (83). The decreased ability of cells with a compromised PERK-eIF2α pathway to tolerate moderate to extreme hypoxia correlated with higher levels of caspase-12 cleavage under hypoxia; however, caspase-$12^{-/-}$ mouse embryonic fibroblasts were as sensitive as caspase-$12^{+/+}$ mouse embryonic fibroblasts under hypoxia (results not shown), indicating that although caspase-12 processing is a marker of hypoxia-induced ER stress it cannot account for the higher sensitivity of $PERK^{-/-}$ cells to hypoxia. Activation of caspase-3 and proteolytic processing of poly(ADP-ribose) polymerase, a marker for late-stage apoptosis, followed a similar pattern with higher levels of cleaved caspase-3 and poly(ADP-ribose) polymerase in the $PERK^{-/-}$ mouse embryonic fibroblasts and S51A as well as in HT29-PERKΔC cells compared with corresponding WT cells. These results indicate that PERK and eIF2α phosphorylation contribute to cellular survival under prolonged hypoxia most likely by increasing the threshold to ER-dependent apoptosis. This hypothesis is further supported by recent findings that expression of ER-targeted bcl-2 (cb5-bcl-2), but not mitochondrial-targeted bcl-2 (maob-bcl-2), confers increased resistance to hypoxia and thapsigargin-mediated apoptosis.[4]

These findings were extended *in vivo* when we showed that transformed cells with an intact PERK-eIF2α pathway form tumors that grow faster and larger (~6-fold) compared with those from corresponding transformed cells with abrogated PERK ($PERK^{-/-}$, PERKΔC) or eIF2a phosphorylation (S51A cells), suggesting that the differential response of these cells to the tumor microenvironment must play a critical role in tumor development. Moreover, tumors grown from cells with a compromised PERK-eIF2α pathway had fewer hypoxic areas that contained a higher percentage of apoptotic cells, indicating that inhibition of the integrated stress response compromises the ability of these tumor cells to tolerate hypoxia *in vivo*. Analysis of HIF and vascular endothelial growth factor levels revealed no overt differences in the inducibility of these major angiogenesis players; however, these results do not exclude the possibility that some other angiogenic pathways are affected in the cell lines with compromised integrated stress response and also contribute to slower tumor growth.

Importance of Hypoxic Control of eIF4F in Cancer

As discussed earlier, two recent reports suggest that cancer cells may display a differential ability to inhibit mTOR under hypoxia and to inhibit translation (30, 78). This may be explained by the fact that several genes that participate in signaling pathways upstream of mTOR are frequently altered in cancer. This includes the commonly disrupted phosphatidylinositol 3-kinase/AKT pathway. Mutations in the tumor suppressor gene PTEN (84, 85) result in deregulation of AKT and subsequent activation of mTOR and mRNA translation. Similarly, receptors, such as HER-2/*neu*, which signal to this pathway, are also frequent targets of amplification or mutation in cancer (86). Mutations in either TSC1 or TSC2 result in formation of benign tumors called hamartomas, which consist of multiple cell types (87). Mutations in the LBK1 tumor suppressor gene, which regulates TSC1/TSC2 in response to reductions in energy levels, causes Peutz-Jeghers syndrome (88). This disease is characterized by the development of hamartomas and various neoplasms, which results as a direct consequence of mTOR deregulation (89).

Downstream of mTOR several studies linking eIF4E to cancer provide more direct evidence for the role of mRNA translation in transformation and tumor progression. Activation of mTOR stimulates translation by increasing eIF4E availability. eIF4E is frequently overexpressed in human cancer and can directly transform immortalized 3T3 fibroblasts (90, 91). Recently, two genetic studies in mice have shown a clear role for eIF4E in tumorigenesis (92, 93). Transgenic mice overexpressing eIF4E showed significantly increased tumorigenesis (92). Modest overexpression of eIF4E (2.5-fold) also considerably accelerated lymphomagenesis in the Eμ-Myc transgenic B-cell lymphoma model (92, 93). In this system, eIF4E accelerated tumor development through the inhibition of apoptosis (93).

Implications of Translational Control on Hypoxia-Regulated Gene Expression

The consequences of hypoxia-dependent changes in overall translation that occur through either eIF2α or eIF4F on the translation of any particular gene are expected to be highly variable. Translation efficiency of a given mRNA transcript is influenced by both positive and negative regulatory elements within the 5′ and 3′ untranslated regions (UTR). For example, mRNA translation is stimulated by interactions between the eIF4F complex and the 3′ polyadenylate tail via binding to the polyadenylate-binding protein (94). It has been proposed that this interaction allows circularization of the mRNA and recycling of ribosomes completing translation back on to the 5′ end of the transcript. Conversely, translation is suppressed by the presence of mRNA stem loops, upstream AUG codons, and their associated upstream open reading frames (uORF) and by RNA-binding proteins present in either 3′ or 5′ UTRs (95-97).

[4] Fels et al., unpublished observations.

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Translation initiation can also be stimulated through direct binding of ribosomes within the 5′ UTR to an internal ribosome entry site (IRES), bypassing the need for assembly of the eIF4F complex at the cap (98). The importance of the UTRs in regulating protein expression is exemplified by the fact that these noncoding regions are often highly conserved and such regions have been proposed to act as sensors of environmental stress (99).

Translational control elements in the UTRs show differential sensitivity to changes in the availability and activity of various initiation factors, which confers on them an ability to alter translation efficiency under specific circumstances. This has been reported to occur in response to a variety of different forms of cell stress (98). Thus, although both moderate and severe hypoxia can inhibit overall mRNA translation, selective translation of a subset of genes under these conditions can occur and may play important roles in the hypoxic phenotype. One might also expect that the genes that are important for adaptation to hypoxia may harbor UTRs that confer a selective translational advantage. This is the case for important factors in the biological response to hypoxia, such as HIF-1α and vascular endothelial growth factor (see below). Furthermore, because the changes in translational control are highly dependent on both severity of hypoxia and exposure time, the consequences for individual gene expression will also be dependent on these factors.

Selective Gene Expression Mediated by eIF2 Regulation during Hypoxia

The rapid activation of PERK and phosphorylation of eIF2α during severe hypoxia prevents formation of an active ternary complex and represses the global initiation of mRNA translation. We and others have shown that under conditions of severe hypoxia the synthesis of ATF4 is increased in a PERK/eIF2α-dependent manner (24, 27, 83). ATF4 is a transcription factor that initiates a crucial response during the UPR, being responsible for the activation of a large set of genes important for recovery and tolerance to ER and oxidative stress (39). Selective translation of ATF4 results from the presence of two conserved uORFs in its 5′ UTR (100, 101). uORFs act as a barrier to translation of the correct ORF because ribosome initiation at the start codon of the uORF prevents the ribosome from reaching the correct start codon. In ATF4, ribosomes initiate translation at the first uORF and seem to reinitiate translation at either the second uORF or further downstream at the correct ORF. Reinitiation following translation of a uORF is considered a rare event and is thought to be influenced by sequences around its stop codon. Reinitiation also requires acquisition of a new ternary complex containing eIF2α and an initiator Met-tRNA. When these complexes are available in high amounts, translation is likely to begin from the next uORF, preventing translation of the ATF4 coding region (reinitiation following translation of the second uORF does not occur). During hypoxia, when ternary complex formation is inhibited, ribosome reinitiation is delayed allowing scanning through the second uORF and initiation at the proper start site of ATF4.

Upstream start codons and uORFs thus represent candidate RNA elements that may stimulate translation of a subset of mRNAs during conditions of severe hypoxia. Although the sequence databases do not contain good information on the 5′ ends of most genes, compilations of genes with known 5′ UTRs indicate that these elements are relatively common. It has been estimated that 10% to 30% of genes contain one or more uORFs and 15% to 53% of mRNAs contain upstream start codons (102, 103). Thus, a large number of transcripts can be potentially influenced through this type of mechanism. Interestingly, CHOP and GADD34, both of which are transcriptionally activated during the UPR, also contain uORFs within their 5′ UTR. By analyzing the distribution of CHOP and GADD34 mRNA with polysomes, we found that both of these genes displayed preferential translation during severe hypoxia (24, 83). The selective translation of these genes was completely dependent on the ability of PERK to phosphorylate and inactivate eIF2α. ATF4 has been reported previously to be expressed higher in breast tumors than in normal breast tissue and to be present near necrotic areas that were expected to be severely hypoxic (104). We have also observed increased levels of ATF4 and CHOP in hypoxic areas of human cervical tumors obtained from patients that had been injected with the hypoxia-sensitive dye pimonidazole (83). Furthermore, ATF4 expression was higher in malignant tissues obtained from patients with brain, breast, cervical, and skin cancers compared with the corresponding normal tissues.

The importance of ATF4 induction and its downstream genes during hypoxia is still not well-defined, but ATF4$^{-/-}$ mouse embryonic fibroblasts are significantly more sensitive to severe and moderate hypoxic exposure (83). ATF4 is important in protecting cells against oxidative stress associated with generation of ROS under conditions of protein folding in the ER (39). The hypoxic cell has long been considered to harbor a more reducing environment than a cell with more physiologic oxygen levels. However, recent models based on the use of redox-sensitive dyes and mechanistic studies on HIF-1α induction seem to support a model of increased oxidative stress in the cytosol most likely due to release of reactive oxygen species from the mitochondria following disruption of the mitochondrial electron transport chain (105). Furthermore, reoxygenation following exposure to hypoxia is known to produce oxidative stress severe enough to activate DNA damage response systems (106). It is thus tempting to hypothesize that induction of ATF4 by hypoxia could serve to induce transcriptional up-regulation of antioxidant-related genes to ameliorate oxidative stress. CHOP, itself a target of ATF4, is also a transcription factor with proapoptotic properties (107, 108). Although an increase in CHOP may play a role in apoptosis under prolonged or severe hypoxia, it is clear (as is also the case for induction of CHOP by pharmacologic agents ref. 40) that the effects of PERK ablation and the inhibition of eIF2α phosphorylation are significantly more severe in terms of cellular survival than an inability to induce CHOP. Thus, the significance of CHOP induction by hypoxia/anoxia remains unclear and will have to be elucidated by using tumor cells with compromised CHOP expression or activity.

Selective Gene Expression Mediated by eIF4F Regulation during Hypoxia

Initiation of translation following assembly of eIF4F at the cap is termed ‘‘cap-dependent’’ translation and constitutes the

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majority of mRNA translation. However, genes that contain an IRES in their 5′ UTR can bypass the requirement for eIF4F and proceed through a cap-independent manner of translation (109). These mRNAs allow direct binding of the ribosome within the UTR without requiring eIF4E. IRES-mediated translation was initially discovered in picornaviruses (110), which exploit this to ensure translation of viral RNA. Many mammalian genes have been subsequently identified to also contain IRES elements. In several instances, these cellular IRES elements facilitate translation under stress conditions, including hypoxia, when protein synthesis via cap-dependent translation is reduced (111). Specifically, vascular endothelial growth factor (112), HIF-1α (113), and BiP (114, 115) have all been reported to have IRES activity, although this is still controversial (116). The availability of eIF4E has been proposed to control a "switch" between cap-dependent and IRES-mediated translation (117). An IRES thus constitutes a possible mechanism to ensure the expression of these proteins during hypoxic conditions when the eIF4F complex is disrupted.

In addition to its effect on eIF4F, mTOR stimulates translation by phosphorylation and activation of S6 kinase. S6 kinase promotes the translation of 5′ terminal oligopyrimidine tract mRNAs, which contain a polypyrimidine stretch of nucleotides adjacent to the 5′ cap (118), although this does not seem to occur through its most well-described substrate S6 (119). 5′ Terminal oligopyrimidine tract sequences are found in the components of the translational machinery, including all ribosomal proteins. The presence of these sequences has also been found in HIF-1α and may contribute to its selective expression in response to mTOR deregulation (86, 120). This idea is supported by a recent report suggesting that rapamycin exerts its antitumor activity primarily by inactivating mTOR and thus reducing the ability to translate HIF-1α due to the presence of these sequences in its 5′ UTR (121). Cells engineered to express a HIF-1 lacking the 5′ UTR were resistant to rapamycin-induced growth inhibition.

The importance of translation for mediating HIF levels is also supported by its frequent overexpression in several diseases associated with mTOR deregulation and cancer. For example, activation of growth factor signaling not only increases global translation but also induces the specific expression of HIF-1α. This has been shown for Ras-transformed cells (122), PTEN-defective glioblastoma cells (123, 124) and, after overexpression of heregulin, the HER-2/*neu* ligand (86). Furthermore, mutations in LKB1, TSC1, and TSC2 also lead to increased expression of HIF-1α (125). Stimulation of translation thus provides an oxygen-independent manner of regulating HIF activity that may contribute to its ability to act oncogenically. The consequences of deregulated mTOR activity combined with hypoxic regulation of mTOR further complicate our understanding of HIF regulation. It will be important to sort out the contribution of these various effects on HIF expression in the coming years.

Translational Control of Gene Expression on a Genome-Wide Scale

The influence of translation control to gene expression has received far less attention than that of transcriptional control, but several reports have shown an important role for this process during development (126), host cell response to poliovirus infection (127), T-cell activation (128), and pVHL expression (129). The overall contribution of mRNA translational control to hypoxia-mediated gene expression is just starting to be understood. In this regard, it is important to point out that dramatic differences in the translation of individual genes can occur even without significant changes in the overall levels of translation. This was elegantly shown by Rajasekhar et al. (130) who used microarrays to assess the levels of individual mRNA transcripts associated with polysomes (i.e., the fraction of mRNA that was actively translated into protein). They showed that on inhibition of the Ras and AKT pathways the range and abundance of efficiently translated mRNAs changed dramatically and much earlier than the well-known transcriptional responses controlled by these pathways. Importantly, this occurred with no significant change in the overall level of mRNA translation. Thus, although large differences in translation are expected under conditions of severe hypoxia, which substantially reduce protein synthesis, significant changes in the translation of individual genes may also occur during moderate hypoxia via inhibition of mTOR, whereas overall rates of protein synthesis do not.

A few studies have already begun to assess the genome-wide effects of translation control during hypoxia. Microarray analysis of polysome mRNA from HeLa cells exposed to 16 hours of severe hypoxia by Blais et al. identified several candidate genes, including ATF4, which displayed increased translation efficiency during hypoxia (27). We did a similar study after only 4 hours of severe hypoxia and showed that of the top 50 genes showing the largest induction during hypoxia 20% of these displayed a selective ability to be translated (131). This included several genes that are involved in both HIF-1- and UPR-dependent responses. A genome-wide analysis of translation in *Arabidopsis* during hypoxia also revealed distinct clusters of genes that showed a preferential ability to maintain translation (132). These genes were involved in abiotic and biotic stress responses and were characterized by having a low GC content in their 5′ UTRs. Analysis of polysomal RNA in various cell lines and under different levels and durations of hypoxia will play an important role in assessing the contribution of translation to the hypoxic phenotype. Studies in genetic models that lack the ability to control translation through either eIF2α or eIF4F will also help to clarify the relative importance of these two pathways on the preferential translation of genes during hypoxia.

Potential for Therapy

Tumor hypoxia has long been established as an impediment to tumor therapy. The data implicating the UPR in tumor progression and hypoxia tolerance describe an important adaptive mechanism that cells in established tumors use to survive the tumor microenvironment. Furthermore, the PERK-eIF2α pathway has been reported to be critical for tumor cell dormancy (133), an important clinical phenomenon, which also contributes to chemoresistance. However, the reliance of hypoxic and dormant tumor cells on a functional UPR may provide a unique therapeutic opportunity. One approach to target the UPR in tumors is by developing inhibitors that would

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target the PERK/eIF2α/ATF4 or IRE1/XBP-1 modules. Although currently no specific small-molecule inhibitors have been identified, screening of small-molecule libraries for inhibitors of PERK and IRE1 is currently under way.[5]

A second approach would be to target tumor cells that have an overactive UPR with pharmacologic agents that further induce ER stress. The rationale in this approach is that tumor cells under hypoxia already have an active PERK-eIF2α-ATF4 pathway, surviving close to a threshold limit of ER stress. Thus, it is reasonable to hypothesize that subjecting these hypoxic tumor cells to additional ER stress would force them past their tolerance levels and onto cell death. Interestingly, proteasome inhibitors, such as PS-341 (Velcade), which is Food and Drug Administration approved for treating human malignancies, have been reported to induce ER stress (134-136). In particular, Fribley et al. showed that PS-341 induced PERK activation, which led to a reduction in protein synthesis and an up-regulation of ATF4 and CHOP (135). Moreover, preliminary data from the Koumenis laboratory suggest that proteasome inhibition may indeed be selectively cytotoxic to hypoxic tumor cells.[3]

Concluding Remarks—Future Perspectives

The work described above suggests that control of mRNA translation is an important cellular response to hypoxia. Hypoxia influences translation through several processes that are dependent on both severity and duration of hypoxic stress. These processes seem to play important roles in the tolerance of tumor cells to hypoxia. In particular, the ER may serve as an "early warning" system to cells experiencing severe hypoxia. Although it has not yet been shown, low oxygen availability may lead to protein misfolding and thus the signal for subsequent activation of the UPR. Proper protein folding in the ER involves, among other modifications, the generation of disulfide bonds and glycosylation of client proteins. Precise control of the oxidizing environment of this organelle is critical for proper folding, and disruption of this oxidizing environment by chemical agents (e.g., DTT) or physicochemical perturbations results in protein unfolding or misfolding. Protein folding is a highly controlled process that is driven by coupled reactions in a protein relay that includes Ero1p (a FAD-dependent enzyme) and protein disulfide isomerase, an abundant ER-resident protein that directly oxidizes disulfide bonds in client proteins in a FAD-dependent manner. Work from Tu and Weissman has shown that in yeast molecular dioxygen is the preferred electron acceptor in this electron transfer chain and directly oxidizes Ero1p. Preliminary data suggest that anoxia leads to rapid changes in the oxidation status of protein disulfide isomerase, a marker of misfolding in mammalian cells as well.[3] We propose that the rapid drop in oxygen levels under anoxia/hypoxia results in a less-than-optimal oxidizing environment in the ER, which leads to the unfolding of proteins and eventual UPR activation.

Temporally, activation of PERK and phosphorylation of eIF2α during hypoxia seems to precede IRE1 and XBP-1 activation, in a manner similar to other stresses, such as thapsigargin and tunicamycin. The activation of the PERK-eIF2α pathway serves to rapidly halt global mRNA translation perhaps in part to reduce ATP consumption and maintain energy homeostasis. The importance of this modification is evident by the hypoxic sensitivity and defective tumor growth of cells with inactivating mutations in PERK, eIF2α, and ATF4. However, a causal relationship between UPR activation and eIF4F inhibition on energy conservation (e.g., ATP levels) remains to be formally shown.

Despite our improved understanding of translational inhibition under hypoxic stress, these findings raise several important questions for the future. As discussed above, early investigations suggest that many genes may be subject to specific translational control during hypoxia. However, it is not yet clear what, if any, predominant mechanisms are used by these genes for promoting their synthesis during hypoxia. Examples of uORF, IRES, and 5′ terminal oligopyrimidine tract sequences have been found in some of the better described hypoxia-induced proteins. A second important area that has not been adequately addressed is the degree to which control of mRNA translation during hypoxia may be altered in cancer. Control of mRNA translation via disruption of eIF4F is indeed expected to vary considerably among different tumors because the upstream pathways that control the assembly of this complex are frequently disrupted in cancer. eIF4F has emerged as an important target of phosphatidylinositol 3-kinase signaling during tumor development. It will thus be important to assess the differences in regulation of translation and gene expression during hypoxia in cells with specific defects in these upstream signaling pathways. Furthermore, although eIF4F assembly under hypoxia is disrupted, the importance of this effect on tumor growth or hypoxia tolerance has yet to be addressed. Third, the mechanisms and importance of cell death induced via sustained activation of the UPR during hypoxia is not fully understood. For example, we do not yet know what the nature of the proapoptotic signals initiating from the ER is during hypoxia-induced apoptosis. It is also not clear if the slower growth rate of the tumors from UPR compromised cells is due solely to increased apoptotic sensitivity or if other effects on host responses may also be involved. Although we have shown that the induction of HIF-1α and vascular endothelial growth factor, two critical proangiogenic factors, are not affected by the different status of PERK or eIF2α, it is still possible that that other proangiogenic pathways may be compromised contributing to tumor growth inhibition.

With several groups now actively engaged in studying translational regulation and UPR activation by hypoxia, the next few years look quite promising for providing answers to these questions and for developing new therapeutic approaches for malignancies based on these hypoxic responses.

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References

1. Brown JM, Wilson WR. Exploiting tumour hypoxia in cancer treatment. Nat Rev Cancer 2004;4:437–47.

2. Evans SM, Koch CJ. Prognostic significance of tumor oxygenation in humans. Cancer Lett 2003;195:1–16.

3. Le QT, Denko NC, Giaccia AJ. Hypoxic gene expression and metastasis. Cancer Metastasis Rev 2004;23:293–310.

[5] Koong et al., personal communication.

4. Teicher BA. Hypoxia and drug resistance. Cancer Metastasis Rev 1994;13:139–68.

5. Teicher BA. Physiologic mechanisms of therapeutic resistance. Blood flow and hypoxia. Hematol Oncol Clin North Am 1995;9:475–506.

6. Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res 1996;56:4509–15.

7. Airley R, Loncaster J, Davidson S, et al. Glucose transporter glut-1 expression correlates with tumor hypoxia and predicts metastasis-free survival in advanced carcinoma of the cervix. Clin Cancer Res 2001;7:928–34.

8. Hockel M, Vaupel P. Biological consequences of tumor hypoxia. Semin Oncol 2001;28:36–41.

9. Sutherland RM, Ausserer WA, Murphy BJ, Laderoute KR. Tumor hypoxia and heterogeneity: challenges and opportunities for the future. Semin Radiat Oncol 1996;6:59–70.

10. Brown JM, Giaccia AJ. The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. Cancer Res 1998;58:1408–16.

11. Chaplin DJ, Hill SA. Temporal heterogeneity in microregional erythrocyte flux in experimental solid tumours. Br J Cancer 1995;71:1210–3.

12. Cardenas-Navia LI, Yu D, Braun RD, Brizel DM, Secomb TW, Dewhirst MW. Tumor-dependent kinetics of partial pressure of oxygen fluctuations during air and oxygen breathing. Cancer Res 2004;64:6010–7.

13. Janssen HL, Haustermans KM, Sprong D, et al. HIF-1A, pimonidazole, and iododeoxyuridine to estimate hypoxia and perfusion in human head-and-neck tumors. Int J Radiat Oncol Biol Phys 2002;54:1537–49.

14. Durand RE, Aquino-Parsons C. Clinical relevance of intermittent tumour blood flow. Acta Oncol 2001;40:929–36.

15. Lanzen J, Braun RD, Klitzman B, Brizel D, Secomb TW, Dewhirst MW. Direct demonstration of instabilities in oxygen concentrations within the extravascular compartment of an experimental tumor. Cancer Res 2006;66:2219–23.

16. Graeber TG, Osmanian C, Jacks T, et al. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours [see comments]. Nature 1996;379:88–91.

17. Kim CY, Tsai MH, Osmanian C, et al. Selection of human cervical epithelial cells that possess reduced apoptotic potential to low-oxygen conditions. Cancer Res 1997;57:4200–4.

18. Epstein AC, Gleadle JM, McNeill LA, et al. *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 2001;107:43–54.

19. Dever TE. Translation initiation: adept at adapting. Trends Biochem Sci 1999;24:398–403.

20. Sheikh MS, Fornace AJ, Jr. Regulation of translation initiation following stress. Oncogene 1999;18:6121–8.

21. Kozak M. Initiation of translation in prokaryotes and eukaryotes. Gene 1999;234:187–208.

22. Preiss T, Hentze MW. From factors to mechanisms: translation and translational control in eukaryotes. Curr Opin Genet Dev 1999;9:515–21.

23. Koumenis C, Naczki C, Koritzinsky M, et al. Regulation of protein synthesis by hypoxia via activation of the endoplasmic reticulum kinase PERK and phosphorylation of the translation initiation factor eIF2α. Mol Cell Biol 2002;22:7405–16.

24. Koritzinsky M, Magagnin MG, van den Beucken T, et al. Gene expression during acute and prolonged hypoxia is regulated by distinct mechanisms of translational control. EMBO J 2006;25:1114–25.

25. Donze O, Jagus R, Koromilas AE, Hershey JW, Sonenberg N. Abrogation of translation initiation factor eIF-2 phosphorylation causes malignant transformation of NIH 3T3 cells. EMBO J 1995;14:3828–34.

26. Scheuner D, Song B, McEwen E, et al. Translational control is required for the unfolded protein response and *in vivo* glucose homeostasis. Mol Cell 2001;7:1165–76.

27. Blais JD, Filipenko V, Bi M, et al. Activating transcription factor 4 is translationally regulated by hypoxic stress. Mol Cell Biol 2004;24:7469–82.

28. Liu L, Cash TP, Jones RG, Keith B, Thompson CB, Simon MC. Hypoxia-induced energy stress regulates mRNA translation and cell growth. Mol Cell 2006;21:521–31.

29. Feldman DE, Chauhan V, Koong AC. The unfolded protein response: a novel component of the hypoxic stress response in tumors. Mol Cancer Res 2005;3:597–605.

30. Connolly E, Braunstein S, Formenti S, Schneider RJ. Hypoxia inhibits protein synthesis through a 4E-BP1 and elongation factor 2 kinase pathway controlled by mTOR and uncoupled in breast cancer cells. Mol Cell Biol 2006;26:3955–65.

31. Kimball SR. Eukaryotic initiation factor eIF2. Int J Biochem Cell Biol 1999;31:25–9.

32. Brewer JW, Diehl JA. PERK mediates cell-cycle exit during the mammalian unfolded protein response. Proc Natl Acad Sci U S A 2000;97:12625–30.

33. Harding HP, Zhang Y, Ron D. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase [see comments]. Nature 1999;397:271–74. Erratum in: Nature 1999;398:90.

34. Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. Nat Cell Biol 2000;2:326–32.

35. Koong AC, Auger EA, Chen EY, Giaccia AJ. The regulation of GRP78 and messenger RNA levels by hypoxia is modulated by protein kinase C activators and inhibitors. Radiat Res 1994;138:S60–3.

36. Dorner AJ, Wasley LC, Kaufman RJ. Protein dissociation from GRP78 and secretion are blocked by depletion of cellular ATP levels. Proc Natl Acad Sci U S A 1990;87:7429–32.

37. Shi Y, Vattem KM, Sood R, et al. Identification and characterization of pancreatic eukaryotic initiation factor 2α-subunit kinase, PEK, involved in translational control. Mol Cell Biol 1998;18:7499–509.

38. Harding HP, Zhang Y, Bertolotti A, Zeng H, Ron D. Perk is essential for translational regulation and cell survival during the unfolded protein response. Mol Cell 2000;5:897–904.

39. Harding HP, Zhang Y, Zeng H, et al. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol Cell 2003;11:619–33.

40. Harding HP, Novoa I, Zhang Y, et al. Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol Cell 2000;6:1099–108.

41. Fawcett TW, Martindale JL, Guyton KZ, Hai T, Holbrook NJ. Complexes containing activating transcription factor (ATF)/cAMP-responsive-element-binding protein (CREB) interact with the CCAAT/enhancer-binding protein (C/EBP)-ATF composite site to regulate Gadd153 expression during the stress response. Biochem J 1999;339:135–41.

42. Lu PD, Jousse C, Marciniak SJ, et al. Cytoprotection by pre-emptive conditional phosphorylation of translation initiation factor 2. EMBO J 2004;23:169–79.

43. Ron D. Translational control in the endoplasmic reticulum stress response. J Clin Invest 2002;110:1383–8.

44. Cullinan SB, Zhang D, Hannink M, Arvisais E, Kaufman RJ, Diehl JA. Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. Mol Cell Biol 2003;23:7198–209.

45. Rutkowski DT, Kaufman RJ. A trip to the ER: coping with stress. Trends Cell Biol 2004;14:20–8.

46. Hetz C, Bernasconi P, Fisher J, et al. Proapoptotic BAX and BAK modulate the unfolded protein response by a direct interaction with IRE1α. Science 2006;312:572–6.

47. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 2001;107:881–91.

48. Calfon M, Zeng H, Urano F, et al. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. Nature 2002;415:92–6.

49. Urano F, Bertolotti A, Ron D. IRE1 and efferent signaling from the endoplasmic reticulum. J Cell Sci 2000;113 Pt 21:3697–702.

50. Romero-Ramirez L, Cao H, Nelson D, et al. XBP1 is essential for survival under hypoxic conditions and is required for tumor growth. Cancer Res 2004;64:5943–7.

51. Haze K, Yoshida H, Yanagi H, Yura T, Mori K. Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. Mol Biol Cell 1999;10:3787–99.

52. Paschen W. Disturbances of calcium homeostasis within the endoplasmic reticulum may contribute to the development of ischemic-cell damage. Med Hypotheses 1996;47:283–8.

53. Ferrari D, Pinton P, Szabadkai G, et al. Endoplasmic reticulum, Bcl-2 and Ca^{2+} handling in apoptosis. Cell Calcium 2002;32:413–20.

54. Kaufman RJ. Orchestrating the unfolded protein response in health and disease. J Clin Invest 2002;110:1389–98.

55. Zong WX, Li C, Hatzivassiliou G, et al. Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. J Cell Biol 2003;162:59–69.

56. Scorrano L, Oakes SA, Opferman JT, et al. BAX and BAK regulation of

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endoplasmic reticulum Ca^{2+}: a control point for apoptosis. Science 2003;300:135–9.

57. Nutt LK, Pataer A, Pahler J, et al. Bax and Bak promote apoptosis by modulating endoplasmic reticular and mitochondrial Ca^{2+} stores. J Biol Chem 2002;277:9219–25.

58. Lam M, Dubyak G, Chen L, Nunez G, Miesfeld RL, Distelhorst CW. Evidence that BCL-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca^{2+} fluxes. Proc Natl Acad Sci U S A 1994;91:6569–73.

59. Breckenridge DG, Germain M, Mathai JP, Nguyen M, Shore GC. Regulation of apoptosis by endoplasmic reticulum pathways. Oncogene 2003;22:8608–18.

60. Pause A, Belsham GJ, Gingras AC, et al. Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5′-cap function. Nature 1994;371:762–7.

61. Poulin F, Gingras AC, Olsen H, Chevalier S, Sonenberg N. 4E-BP3, a new member of the eukaryotic initiation factor 4E-binding protein family. J Biol Chem 1998;273:14002–7.

62. Tinton SA, Buc-Calderon PM. Hypoxia increases the association of 4E-binding protein 1 with the initiation factor 4E in isolated rat hepatocytes. FEBS Lett 1999;446:55–9.

63. Arsham AM, Howell JJ, Simon MC. A novel hypoxia-inducible factor-independent hypoxic response regulating mammalian target of rapamycin and its targets. J Biol Chem 2003;278:29655–60.

64. Gingras AC, Raught B, Gygi SP, et al. Hierarchical phosphorylation of the translation inhibitor 4E-BP1. Genes Dev 2001;15:2852–64.

65. Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev 2004;18:1926–45.

66. Dilling MB, Germain GS, Dudkin L, et al. 4E-binding proteins, the suppressors of eukaryotic initiation factor 4E, are down-regulated in cells with acquired or intrinsic resistance to rapamycin. J Biol Chem 2002;277:13907–17.

67. Crew JP, Fuggle S, Bicknell R, Cranston DW, de Benedetti A, Harris AL. Eukaryotic initiation factor-4E in superficial and muscle invasive bladder cancer and its correlation with vascular endothelial growth factor expression and tumour progression. Br J Cancer 2000;82:161–6.

68. De Benedetti A, Harris AL. eIF4E expression in tumors: its possible role in progression of malignancies. Int J Biochem Cell Biol 1999;31:59–72.

69. Cai SL, Tee AR, Short JD, et al. Activity of TSC2 is inhibited by AKT-mediated phosphorylation and membrane partitioning. J Cell Biol 2006;173:279–89.

70. Brugarolas J, Lei K, Hurley RL, et al. Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. Genes Dev 2004;18:2893–904.

71. Sofer A, Lei K, Johannessen CM, Ellisen LW. Regulation of mTOR and cell growth in response to energy stress by REDD1. Mol Cell Biol 2005;25:5834–45.

72. Schwarzer R, Tondera D, Arnold W, Giese K, Klippel A, Kaufmann J. REDD1 integrates hypoxia-mediated survival signaling downstream of phosphatidylinositol 3-kinase. Oncogene 2005;24:1138–49.

73. Reiling JH, Hafen E. The hypoxia-induced paralogs Scylla and Charybdis inhibit growth by down-regulating S6K activity upstream of TSC in *Drosophila*. Genes Dev 2004;18:2879–92.

74. Corradetti MN, Inoki K, Bardeesy N, DePinho RA, Guan KL. Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome. Genes Dev 2004;18:1533–8.

75. Shaw RJ, Bardeesy N, Manning BD, et al. The LKB1 tumor suppressor negatively regulates mTOR signaling. Cancer Cell 2004;6:91–9.

76. Browne GJ, Proud CG. A novel mTOR-regulated phosphorylation site in elongation factor 2 kinase modulates the activity of the kinase and its binding to calmodulin. Mol Cell Biol 2004;24:2986–97.

77. Browne GJ, Finn SG, Proud CG. Stimulation of the AMP-activated protein kinase leads to activation of eukaryotic elongation factor 2 kinase and to its phosphorylation at a novel site, serine 398. J Biol Chem 2004;279:12220–31.

78. Kaper F, Dornhoefer N, Giaccia AJ. Mutations in the PI3K/PTEN/TSC2 pathway contribute to mammalian target of rapamycin activity and increased translation under hypoxic conditions. Cancer Res 2006;66:1561–9.

79. Dostie J, Ferraiuolo M, Pause A, Adam SA, Sonenberg N. A novel shuttling protein, 4E-T, mediates the nuclear import of the mRNA 5′ cap-binding protein, eIF4E. EMBO J 2000;19:3142–56.

80. Andrei MA, Ingelfinger D, Heintzmann R, Achsel T, Rivera-Pomar R, Luhrmann R. A role for eIF4E and eIF4E-transporter in targeting mRNPs to mammalian processing bodies. RNA 2005;11:717–27.

81. Lejbkowicz F, Goyer C, Darveau A, Neron S, Lemieux R, Sonenberg N. A fraction of the mRNA 5′ cap-binding protein, eukaryotic initiation factor 4E, localizes to the nucleus. Proc Natl Acad Sci U S A 1992;89:9612–6.

82. Liu J, Valencia-Sanchez MA, Hannon GJ, Parker R. MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. Nat Cell Biol 2005;7:719–23.

83. Bi M, Naczki C, Koritzinsky M, et al. ER stress-regulated translation increases tolerance to extreme hypoxia and promotes tumor growth. EMBO J 2005;24:3470–81.

84. Li J, Yen C, Liaw D, et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 1997;275:1943–7.

85. Steck PA, Pershouse MA, Jasser SA, et al. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat Genet 1997;15:356–62.

86. Laughner E, Taghavi P, Chiles K, Mahon PC, Semenza GL. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1α (HIF-1α) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol Cell Biol 2001;21:3995–4004.

87. Young J, Povey S. The genetic basis of tuberous sclerosis. Mol Med Today 1998;4:313–9.

88. Boudeau J, Sapkota G, Alessi DR. LKB1, a protein kinase regulating cell proliferation and polarity. FEBS Lett 2003;546:159–65.

89. Kwiatkowski DJ. Tuberous sclerosis: from tubers to mTOR. Ann Hum Genet 2003;67:87–96.

90. Lazaris-Karatzas A, Montine KS, Sonenberg N. Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5′ cap. Nature 1990;345:544–7.

91. Zimmer SG, DeBenedetti A, Graff JR. Translational control of malignancy: the mRNA cap-binding protein, eIF-4E, as a central regulator of tumor formation, growth, invasion and metastasis. Anticancer Res 2000;20:1343–51.

92. Ruggero D, Montanaro L, Ma L, et al. The translation factor eIF-4E promotes tumor formation and cooperates with c-Myc in lymphomagenesis. Nat Med 2004;10:484–6.

93. Wendel HG, De Stanchina E, Fridman JS, et al. Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy. Nature 2004;428:332–7.

94. Richter JD, Sonenberg N. Regulation of cap-dependent translation by eIF4E inhibitory proteins. Nature 2005;433:477–80.

95. Kuersten S, Goodwin EB. The power of the 3′ UTR: translational control and development. Nat Rev Genet 2003;4:626–37.

96. Meijer HA, Thomas AA. Control of eukaryotic protein synthesis by upstream open reading frames in the 5′-untranslated region of an mRNA. Biochem J 2002;367:1–11.

97. Sachs MS1, Geballe AP. Downstream control of upstream open reading frames. Genes Dev 2006;20:915–21.

98. Holcik M, Sonenberg N. Translational control in stress and apoptosis. Nat Rev Mol Cell Biol 2005;6:318–27.

99. Spicher A, Guicherit OM, Duret L, et al. Highly conserved RNA sequences that are sensors of environmental stress. Mol Cell Biol 1998;18:7371–82.

100. Lu PD, Harding HP, Ron D. Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. J Cell Biol 2004;167:27–33.

101. Vattem KM, Wek RC. Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. Proc Natl Acad Sci U S A 2004;101:11269–74.

102. Churbanov A, Rogozin IB, Babenko VN, Ali H, Koonin EV. Evolutionary conservation suggests a regulatory function of AUG triplets in 5′-UTRs of eukaryotic genes. Nucleic Acids Res 2005;33:5512–20.

103. Suzuki Y, Ishihara D, Sasaki M, et al. Statistical analysis of the 5′ untranslated region of human mRNA using "oligo-capped" cDNA libraries. Genomics 2000;64:286–97.

104. Ameri K, Lewis CE, Raida M, Sowter H, Hai T, Harris AL. Anoxic induction of ATF-4 through HIF-1-independent pathways of protein stabilization in human cancer cells. Blood 2004;103:1876–82.

105. Schumacker PT. Current paradigms in cellular oxygen sensing. Adv Exp Med Biol 2003;543:57–71.

106. Hammond EM, Dorie MJ, Giaccia AJ. ATR/ATM targets are phosphorylated by ATR in response to hypoxia and ATM in response to reoxygenation. J Biol Chem 2003;278:12207–13.

107. McCullough KD, Martindale JL, Klotz LO, Aw TY, Holbrook NJ. Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. Mol Cell Biol 2001;21:1249–59.

Published OnlineFirst July 6, 2006; DOI: 10.1158/1541-7786.MCR-06-0150

108. Zinszner H, Kuroda M, Wang X, et al. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. Genes Dev 1998;12:982–95.

109. Hellen CU, Sarnow P. Internal ribosome entry sites in eukaryotic mRNA molecules. Genes Dev 2001;15:1593–612.

110. Jang SK, Krausslich HG, Nicklin MJ, Duke GM, Palmenberg AC, Wimmer E. A segment of the 5′ nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during *in vitro* translation. J Virol 1988;62:2636–43.

111. Bonnal S, Boutonnet C, Prado-Lourenco L, Vagner S. IRESdb: the Internal Ribosome Entry Site database. Nucleic Acids Res 2003;31:427–8.

112. Stein I, Itin A, Einat P, Skaliter R, Grossman Z, Keshet E. Translation of vascular endothelial growth factor mRNA by internal ribosome entry: implications for translation under hypoxia. Mol Cell Biol 1998;18:3112–9.

113. Lang KJ, Kappel A, Goodall GJ. Hypoxia-inducible factor-1α mRNA contains an internal ribosome entry site that allows efficient translation during normoxia and hypoxia. Mol Biol Cell 2002;13:1792–801.

114. Macejak DG, Sarnow P. Internal initiation of translation mediated by the 5′ leader of a cellular mRNA. Nature 1991;353:90–4.

115. Thoma C, Bergamini G, Galy B, Hundsdoerfer P, Hentze MW. Enhancement of IRES-mediated translation of the c-myc and BiP mRNAs by the poly(A) tail is independent of intact eIF4G and PABP. Mol Cell 2004;15:925–35.

116. Bert AG, Grepin R, Vadas MA, Goodall GJ. Assessing IRES activity in the HIF-1α and other cellular 5′ UTRs. RNA 2006;12:1074–83.

117. Svitkin YV, Herdy B, Costa-Mattioli M, Gingras AC, Raught B, Sonenberg N. Eukaryotic translation initiation factor 4E availability controls the switch between cap-dependent and internal ribosomal entry site-mediated translation. Mol Cell Biol 2005;25:10556–65.

118. Jefferies HB, Fumagalli S, Dennis PB, Reinhard C, Pearson RB, Thomas G. Rapamycin suppresses 5′TOP mRNA translation through inhibition of p70s6k. EMBO J 1997;16:3693–704.

119. Ruvinsky I, Meyuhas O. Ribosomal protein S6 phosphorylation: from protein synthesis to cell size. Trends Biochem Sci 2006;31:342–8.

120. Schepens B, Tinton SA, Bruynooghe Y, Beyaert R, Cornelis S. The polypyrimidine tract-binding protein stimulates HIF-1α IRES-mediated translation during hypoxia. Nucleic Acids Res 2005;33:6884–94.

121. Thomas GV, Tran C, Mellinghoff IK, et al. Hypoxia-inducible factor determines sensitivity to inhibitors of mTOR in kidney cancer. Nat Med 2006;12:122–7.

122. Mazure NM, Chen EY, Laderoute KR, Giaccia AJ. Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidylinositol 3-kinase/Akt signaling pathway in Ha-ras-transformed cells through a hypoxia inducible factor-1 transcriptional element. Blood 1997;90:3322–31.

123. Zhong H, De Marzo AM, Laughner E, et al. Overexpression of hypoxia-inducible factor 1α in common human cancers and their metastases. Cancer Res 1999;59:5830–5.

124. Zundel W, Schindler C, Haas-Kogan D, et al. Loss of PTEN facilitates HIF-1-mediated gene expression. Genes Dev 2000;14:391–6.

125. Brugarolas J, Kaelin WG, Jr. Dysregulation of HIF and VEGF is a unifying feature of the familial hamartoma syndromes. Cancer Cell 2004;6:7–10.

126. Richter JD. Translational control in development: a perspective. Dev Genet 1993;14:407–11.

127. Johannes G, Carter MS, Eisen MB, Brown PO, Sarnow P. Identification of eukaryotic mRNAs that are translated at reduced cap binding complex eIF4F concentrations using a cDNA microarray. Proc Natl Acad Sci U S A 1999;96:13118–23.

128. Mikulits W, Pradet-Balade B, Habermann B, Beug H, Garcia-Sanz JA, Mullner EW. Isolation of translationally controlled mRNAs by differential screening. FASEB J 2000;14:1641–52.

129. Galban S, Fan J, Martindale JL, et al. von Hippel-Lindau protein-mediated repression of tumor necrosis factor α translation revealed through use of cDNA arrays. Mol Cell Biol 2003;23:2316–28.

130. Rajasekhar VK, Viale A, Socci ND, Wiedmann M, Hu X, Holland EC. Oncogenic Ras and Akt signaling contribute to glioblastoma formation by differential recruitment of existing mRNAs to polysomes. Mol Cell 2003;12:889–901.

131. Koritzinsky M, Seigneuric R, Magagnin MG, van den Beucken T, Lambin P, Wouters BG. The hypoxic proteome is influenced by gene-specific changes in mRNA translation. Radiother Oncol 2005;76:177–86.

132. Branco-Price C, Kawaguchi R, Ferreira RB, Bailey-Serres J. Genome-wide analysis of transcript abundance and translation in *Arabidopsis* seedlings subjected to oxygen deprivation. Ann Bot (Lond) 2005;96:647–60.

133. Ranganathan AC, Zhang L, Adam AP, Aguirre-Ghiso JA. Functional coupling of p38-induced up-regulation of BiP and activation of RNA-dependent protein kinase-like endoplasmic reticulum kinase to drug resistance of dormant carcinoma cells. Cancer Res 2006;66:1702–11.

134. Lee C, Waldman T. Human somatic cell knockouts reveal determinants of sensitivity and resistance to proteasome inhibitor PS-341. Cancer Biol Ther 2003;2:700–1.

135. Fribley A, Zeng Q, Wang CY. Proteasome inhibitor PS-341 induces apoptosis through induction of endoplasmic reticulum stress-reactive oxygen species in head and neck squamous cell carcinoma cells. Mol Cell Biol 2004;24:9695–704.

136. Obeng EA, Carlson LM, Gutman DM, Harrington WJ, Jr., Lee KP, Boise LH. Proteasome inhibitors induce a terminal unfolded protein response in multiple myeloma cells. Blood 2006;107:4907–16.

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"Translating" Tumor Hypoxia: Unfolded Protein Response (UPR)–Dependent and UPR-Independent Pathways

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