

Science  
from the  
Source**CELLULAR ADAPTATION TO LOW OXYGEN AVAILABILITY BY  
A SWITCH IN THE PROTEIN SYNTHESIS MACHINERY**

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**ABSTRACT**

Protein synthesis is a classic molecular mechanism of cell biology that is taught in introductory biology classrooms. It involves the translation of messenger ribonucleic acid (mRNA) information into proteins, the building blocks of life. The initial step of protein synthesis consists of the eukaryotic translation initiation factor 4E (eIF4E) binding to the 5' cap of mRNAs. A variety of stresses repress translation to conserve energy because protein synthesis requires over half of a cell's energy supply. An important stress for multicellular animals is low oxygen availability (hypoxia). This causes a repression of cap-directed translation by inhibiting eIF4E. This raises a fundamental question in cell biology as to how proteins are synthesized in periods of oxygen scarcity and eIF4E inhibition. Here, we describe an oxygen-regulated translation initiation complex that mediates selective cap-dependent protein synthesis. Hypoxia stimulates the formation of a complex that includes the oxygen-regulated hypoxia-inducible factor 2 $\alpha$  (HIF-2 $\alpha$ ), the RNA binding protein RBM4 and the cap-binding eIF4E2, an eIF4E homologue. We also identified an RNA hypoxia response element (rHRE) that recruits this complex to a wide array of mRNAs, including the epidermal growth factor receptor (EGFR), which plays a role in growth signaling and proliferation. Once assembled at the rHRE, HIF-2 $\alpha$ /RBM4/eIF4E2 captures the 5' cap and targets mRNAs for active translation thereby evading hypoxia-induced repression of protein synthesis. These findings demonstrate that cells have evolved a program whereby oxygen availability switches the basic translation initiation machinery.

**INTRODUCTION**

The appearance of oxygen in Earth's atmosphere 3 billion years ago was a key event in evolution. It gave rise to eukaryotic cells that utilized aerobic metabolism to produce 16-18 times more energy than anaerobic metabolism. This higher energy supply allowed complex multicellular organisms such as mammals to emerge. With increased size came the requirement of sophisticated oxygen-delivery systems including blood and blood vessels. The cells of the human body have intricate oxygen-sensing mechanisms which closely monitor fluctuations. One of the major cellular responses to low oxygen availability (hypoxia) is through the stabilization of Hypoxia Inducible Factors (HIFs). HIFs are composed of oxygen-regulated alpha subunits HIF-1 $\alpha$  or HIF-2 $\alpha$  and a beta subunit (HIF-1 $\beta$ ), which is always expressed. The HIFs are transcription

factors, transcribing DNA into mRNA that codes for proteins which will allow the cell to adapt to hypoxia<sup>1</sup>. Transcription is the first step in gene expression whereby DNA is transcribed into mRNA using the cellular language of nucleic acids. This is typically followed by translation of these mRNAs into another language, amino acids, which are the building blocks of proteins. Proteins make up most of the functional macromolecules in the cell. A major question in cell biology has been how these hypoxia-response mRNAs are translated into proteins because as hypoxia induces a robust transcriptional response, it also represses translation (protein synthesis) to conserve energy.

Most (>95%) of protein synthesis is initiated at one end of the mRNA (the 5' end), by a complex of proteins that includes one protein called eIF4E. Reduction of the activity of eIF4E

drastically reduces translation rates. Such is the case during exposure to hypoxia, which reduces eIF4E activity, and therefore drastically reduces translation rates. Since translation of certain proteins is required for the adaptation to hypoxia to take place, other mechanisms of translation must occur when eIF4E is repressed.

This is an important question because hypoxia has implications in many physiological processes, as well as in many of today's most deadly diseases. Proper embryonic development depends on the hypoxic response to build a thorough blood vessel network to fully oxygenate the foetus. Intense muscle exercise requires a switch to anaerobic metabolism and the synthesis of key proteins involved in the glycolytic pathway. Populations that live at high altitudes or temporary visitors to these locations must deal with lower atmospheric oxygen levels to avoid mountain sickness, which could be fatal. Many of the most deadly diseases in North America are driven by the hypoxic response such as stroke, heart disease and cancer. Stroke and heart disease are the result of a blockage in a blood vessel, restricting the delivery of oxygen to the affected organs often causing irreversible tissue damage. Cancer can result in the development of a tumor, a cellular mass that quickly outgrows its blood supply resulting in massive regions of hypoxia. Tumor hypoxia has been linked to poor prognosis due to the resistance to chemo and radiation therapy and has been a primary target of cancer therapy for decades. Our research goals were to better understand the mechanisms behind how human cells translate mRNA into protein during the harsh conditions of hypoxia, which would allow us to better appreciate how the abovementioned processes are regulated.

#### HOW WE DID IT

**Cell lines and reagents.** All human cell lines (U87MG glioblastoma and human renal proximal tubular epithelial cells) were obtained from the American Type Culture Collection and propagated grown as suggested. Cells were incubated at 37 °C in a 5% CO<sub>2</sub> environment. Hypoxia was induced by incubation at 1% O<sub>2</sub>, 5% CO<sub>2</sub> and N<sub>2</sub>-balanced atmosphere for 24 h.

actinomycinD, a transcription inhibitor (10 µg/mL) was added 30 min prior to hypoxic exposure.

**Protein synthesis by 35S-Methionine incorporation.** Prior to cell lysis, cells were labeled with radioactive [<sup>35</sup>S]Methionine for 15 min to visualize newly-synthesized proteins (proteins are the only macromolecules to contain sulphur). Cells were lysed in 1 mL RIPA lysis buffer. Samples were separated by a method known as SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins by size, and then exposed to X-ray film overnight and visualized by autoradiography.

**RNA Immunoprecipitation.** Messenger RNAs were crosslinked to proteins with formaldehyde and cells were lysed in 1 mL RIPA. HIF-2α and RBM4 were precipitated with 2 µg/mL of primary antibody overnight. Target proteins were captured with Dynabeads, which were washed five times with RIPA. Crosslinking was reversed by incubating overnight at 65 °C. RNAs associated with HIF-2α or RBM4 were extracted with TRIzol and amplified with reverse-transcription-polymerase chain reaction (RT-PCR) analysis.

**Cap-binding assays.** Cells were lysed and incubated with 50 µL 7-methyl GTP-Sepharose 4B beads (5' cap linked to beads) for 1 h. Beads were washed 4 times with lysis buffer and resuspended in 1 mM GTP for 1 h (GTP is a competitor for the beads and will dislodge the bound proteins). Samples were separated on an SDS-PAGE.

**Polysome analysis.** Polysome analysis was performed by trapping ribosomes on mRNAs with the inhibitor of translocation cycloheximide, lysing cells, and then loading them onto a linear 10-50% sucrose density gradient (which will separate cellular components by density). The dense polysomes will be isolated and probed by Western blot for various components of the translation apparatus such as eIF4E and eIF4E2.

**PAR-CLIP.** Cells were irradiated with 365 nm UV light and lysed. Immunoprecipitations of HIF-2α and RBM4 were performed as described above. Radioactive <sup>32</sup>P-ATP was added to label the ends of the associated mRNAs so



that they could be detected by SDS-PAGE and autoradiography. The radioactive RNA-protein complex migrating at the expected molecular weight of the target protein was excised from the gel. The proteins were removed by Proteinase K digestion and the RNA was recovered by acidic phenol/chloroform extraction followed by a chloroform extraction and ethanol precipitation. The RNA pellet was dissolved in 10  $\mu$ L of water and amplified by RT-PCR after binding of the appropriate adapter sequences for Next Generation Sequencing. Samples were sequenced by Illumina next generation sequencing at the Next Generation Sequencing Facility (Hospital for Sick Children, Toronto, Ontario). Sequence reads were analyzed with CLC Genomics Workbench 4.7.1. Associated mRNA sequences were identified using Basic Local Alignment Search Tool mapped against the human genomic transcript database downloaded from Genbank.

#### WHAT WE FOUND

*HIF-2 $\alpha$  has an alter ego as a translation factor.*  
The major finding that led us down the path of translational control was that many key hypoxia-induced proteins that were HIF-2 $\alpha$ -dependent accumulated in the presence of transcription inhibitors. This was consistent with a role of HIF-2 $\alpha$  outside of transcription. We further investigated the role of HIF-2 $\alpha$  as a translation factor by monitoring its association with hypoxic polysomes (mRNAs that are being actively translated). Indeed, HIF-2 $\alpha$  associated with hypoxic polysomes and directly with the epidermal growth factor receptor (EGFR) mRNA, which is a key hypoxia-induced protein that allows cells to survive hypoxic stress. Furthermore, when we reduced the levels of HIF-2 $\alpha$  in the cell by RNA interference, we observed no interaction of HIF-2 $\alpha$  with the EGFR mRNA and a drastic reduction in global hypoxic protein synthesis rates by radioactive sulphur incorporation even in the presence of transcription inhibitors. This decrease in overall protein synthesis suggests that HIF-2 $\alpha$  plays a role in global hypoxic translation. Interestingly, reducing the levels of its HIF-1 $\alpha$  or HIF-1 $\beta$  had no effect on hypoxic protein synthesis

rates highlighting the role of HIF-2 $\alpha$  outside of transcription and independent activities from its closely related homolog HIF-1 $\alpha$ . We next set out to investigate how HIF-2 $\alpha$  could associate with mRNA without a classical RNA recognition motif in its protein sequence.

*An interaction with RBM4 allows HIF-2 $\alpha$  to associate with hundreds of mRNAs.*

We performed immunoprecipitations (IPs) of HIF-2 $\alpha$  and HIF-1 $\alpha$  followed by mass spectrometry to identify proteins that preferentially associated with HIF-2 $\alpha$  under hypoxia since we knew that HIF-1 $\alpha$  was not involved in protein synthesis. A protein of interest was identified as RNA binding motif protein 4 (RBM4). RBM4 had been previously shown to be involved in mRNA splicing and translation activation during cellular stress. Consistent with a role in translation, reducing levels of cellular RBM4 prevented HIF-2 $\alpha$  from associating with the EGFR mRNA and repressed global hypoxic protein synthesis rates. To gain a better understanding of which mRNAs, and how many, were being recruited for active translation by HIF-2 $\alpha$  and RBM4, we utilized a technique named photoactivatable ribonucleoside cross-linking and IP (PAR-CLIP). This technique allowed us to specifically isolate the HIF-2 $\alpha$  molecules that interacted with RBM4 and to identify the mRNAs that were associated with this complex. More importantly, PAR-CLIP also reveals which sequences within the mRNAs recruit this complex. We were able to identify hundreds of mRNAs that bound the HIF-2 $\alpha$ /RBM4 complex at specific sequences located in the 3' untranslated region (3' UTR). Many of these mRNAs code for proteins involved in the hypoxic response including a few key cancer hallmarks expressed in the cores of hypoxic tumors such as EGFR, platelet-derived growth factor receptor alpha (PDGFRA), and insulin growth factor 1 receptor (IGF1R). Interestingly, this 3' UTR sequence could be attached to any mRNA (even non-human mRNA) and transform it into a hypoxia-induced molecule. We termed this sequence the RNA Hypoxia Response Element (rHRE). Our next goal was to identify whether this was a cap-dependent

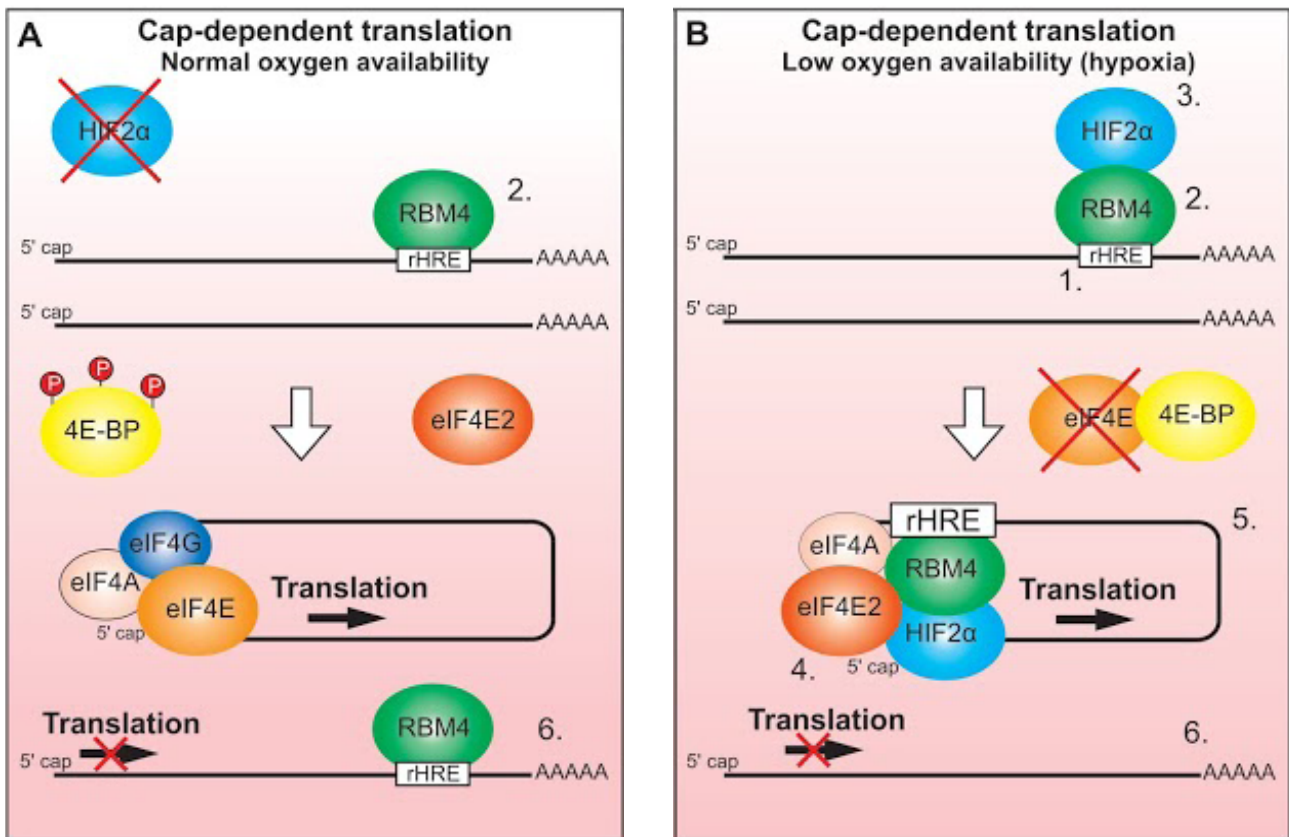


or cap-independent mechanism. A clue lay in our observation that, while mRNAs required rHREs in their 3' UTR to be translated in hypoxia, any 5' UTR sequence could be attached to these mRNAs, but they did require a 5' UTR.

*An alternative cap-binding protein is responsible for a robust cap-dependent translation program that escapes mTOR repression during hypoxia.*

A universal feature of mRNA 5' UTRs is a 5' cap. Therefore we first wanted to test the possibility that cap-dependent translation could occur in the absence of eIF4E. A thorough literature review revealed that only the eIF4E family of proteins could bind the 5' cap. There

are three members of this family: eIF4E, eIF4E2, and eIF4E3. eIF4E is inactive in hypoxia while eIF4E3 expression is restricted to muscle, lung and spleen. eIF4E2 is a good candidate for stress-mediated translation because it is not recognized by the 4E-BPs. We monitored the association of eIF4E2 with HIF-2 $\alpha$  and RBM4 by performing IPs. eIF4E2 associated with HIF-2 $\alpha$  and RBM4 only during hypoxia, and was required for the accumulation of key hypoxia-response proteins such as EGFR, PDGFRA, and IGF1R. Moreover, mRNA targets identified by PAR-CLIP associated with hypoxic polysomes in an eIF4E2-dependent manner. To test whether eIF4E2 allows for HIF-2 $\alpha$  and RBM4



**Figure 1** (Model of normoxic and hypoxic cap-dependent translation). A novel translation initiation complex mediates selective cap-dependent protein synthesis during hypoxia and eIF4E inactivation.

1. The mRNAs containing RNA hypoxia response elements (rHRE) are targeted for translation under hypoxia. PAR-CLIP (Photoactivatable ribonucleoside-enhanced crosslinking-immunoprecipitation) analysis indentified rHREs in the 3'UTR of hundreds of mRNAs.
2. The RNA binding motif protein 4 (RBM4) constitutively associates with rHRE-containing transcripts.
3. Hypoxia inducible factor 2 $\alpha$  (HIF-2 $\alpha$ ) associates with RBM4 in response to low oxygen.
4. The HIF-2 $\alpha$ /RBM4 complex recruits eIF4E2 to rHRE-containing mRNAs.
5. The eIF4E2/HIF-2 $\alpha$ /RBM4 complex binds the 5' cap, targets the mRNAs to polysomes, and initiates translation.
6. The rHRE enables selective expression of the hypoxic cell proteome; other transcripts are not translated.



to bind to the cap, we performed cap-binding assays with beads that have a 5' cap attached, mimicking an mRNA 5' cap. When eIF4E2, but not eIF4E, was depleted from cells, HIF-2 $\alpha$  and RBM4 could no longer bind to the 5' cap-linked beads indicating that eIF4E2 was the cap-binding component that allowed for hypoxic cap-dependent translation to occur. Furthermore, reducing cellular eIF4E2 caused a global reduction in newly synthesized proteins specifically during hypoxia, but not in normal oxygen conditions. A switch between the association of eIF4E and eIF4E2 with polysomes could be observed between normal oxygen conditions and hypoxia. eIF4E was mostly associated with polysomes under normal oxygen conditions, but shifted away from these during hypoxia. Conversely, eIF4E2 was enriched in polysomes only during hypoxia. This switch of eIF4E2 into polysomes was dependent on the presence of HIF-2 $\alpha$  (Fig. 1B).

#### WHAT DOES IT MEAN?

We have identified a translation mechanism that functions independently of eIF4E. This alternative cap-dependent translation mechanism utilizes a related protein to eIF4E (eIF4E2) that escapes global translation repression by hypoxia. This finding furthers our understanding of how cells adapt to low oxygen availability. As mentioned in the introduction, many physiological processes depend on these hypoxic responses. Evidence in the literature suggests the importance of this eIF4E2-directed translation pathway in many of these processes. Mice that lack HIF-2 $\alpha$  or eIF4E2 die during foetal development. Muscles engaged in intense activity require proteins involved in aerobic metabolism, which are mRNA targets of HIF-2 $\alpha$  and RBM4. Populations that live in the mountains of Tibet, altitudes that would be lethal to most people, have acquired a point mutation in the HIF-2 $\alpha$  gene that allows them to thrive at these altitudes. Interestingly, this is the most rapid evidence of natural selection in a human population. It took under 3,000 years since the Tibetan highlanders separated from the lowlanders and migrated up the mountain. The previous record was the appearance of

the lactase gene (the ability to digest milk) in Northern Europeans. Diseases such as stroke may depend on eIF4E2-dependent translation pathway. There is evidence that overexpressing HIF-2 $\alpha$  in stroke patients is neuro-protective. Tumors with elevated HIF-2 $\alpha$  are more aggressive, and eIF4E2 is one of six genes that are strong predictors of metastasis in a variety of cancers.

eIF4E2 is conserved across most eukaryotes. In the fruit fly *Drosophila melanogaster*, eIF4E2 is named 4EHP and participates in the translation regulation of specific mRNAs involved in embryonic development. In the roundworm *Caenorhabditis elegans*, an important part of its life cycle is the burrowing for food deep in the earth where there is a lack of oxygen. In this animal, eIF4E2 is named *ife-4*, and it has been shown to be involved in the selective translation of key mRNAs required for survival. In the fission yeast, *Schizosaccharomyces pombe*, eIF4E2 is required for the adaptation to heat shock, salt stress and glucose starvation. eIF4E2 is a poor competitor for the 5' cap of mRNA in normal conditions, but this changes during hypoxia when eIF4E is inhibited allowing eIF4E2 the opportunity to interact with mRNA. We have identified a protein synthesis pathway that could be important across the animal kingdom for the adaptation to stress, namely low oxygen availability, which is a stress that plagues most multicellular animals.

#### FUTURE DIRECTIONS

Many questions still remain unanswered. How does eIF4E2 activate translation without interacting with a scaffolding protein like the eIF4G that eIF4E interacts with? To address this question, it would be necessary to identify all the proteins that interact with eIF4E2 during hypoxia. Another point that needs to be addressed is how diseases such as cancer utilize hypoxic translation to their advantage. Do cancers require eIF4E2-directed hypoxic translation to progress into large deadly hypoxic tumors that resist standard treatments? Studies should be performed across a variety of tumor-forming cancers to test whether these rely on eIF4E2 en



route to tumor progression. Cancer cells depleted in eIF4E2 could be monitored for their ability to form tumors in a test tube and then in a mouse model, for example. It is exciting times in science when a classical molecular mechanism of cell biology such as protein synthesis still has many new discoveries on the horizon.

#### RESOURCES

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#### Q&A WITH THE AUTHOR

What is your day-to-day work life like as a research scientist in Molecular & Cellular Biology?

As an Assistant Professor in the Department of Molecular and Cellular Biology at the University of Guelph, my day-to-day work life involves managing a group of lab technicians, graduate students and undergraduate research project students. We all have the same goals to better understand how cells adapt to low oxygen availability and how this relates to human health. Many of the ongoing projects in my lab are attempting to answer some of the questions brought up in the Future Directions section of this article. Every lab member has his or her own project and we hold weekly lab meetings to discuss our findings or get advice from the rest of the group.

What is the most fulfilling aspect of working in your research field?

The most fulfilling aspects of working in the fields of molecular biology of human health are the partnerships with industry that can be built to achieve drug development or policy change. Seeing your research put into action and positively affecting human life is what we strive for in our field.

What are the biggest challenges faced by your field of research today?

The biggest challenge facing my field of research is funding. There have been budget cuts across most government and private funding agencies that have had significant impacts on research in the human health field. More than ever, we have competent up-and-coming young scientists and sophisticated technology to perform research, but available money has been the limiting factor in recent history.

What advice would you give to high school and undergraduate students interested in Molecular and Cellular Biology?

I would recommend choosing a major that will allow you to develop your lab skills in molecular and cellular biology techniques. I would suggest speaking with professors about their research and asking about volunteering or research project positions in their labs. It is important to get into a lab early on to see what type of research you enjoy or what field inspires you. Being in a lab early will also give you access to mentors such as graduate students and postdoctoral fellows to guide you in your early career.