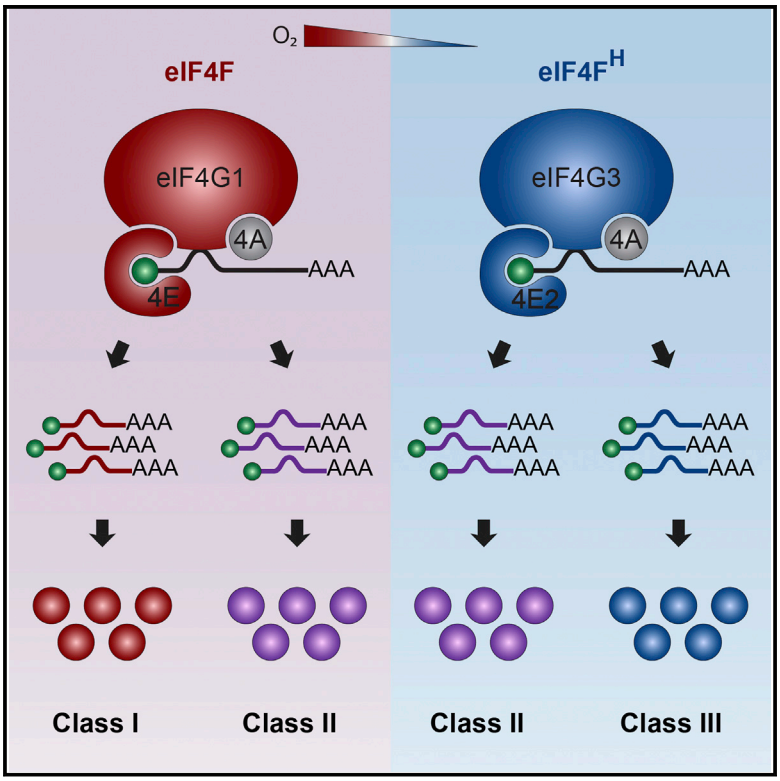
Report

Cell Reports

Systemic Reprogramming of Translation Efficiencies on Oxygen Stimulus

Graphical Abstract



Highlights

- O_2 stimulus reprograms protein output by altering mRNA translation efficiency
- eIF4F^H mediates hypoxic cap-dependent protein synthesis
- eIF4F and eIF4F^H triage mRNAs to generate O_2-responsive translatomes
- Hypoxia-inducible proteins are controlled by translation efficiency, not mRNA levels

Authors

J.J. David Ho, Miling Wang, Timothy E. Audas, ..., Steven Chen, James Uniacke, Stephen Lee

Correspondence

stephenlee@med.miami.edu

In Brief

Ho et al. show that cells rely on a switch in mRNA translation efficiency, and not mRNA levels, to alter protein output on O_2 stimulus. Two distinct cap-dependent protein synthesis machineries mediate this process: the normoxic eIF4F and the hypoxic eIF4F^H. The O_2-regulated eIF4F and eIF4F^H generate complex and adaptive translatomes.

Ho et al., 2016, Cell Reports *14*, 1293–1300
February 16, 2016
http://dx.doi.org/10.1016/j.celrep.2016.01.036

Cell Reports
Report

Systemic Reprogramming of Translation Efficiencies on Oxygen Stimulus

J.J. David Ho,[1,2] Miling Wang,[1,2] Timothy E. Audas,[1,2] Deukwoo Kwon,[2,3] Steven K. Carlsson,[4] Sara Timpano,[5] Sonia L. Evagelou,[5] Shaun Brothers,[4,6] Mark L. Gonzalgo,[2,7] Jonathan R. Krieger,[8] Steven Chen,[2,3,9] James Uniacke,[5,11] and Stephen Lee[1,2,10,11,*]

[1]Department of Biochemistry and Molecular Biology, Miller School of Medicine, University of Miami, Miami, FL 31336, USA
[2]Sylvester Comprehensive Cancer Center, Miller School of Medicine, University of Miami, Miami, FL 31336, USA
[3]Biostatistics and Bioinformatics Core, Miller School of Medicine, University of Miami, Miami, FL 31336, USA
[4]Center for Therapeutic Innovation, Miller School of Medicine, University of Miami, Miami, FL 31336, USA
[5]Department of Molecular and Cellular Biology, College of Biological Science, University of Guelph, Guelph, ON N1G 2W1, Canada
[6]Department of Psychiatry, Miller School of Medicine, University of Miami, Miami, FL 31336, USA
[7]Department of Urology, Miller School of Medicine, University of Miami, Miami, FL 31336, USA
[8]SPARC BioCentre, The Hospital for Sick Children, Toronto, ON M5G 1X8, Canada
[9]Division of Biostatistics, Department of Public Health Sciences, Miller School of Medicine, University of Miami, Miami, FL 31336, USA
[10]Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, Ottawa, ON K1H 8M5, Canada
[11]Co-senior author
*Correspondence: stephenlee@med.miami.edu
http://dx.doi.org/10.1016/j.celrep.2016.01.036

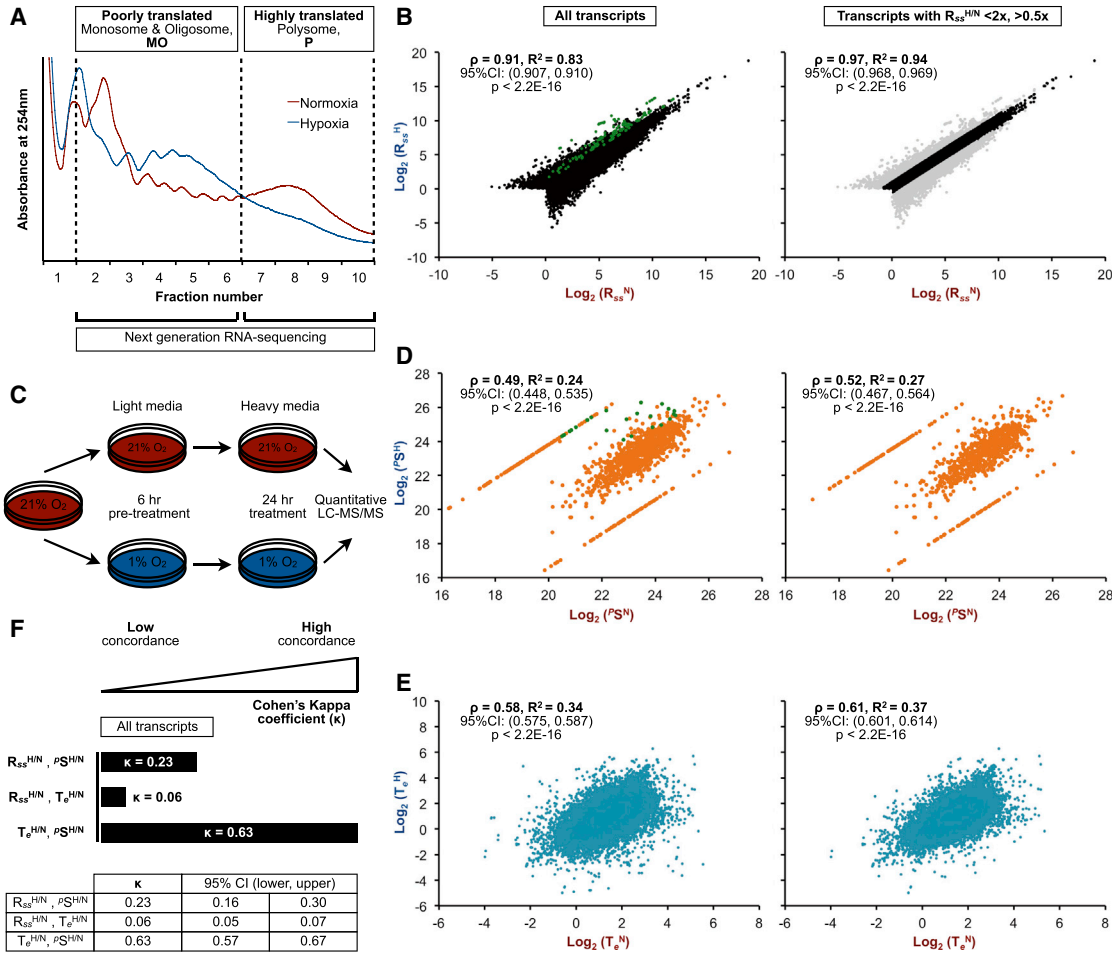
SUMMARY

Protein concentrations evolve under greater evolutionary constraint than mRNA levels. Translation efficiency of mRNA represents the chief determinant of basal protein concentrations. This raises a fundamental question of how mRNA and protein levels are coordinated in dynamic systems responding to physiological stimuli. This report examines the contributions of mRNA abundance and translation efficiency to protein output in cells responding to oxygen stimulus. We show that changes in translation efficiencies, and not mRNA levels, represent the major mechanism governing cellular responses to [O_2] perturbations. Two distinct cap-dependent protein synthesis machineries select mRNAs for translation: the normoxic eIF4F and the hypoxic eIF4F^H. O_2-dependent remodeling of translation efficiencies enables cells to produce adaptive translatomes from preexisting mRNA pools. Differences in mRNA expression observed under different [O_2] are likely neutral, given that they occur during evolution. We propose that mRNAs contain translation efficiency determinants for their triage by the translation apparatus on [O_2] stimulus.

INTRODUCTION

It is assumed that steady-state mRNA levels represent an accurate proxy for protein expression. In most studies, the protein synthesis machinery is perceived as a passive participant in the regulation of gene expression that reflexively translates mRNA abundance into protein output. Recent studies have challenged this assumption by demonstrating a lack of correlation between protein and mRNA levels (Schwanhäusser et al., 2011; Tian et al., 2004; Vogel et al., 2010; Wang et al., 2013). These studies provide strong evidence that translation efficiency (T_e) is a superior predictor of steady-state protein levels compared to mRNA levels, mRNA stability, and protein stability (Schwanhäusser et al., 2011). Interestingly, a comparison of primates established that protein expression evolved under stronger constraints than mRNA levels, the latter being effectively neutral (Khan et al., 2013). These findings point to the evolution of complex regulatory processes of the translation apparatus to titrate protein output from highly divergent levels of cellular mRNAs. A biological role for alternative T_e was recently reported for the transcriptionally silent system of *Drosophila* oocyte-to-embryo transition (Kronja et al., 2014) and in stem cell differentiation (Lu et al., 2009). How mRNA and protein abundance are coordinated in dynamic systems responding to a stimulus remains a fundamental question (Vogel, 2013).

Perturbations in environmental [O_2] are observed in a wide array of physiological and pathological conditions including development, cardiovascular disease and cancer (Ratcliffe, 2013; Semenza, 2014). Cells exposed to hypoxia (i.e., low [O_2]) activate a robust transcription program by the hypoxia-inducible factor (HIF) (Wang et al., 1995). HIF promotes the synthesis of key mRNAs that encode proteins involved in cellular O_2 homeostasis. Hypoxia also elicits a fundamental reorganization of the cellular translation apparatus. In normoxia, the eIF4F complex typically initiates protein synthesis (Sonenberg and Hinnebusch, 2009). The cap-binding eIF4E, the RNA helicase eIF4A, and the scaffold eIF4G constitute the three major components of eIF4F



	κ	95% CI (lower, upper)	
$R_{ss}^{H/N}$, ${}^{p}S^{H/N}$	0.23	0.16	0.30
$R_{ss}^{H/N}$, $T_e^{H/N}$	0.06	0.05	0.07
$T_e^{H/N}$, ${}^{p}S^{H/N}$	0.63	0.57	0.67

Figure 1. O_2-Dependent Remodeling of the Cellular Translatome

(A) RNA-seq was performed on MO and P fractions of normoxic and hypoxic U87MG. MO/P demarcation was selected based on the induction pattern of hypoxic translation of several mRNAs (Uniacke et al., 2012, 2014).

(B) Plots of R_{ss} in hypoxic (R_{ss}^{H}) versus normoxic (R_{ss}^{N}) (left) U87MG (left) and transcripts with R_{ss}^{H}/R_{ss}^{N} ($R_{ss}^{H/N}$) ratios between <2× and >0.5× (right, black). Transcripts with R_{ss} < 1 were excluded from analysis. CI, confidence interval.

(C) Schematic of pSILAC workflow.

(D) Normalized heavy intensities of newly synthesized proteins determined by pSILAC in hypoxic (${}^{p}S^{H}$) and normoxic (${}^{p}S^{N}$) U87MG (left) and from transcripts with $R_{ss}^{H/N}$ ratios between <2× and >0.5× (right). Proteins that were only detected in normoxic (red) and hypoxic (blue) U87MG were given the maximum fold change observed. Canonical hypoxia-inducible genes are highlighted in green.

(E) Plots of T_e in hypoxic (T_e^{H}) versus normoxic (T_e^{N}) U87MG (left), and transcripts with $R_{ss}^{H/N}$ ratios between <2× and >0.5× (right).

(F) Concordance analysis between $R_{ss}^{H/N}$, ${}^{p}S^{H/N}$, and $T_e^{H/N}$.

(Jackson et al., 2010). Hypoxia prevents binding of eIF4E to eIF4G, thereby inhibiting eIF4F activity (Connolly et al., 2006; Koritzinsky et al., 2006; Liu et al., 2006). Hypoxic cells activate an alternative translation pathway that relies on the cap-binding eIF4E2 and the O_2-regulated HIF-2α (Uniacke et al., 2012, 2014). Additional eIF4E-dependent and -independent pathways, such as an internal ribosome entry site (IRES), can be activated during hypoxia (Braunstein et al., 2007; Yi et al., 2013; Young et al., 2008). The profound reorganization of essential cellular pathways by [O_2] provides an ideal system to examine the contributions of the transcription and translation machineries to protein output in response to a physiological stimulus. In this report, we present evidence that an O_2-regulated global remodeling of T_es, rather than changes in transcript abundance, is the principal determinant of protein output to O_2 deprivation.

RESULTS

Widespread Remodeling of the Translatome by O_2

We investigated the role of mRNA expression and T_e in a dynamic system associated with a robust transcription response to stimulus: oxygen tension. First, we isolated transcripts engaged by the protein synthesis machineries of cells maintained in normoxia (21% O_2) or hypoxia (1% O_2, 24 hr) (Figures 1A and S1A). Poorly translated mRNAs accumulate in the monosome and oligosome (MO) fractions, while highly translated

mRNAs are found in polysome (P) fractions (Figures 1A and S1A). Total RNA isolated from the MO and P fractions were subjected to high-throughput RNA sequencing (RNA-seq) (Figure 1A). Cellular RNA steady-state level (R_{ss}) was defined as the total read count (RC) from sequenced fractions: $R_{ss} = P^{RC} + MO^{RC}$. RNA-seq analysis identified approximately 46,500 and 45,000 different transcripts in normoxic (R_{ss}^{N}) and hypoxic (R_{ss}^{H}) cells, respectively (Figures S1B and S1C). R_{ss}^{H} and R_{ss}^{N} displayed a high correlation ($R^2 = 0.83$) (Figures 1B and S1D, left), with more than 77% of mRNAs within a range of 0.5-fold to 2-fold difference (Figures 1B and S1D, right). Targets of the HIF transcription program have high R_{ss}^{H}/R_{ss}^{N} ratios, as expected (Figures 1B and S1D, left, green). To determine protein output, we performed pulse-stable isotope labeling with amino acids in cell culture (pSILAC) analyses (Selbach et al., 2008) (Figure 1C). pSILAC identified more than 1,000 different newly synthesized proteins in normoxic ($^{p}S^{N}$) and hypoxic ($^{p}S^{H}$) cells. ~20% of proteins displayed $^{p}S^{H}/^{p}S^{N}$ ratios of ~1.0, whereas HIF targets exhibited high ratios (Figure S1E), confirming that this assay was capable of distinguishing between proteins with similar and different rates of synthesis. Interestingly, $^{p}S^{H}/^{p}S^{N}$ displayed lower correlation ($R^2 = 0.24$) (Figures 1D and S1F, left) than what would be predicted by R_{ss}^{H}/R_{ss}^{N}. The weak relationship between mRNA levels and protein output suggests that a switch in T_e, rather than changes in transcript levels, may be the primary cellular response to O_2 availability. To explore this possibility, we examined the T_e of mRNAs identified by RNA-seq of MO and P fractions ($T_e = P^{RC}/MO^{RC}$). T_e^{H}/T_e^{N} correlation ($R^2 = 0.34$) (Figures 1E and S1G, left) was in good agreement with $^{p}S^{H}/^{p}S^{N}$. R_{ss}^{H}/R_{ss}^{N} had low concordance with T_e^{H}/T_e^{N}, indicating that polysomal capture of transcripts cannot be simply implied by O_2-regulated changes in steady-state mRNA (Figures 1F and S1H). In contrast to R_{ss}^{H}/R_{ss}^{N}, T_e^{H}/T_e^{N} displayed higher concordance with $^{p}S^{H}/^{p}S^{N}$ (Figure 1F). As the cellular response to O_2 stimulus does encompass changes in mRNA steady-state levels, we measured the relationship between R_{ss}^{H}/R_{ss}^{N}, $^{p}S^{H}/^{p}S^{N}$, and T_e^{H}/T_e^{N} for transcripts displaying minimal variations in expression as a function of $[O_2]$. Transcripts that display a <2-fold difference between hypoxic and normoxic cells (Figures 1B and S1D, right) also produced highly variable protein outputs (Figures 1D and S1F, right) and T_e^{H}/T_e^{N} (Figures 1E and S1G, right) with concordances similar to those observed for the total mRNA population (Figure S1I). These results suggest that changes in $[O_2]$ cause a widespread remodeling of protein output that relies mostly on a systemic switch in T_e and not on mRNA levels.

The eIF4F and eIF4F^{H} Protein Synthesis Machineries Coordinate the O_2-Regulated Translatomes

KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis revealed that hypoxic cells populate essentially the same functional pathways as their normoxic counterparts with different proteins, while prioritizing certain processes over others (Figures S2A–S2C). Silencing elements of the canonical eIF4F translation initiation complex—namely, eIF4E and eIF4G1—prevents the bulk of protein synthesis in normoxic cells (Figures 2A and S2C), indicating that T_e^{N} (Figure S1H, left) and the normoxic translatome (Figure 1D, left) rely on this complex. On the other hand, eIF4E or eIF4G1 silencing had little effect on hypoxic global translation rates, likely because of eIF4F inactivation by low $[O_2]$ (Connolly et al., 2006; Liu et al., 2006) (Figure 2A and S2C). This implies the existence of a broad alternative hypoxic translation initiation complex that sustains T_e^{H}, which we term the hypoxic eIF4F (eIF4F^{H}) (Figure 2B). Pull-down analysis revealed that eIF4F^{H} consists of eIF4E2, eIF4A (Uniacke et al., 2012), and eIF4G3, a functional homolog of eIF4G1 (Figure 2C and S2D). Silencing elements specific to eIF4F^{H} essentially abolished the global rate of translation in hypoxic cells, with little effect on normoxic cells (Figures 2A and S2C). Recruitment of mRNAs to polysomes of hypoxic cells was significantly impaired in eIF4E2-depleted cells (Figure 2D) or eIF4G3-depleted cells (Figure S2E). While this prevented a T_e^{H} analysis in eIF4F^{H}-defective cells, pSILAC revealed that more than 90% of produced proteins observed in hypoxic cells were either not detectable or considerably reduced in eIF4E2-impaired cells (Figure 2E). These results demonstrate the existence of two major cap-dependent protein synthesis pathways (Figure 2B)—the normoxic eIF4F (eIF4E-eIF4A-eIF4G1) and the hypoxic eIF4F^{H} (eIF4E2-eIF4A-eIF4G3)—that remodel T_e in response to O_2 stimulus.

Classification of Three Major mRNA Classes Based on O_2-Dependent T_es

A closer examination of T_e^{H}/T_e^{N} ratios suggests that mRNAs can be divided into three O_2-responsive classes. (Figure 3A, top; Figures S3A and S3B, top). Class I mRNAs are efficiently translated in normoxia but less in hypoxia. Class II mRNAs are efficiently translated independently of $[O_2]$. Class III mRNAs maintain or increase T_e in hypoxia. Overall, classes I, II, and III represent ~25%, ~60%, and ~15% of the combined normoxic and hypoxic translatomes, respectively. The presence and relative size of the three classes were maintained even for mRNAs that exhibited minimal R_{ss}^{H}/R_{ss}^{N} differences (Figures 3B and S3B, bottom; Figure 1B, right). Five representative transcripts from each class were validated by qRT-PCR (Figure S3B). These results raise the intriguing possibility that cells express mRNA populations that are hard-wired for either normoxic eIF4F (classes I and II) or hypoxic eIF4F^{H} (classes II and III) translation. Immunoblot analysis revealed that proteins derived from class I mRNAs, e.g., RBM3 and RPL32, accumulate preferentially under normoxia and are predominantly dependent on eIF4F (Figure 3C). Class II proteins, e.g., RBM5 and MDM4, can be synthesized by eIF4F and eIF4F^{H}, respectively (Figure 3C). Proteins of class III mRNAs, e.g., EGFR (epidermal growth factor receptor) and IGF1R (insulin-like growth factor 1 receptor) accumulate preferentially under hypoxia and are synthesized by eIF4F^{H} (Figure 3C). To confirm the validity of class I–III mRNAs, we tested our model in the renal carcinoma cell line 786-O. The eIF4E2/eIF4F^{H} activator HIF-2α, which is normally degraded in normoxia, is constitutively active in 786-O as a consequence of von Hippel-Lindau (VHL) deficiency (Maxwell et al., 1999). This provides the opportunity to examine eIF4F and eIF4F^{H} operating in parallel within the same normoxic cellular context. Silencing of both eIF4E and eIF4E2 was required to reduce global translation to below 20% of control, confirming that both translation machineries are operative in

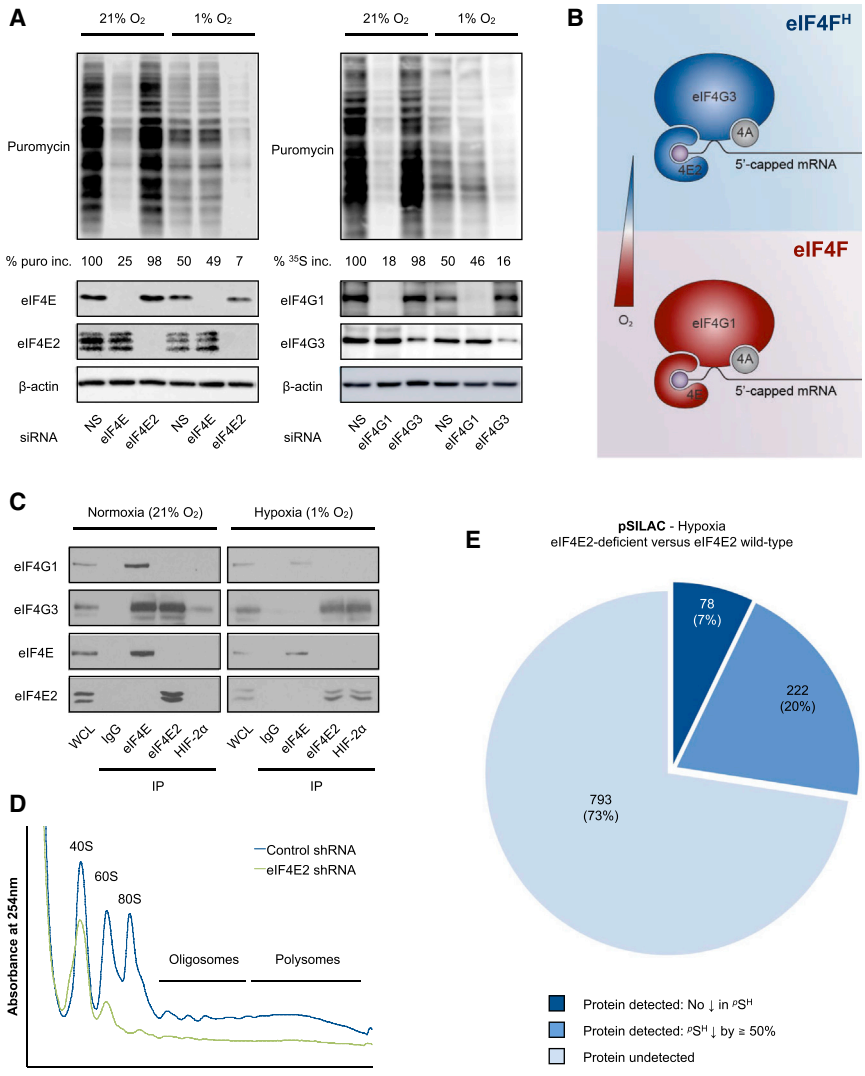


Figure 2. The eIF4F and eIF4F^H Translation Machineries Govern O_2-Dependent Translatome Remodeling

(A) Global translation rates of U87MG transiently transfected with siRNA against the indicated proteins and non-silencing (NS) control siRNA were measured by puromycin incorporation. Loading was performed on an equal cell basis. Immunoblots of silenced proteins are shown. β-actin was used as a loading control. % puro inc., percent puromycin incorporation; % ^{35}S, percent ^{35}S incorporation.

(B) Schematic of eIF4F^H and eIF4F.

(C) Immunoblots of eIF4E, eIF4E2, and HIF-2α endogenous immunoprecipitates in normoxic and hypoxic U87MG. IP, immunoprecipitation; WCL, 5% whole cell lysate; IgG, immunoglobulin G.

(D and E) Polysome profiles (D) and pSILAC analysis (E) of hypoxic U87MG stably expressing eIF4E2-specific or non-silencing control shRNA.

normoxic 786-0 cells (Figure 3D). Protein accumulation of the class-I-mRNA-derived RPL32 occurred in normoxic 786-O in an eIF4E- but not eIF4E2-dependent manner (Figure 3E). Class II protein RBM5 was dependent on both eIF4E and eIF4E2 under normoxic conditions (Figure 3E), as expected. Finally, the class III protein EGFR was sensitive to eIF4E2, but not eIF4E, depletion (Figure 3E). Only class II and III proteins were produced in hypoxic 786-O due to the loss of eIF4F activity. These results suggest the existence of T_e determinants that are hardwired in mRNAs, which provides the basis for differential recruitment by the O_2-regulated protein synthesis machineries eIF4F and eIF4F^H.

T_e Controls Protein Production from HIF Target mRNAs

Hypoxia elicits a robust transcriptional response by HIF, which promotes the synthesis of genes involved in O_2 homeostasis (Schödel et al., 2011; Wang et al., 1995). Exactly 50 canonical transcription targets of the HIF pathway were identified to exhibit an $R_{ss}{}^H/R_{ss}{}^N$ ratio $\geq$ 2 (Figure 1B, left), considerably less than proteins derived from class III mRNAs with minimal change in $R_{ss}{}^H/R_{ss}{}^N$ (Figure 4A). Interestingly, the majority of HIF targets showed decreased $T_e{}^H/T_e{}^N$ (Figures 4B and S4A). Therefore, we suspected that the substantial increase in HIF target proteins in hypoxia (Figure S4B) might be explained by the class III property rather than absolute increases in their respective mRNAs levels (Figure S4C). To test this, we examined the effect of silencing HIF-1β (ARNT), a mandatory subunit of the HIF transcription factor (Wang et al., 1995). HIF target proteins accumulated in HIF-1β-impaired hypoxic cells to levels similar to those observed in their control counterparts (Figure 4C), even though their corresponding mRNAs were not induced upon hypoxia (Figures 4D and S4D). Likewise, cells treated with the general transcription inhibitor actinomycin D (Act. D) exhibited an accumulation of HIF target proteins to levels undistinguishable from those of untreated controls (Figure 4E), even in the absence of their respective mRNA induction (Figure 4F). T_e analysis revealed a substantial increase in $T_e{}^H/T_e{}^N$ of HIF target mRNAs in transcriptionally silent cells, thereby revealing their identity as class III mRNAs (Figure 4G). In agreement with class III mRNA characteristics (Figure 3), HIF target mRNAs are selectively recruited for translation by the eIF4F^H machinery in hypoxic cells, regardless of cellular transcription competency (Figure 4H) and mRNA induction (Figure 4I), as well as in normoxic 786-0 cells, where eIF4F^H and eIF4F are simultaneously active (Figure 4J). These observations can be generalized on a global scale, as protein output is mostly unaffected in transcription-incompetent hypoxic cells while remaining dependent on eIF4E2 activity (Figure 4K). These results demonstrate that T_e, rather than mRNA expression, is the primary determinant of protein levels in dynamic

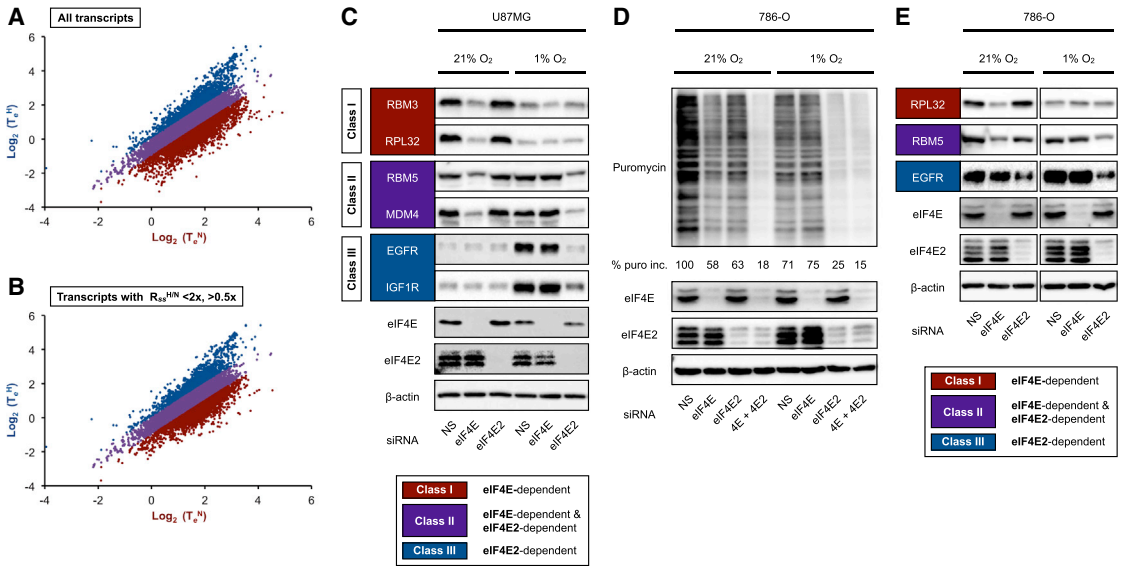


Figure 3. Global Reorganization of T_e during O_2 Deprivation

(A and B) Classification of (A) all transcripts and (B) transcripts with $R_{ss}^{H/N}$ ratios between <2× and >0.5× into three major classes according to T_e^H/T_e^N ratios: class I transcripts ($T_e^H/T_e^N \leq$ 0.5-fold, red); class II transcripts (purple); and class III transcripts ($T_e^H/T_e^N \geq$ 1-fold, blue). Low abundance transcripts (R_{ss} < 10) were excluded from the analysis.

(C) Immunoblots of representative proteins in normoxic and hypoxic U87MG from each class. NS, non-silencing.

(D) Global translation rates in normoxic and hypoxic 786-O transiently transfected with eIF4E-specific, eIF4E2-specific, or NS control siRNA were measured using puromycin incorporation (puro inc.). Immunoblots of silenced proteins are shown.

(E) Immunoblots of representative proteins in normoxic and hypoxic 786-O, as measured in (C).

systems responding to a physiological stimulus, even in the presence of robust transcriptional activity.

DISCUSSION

The demonstration that protein concentration is determined by T_e rather than mRNA abundance (Schwanhäusser et al., 2011) and that changes in mRNA levels are evolutionarily neutral (Khan et al., 2013) represents breakthroughs in our understanding of gene expression. These studies raise the question as to the role of mRNA level changes in response to physiological stimuli (Vogel, 2013). We show that cells reprogram protein output as a function of [O_2] through a systemic switch in mRNA T_es. Two distinct cap-dependent protein synthesis machineries govern this phenomenon: the normoxic eIF4F and the hypoxic eIF4F^H. These two translational programs remodel the cellular translatome by triaging available mRNAs depending on [O_2], with minimal reliance on changes in steady-state transcript levels. Even hypoxia-inducible mRNAs, including HIF targets, are ultimately controlled at the level of T_e and not changes in mRNA levels. We suggest that T_e controls protein output on O_2 stimulus and that changes in mRNA levels may be effectively neutral, as they are during evolution.

We have defined three major classes of O_2-responsive mRNAs. Class I and class III mRNAs are exclusively recruited by eIF4F or eIF4F^H, respectively. Class II mRNAs can be recruited by both and undergo efficient translation regardless of [O_2]. These findings suggest that genes have evolved O_2-regulated T_e determinants that enable their selective recruitment by eIF4F or eIF4F^H. In the case of class III mRNAs, this may be explained, at least in part, by the presence of the RNA hypoxia response elements (rHREs). The rHRE recruits RBM4, which inhibits eIF4F-mediated translation (Lin et al., 2007) but facilitates eIF4F^H-directed hypoxic protein synthesis (Uniacke et al., 2012). It is possible that class I mRNAs encode an RNA element (or elements) that promotes eIF4F activity while opposing eIF4F^H under normoxia. These O_2-regulated T_e determinants enable cells to triage the diverse mRNA populations in order to remodel protein output in response to changes in [O_2]. From a broader perspective, it is tempting to speculate that mRNAs encode an array of T_e determinants that help redefine the translatome on different stimuli. In support of this model, another cap-binding protein, eIF4E3, has been suggested to regulate translation under other settings (Landon et al., 2014). Thus, it is likely that cells have evolved multiple alternative translation initiation machineries that allow them to activate stimulus-specific T_e programs (Andreev et al., 2015; Baudin-Baillieu et al., 2014; Kronja et al., 2014; Lu et al., 2009; McKinney et al., 2014; Ventoso et al., 2012; Young et al., 2008). These alternative machineries would reprogram global mRNA T_es in order to generate distinct, adaptive translatomes/proteomes. Finally, these findings imply that we should revisit the role of transcription-induced changes in mRNA levels in response to stimuli, as we have from the evolutionary perspective. The future challenge will be to decipher the roles of T_e/protein synthesis machineries in fields of research that have been dominated by studies of transcriptional responses to cellular perturbations.

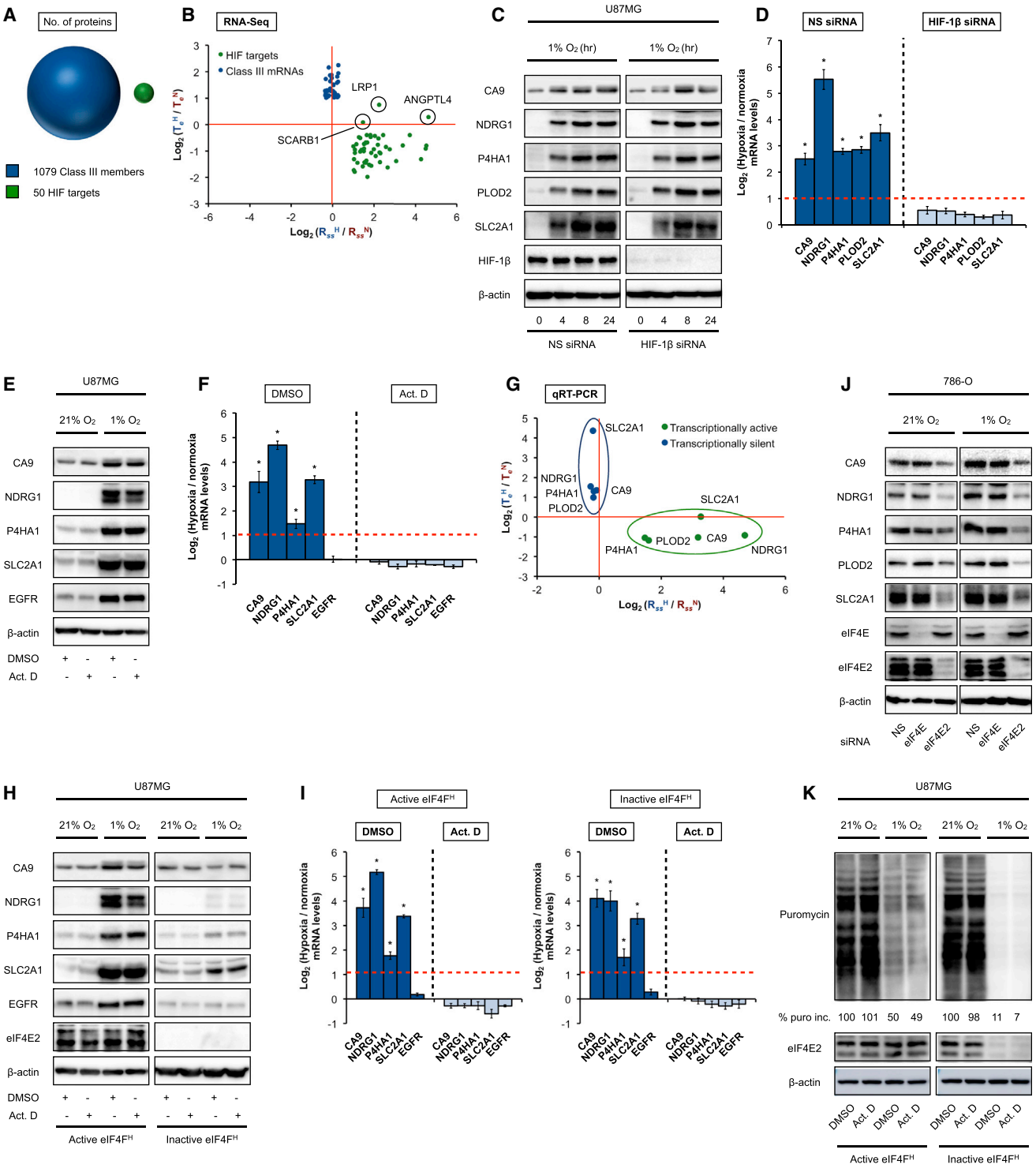


Figure 4. T_e Determines Protein Output in Response to Hypoxia

(A) RNA-seq analysis identified 1,079 different proteins derived from class III mRNAs with $R_{ss}^{H/N}$ ratios between <2× and >0.5× (blue) and 50 proteins derived from HIF target mRNAs, with $R_{ss}^{H/N}$ ratios ≥2 (green) (see Figure 1B).

(B) Plot of change in R_{ss} against change in T_e for 50 HIF target mRNAs in hypoxic versus normoxic U87MG (see Figure 1B). Blue dots indicate representative class III candidates with minimal change in R_{ss} levels.

(C) Immunoblots of HIF target proteins in U87MG transiently transfected with HIF-1β-specific or non-silencing (NS) siRNA and subjected to a hypoxic time course.

(D) Corresponding 24 hr hypoxia/normoxia steady-state mRNA levels of proteins measured in (C). *$p < 0.05$, compared to 0 hr hypoxia.

(legend continued on next page)

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents

U87MG human glioblastoma and 786-O human renal clear cell carcinoma cell lines were obtained from the American Type Culture Collection and propagated as suggested. Cells were maintained at 37°C in a 5% CO_2 humidified incubator. Cells were subjected to hypoxia (1% O_2, 24 hr unless otherwise stated) at 37°C in a 5% CO_2, N_2-balanced, humidified H35 HypOxystation (HypOxygen). Act. D (Amresco) was added to cells at a final concentration of 1 μg/ml.

Polysome Fractionation and RNA-Seq

Polysome fractionations were performed essentially as previously described (Franovic et al., 2007). Total RNA was isolated from individual fractions by standard phenol/chloroform extraction and ethanol precipitation following proteinase K treatment. Equal volumes of individual fractions from four independent experiments were pooled to yield the MO (fractions 2–6) and P (fractions 7–10) samples. cDNA library construction (Ovation RNA-Seq V2, NuGEN), sequencing runs (NextSeq 500, Illumina), and raw data processing were performed by Cofactor Genomics. RNA-seq data are available via the NCBI Sequence Read Archive (SRA) (accession numbers SRP065114 and SRP065127).

pSILAC and Mass Spectrometry

Cells grown in "light" (R_0K_0) media were subjected to 1% O_2 or 21% O_2 pretreatment for 6 hr. Then, we replaced light media with "heavy" ($R_{10}K_8$) media, and cells were left to grow at 1% O_2 or 21% O_2 for 24 hr. Total cellular protein was then harvested, using a 9 M urea lysis buffer, and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. MS data are available via ProteomeXchange (identifier PXD003037).

Global Protein Synthesis Measurements

Global protein synthesis was measured by puromycin (GIBCO, Life Technologies) incorporation (1 μg/ml) for 30 min, followed by immunoblot analysis with an anti-puromycin antibody (Kerafast).

RNA Interference

Small interfering RNA (siRNA) (GE Dharmacon) was transfected at a final concentration of 100 nM using Effectene (QIAGEN). Short hairpin RNA (shRNA) (GE Dharmacon) was stably introduced as previously described (Uniacke et al., 2012).

Statistical Analysis

All experiments were performed at least three independent times, unless otherwise stated. Student's t tests were performed on immunoblot and qRT-PCR measurements (mean ± SEM). Pearson correlation coefficients (ρ) were calculated for RNA-seq and pSILAC analyses. Cohen's kappa coefficients (κ) were calculated to assess concordance between changes in T_e, R_{ss}, and ^{P}S.

Additional details are provided in the Supplemental Experimental Procedures.

ACCESSION NUMBERS

RNA-seq data reported in this paper are available via the NCBI SRA under accession numbers SRA: SRP065114 and SRA: SRP065127. The accession number for the mass spectrometry data reported in this paper is ProteomeXchange: PXD003037.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.01.036.

AUTHOR CONTRIBUTIONS

J.J.D.H., M.W., S.K.C., S.T., S.L.E., J.R.K., and J.U. performed the experiments. J.J.D.H., T.E.A., J.U., and S.L. conceived the experiments. J.J.D.H., M.W., S.B., M.L.G., J.U., and S.L. analyzed the data. D.K. and S.C. performed the statistical analysis. J.J.D.H. and S.L. wrote the paper.

ACKNOWLEDGMENTS

S.L. is funded by the Sylvester Comprehensive Cancer Center and grants from the National Institute of General Medical Sciences (1R01GM115342), the National Cancer Institute (1R01CA200676), and the Canadian Institutes of Health Research (CIHR). J.U. is funded by a grant from the Cancer Research Society. J.J.D.H is a recipient of a CIHR Postdoctoral Fellowship.

Received: July 23, 2015
Revised: October 29, 2015
Accepted: January 8, 2016
Published: February 4, 2016

REFERENCES

Andreev, D.E., O'Connor, P.B., Zhdanov, A.V., Dmitriev, R.I., Shatsky, I.N., Papkovsky, D.B., and Baranov, P.V. (2015). Oxygen and glucose deprivation induces widespread alterations in mRNA translation within 20 minutes. Genome Biol. *16*, 90.

Baudin-Baillieu, A., Legendre, R., Kuchly, C., Hatin, I., Demais, S., Mestdagh, C., Gautheret, D., and Namy, O. (2014). Genome-wide translational changes induced by the prion [PSI+]. Cell Rep. *8*, 439–448.

Braunstein, S., Karpisheva, K., Pola, C., Goldberg, J., Hochman, T., Yee, H., Cangiarella, J., Arju, R., Formenti, S.C., and Schneider, R.J. (2007). A hypoxia-controlled cap-dependent to cap-independent translation switch in breast cancer. Mol. Cell *28*, 501–512.

Connolly, E., Braunstein, S., Formenti, S., and Schneider, R.J. (2006). 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. *26*, 3955–3965.

Franovic, A., Gunaratnam, L., Smith, K., Robert, I., Patten, D., and Lee, S. (2007). Translational up-regulation of the EGFR by tumor hypoxia provides a nonmutational explanation for its overexpression in human cancer. Proc. Natl. Acad. Sci. USA *104*, 13092–13097.

Jackson, R.J., Hellen, C.U., and Pestova, T.V. (2010). The mechanism of eukaryotic translation initiation and principles of its regulation. Nat. Rev. Mol. Cell Biol. *11*, 113–127.

(E) Immunoblots of hypoxia-inducible proteins in U87MG treated with Act. D or DMSO for 20 min, followed by 6 hr of hypoxic or normoxic treatment.
(F) Corresponding hypoxia/normoxia steady-state mRNA levels of proteins measured in (E). *$p < 0.05$, compared to the corresponding normoxia control.
(G) Plot of change in mRNA levels against change in T_e for five representative HIF target mRNAs in transcriptionally silent versus active U87MG under hypoxic versus normoxic conditions.
(H) Immunoblots of HIF target proteins in U87MG stably expressing shRNA targeting eIF4E2 (inactive eIF4F^H) or NS shRNA (active eIF4F^H), and treated with Act. D as in (E).
(I) Corresponding hypoxic induction of steady-state mRNA levels of proteins measured in (H). *$p \leq 0.05$ compared to the corresponding normoxia control.
(J) Immunoblots of HIF target proteins in normoxic and hypoxic 786-O transiently transfected with eIF4E-specific, eIF4E2-specific, or NS siRNA.
(K) Global translation rates in normoxic and hypoxic U87MG transiently transfected with eIF4E2-specific or NS siRNA, and treated with Act. D as in (E), were measured using puromycin incorporation (puro inc.). Immunoblots of silenced proteins are shown.
Data are presented as mean ± SEM.

Khan, Z., Ford, M.J., Cusanovich, D.A., Mitrano, A., Pritchard, J.K., and Gilad, Y. (2013). Primate transcript and protein expression levels evolve under compensatory selection pressures. Science *342*, 1100–1104.

Koritzinsky, M., Magagnin, M.G., van den Beucken, T., Seigneuric, R., Savelkouls, K., Dostie, J., Pyronnet, S., Kaufman, R.J., Weppler, S.A., Voncken, J.W., et al. (2006). Gene expression during acute and prolonged hypoxia is regulated by distinct mechanisms of translational control. EMBO J. *25*, 1114–1125.

Kronja, I., Yuan, B., Eichhorn, S.W., Dzeyk, K., Krijgsveld, J., Bartel, D.P., and Orr-Weaver, T.L. (2014). Widespread changes in the posttranscriptional landscape at the Drosophila oocyte-to-embryo transition. Cell Rep. *7*, 1495–1508.

Landon, A.L., Muniandy, P.A., Shetty, A.C., Lehrmann, E., Volpon, L., Houng, S., Zhang, Y., Dai, B., Peroutka, R., Mazan-Mamczarz, K., et al. (2014). MNKs act as a regulatory switch for eIF4E1 and eIF4E3 driven mRNA translation in DLBCL. Nat. Commun. *5*, 5413.

Lin, J.C., Hsu, M., and Tarn, W.Y. (2007). Cell stress modulates the function of splicing regulatory protein RBM4 in translation control. Proc. Natl. Acad. Sci. USA *104*, 2235–2240.

Liu, L., Cash, T.P., Jones, R.G., Keith, B., Thompson, C.B., and Simon, M.C. (2006). Hypoxia-induced energy stress regulates mRNA translation and cell growth. Mol. Cell *21*, 521–531.

Lu, R., Markowetz, F., Unwin, R.D., Leek, J.T., Airoldi, E.M., MacArthur, B.D., Lachmann, A., Rozov, R., Ma'ayan, A., Boyer, L.A., et al. (2009). Systems-level dynamic analyses of fate change in murine embryonic stem cells. Nature *462*, 358–362.

Maxwell, P.H., Wiesener, M.S., Chang, G.W., Clifford, S.C., Vaux, E.C., Cockman, M.E., Wykoff, C.C., Pugh, C.W., Maher, E.R., and Ratcliffe, P.J. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature *399*, 271–275.

McKinney, C., Zavadil, J., Bianco, C., Shiflett, L., Brown, S., and Mohr, I. (2014). Global reprogramming of the cellular translational landscape facilitates cytomegalovirus replication. Cell Rep. *6*, 9–17.

Ratcliffe, P.J. (2013). Oxygen sensing and hypoxia signalling pathways in animals: the implications of physiology for cancer. J. Physiol. *591*, 2027–2042.

Schödel, J., Oikonomopoulos, S., Ragoussis, J., Pugh, C.W., Ratcliffe, P.J., and Mole, D.R. (2011). High-resolution genome-wide mapping of HIF-binding sites by ChIP-seq. Blood *117*, e207–e217.

Schwanhäusser, B., Busse, D., Li, N., Dittmar, G., Schuchhardt, J., Wolf, J., Chen, W., and Selbach, M. (2011). Global quantification of mammalian gene expression control. Nature *473*, 337–342.

Selbach, M., Schwanhäusser, B., Thierfelder, N., Fang, Z., Khanin, R., and Rajewsky, N. (2008). Widespread changes in protein synthesis induced by microRNAs. Nature *455*, 58–63.

Semenza, G.L. (2014). Oxygen sensing, hypoxia-inducible factors, and disease pathophysiology. Annu. Rev. Pathol. *9*, 47–71.

Sonenberg, N., and Hinnebusch, A.G. (2009). Regulation of translation initiation in eukaryotes: mechanisms and biological targets. Cell *136*, 731–745.

Tian, Q., Stepaniants, S.B., Mao, M., Weng, L., Feetham, M.C., Doyle, M.J., Yi, E.C., Dai, H., Thorsson, V., Eng, J., et al. (2004). Integrated genomic and proteomic analyses of gene expression in mammalian cells. Mol. Cell. Proteomics *3*, 960–969.

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

Uniacke, J., Perera, J.K., Lachance, G., Francisco, C.B., and Lee, S. (2014). Cancer cells exploit eIF4E2-directed synthesis of hypoxia response proteins to drive tumor progression. Cancer Res. *74*, 1379–1389.

Ventoso, I., Kochetov, A., Montaner, D., Dopazo, J., and Santoyo, J. (2012). Extensive translatome remodeling during ER stress response in mammalian cells. PLoS ONE *7*, e35915.

Vogel, C. (2013). Evolution. Protein expression under pressure. Science *342*, 1052–1053.

Vogel, C., Abreu, R. de S., Ko, D., Le, S.Y., Shapiro, B.A., Burns, S.C., Sandhu, D., Boutz, D.R., Marcotte, E.M., and Penalva, L.O. (2010). Sequence signatures and mRNA concentration can explain two-thirds of protein abundance variation in a human cell line. Mol. Syst. Biol. *6*, 400.

Wang, G.L., Jiang, B.H., Rue, E.A., and Semenza, G.L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc. Natl. Acad. Sci. USA *92*, 5510–5514.

Wang, T., Cui, Y., Jin, J., Guo, J., Wang, G., Yin, X., He, Q.Y., and Zhang, G. (2013). Translating mRNAs strongly correlate to proteins in a multivariate manner and their translation ratios are phenotype specific. Nucleic Acids Res. *41*, 4743–4754.

Yi, T., Papadopoulos, E., Hagner, P.R., and Wagner, G. (2013). Hypoxia-inducible factor-1α (HIF-1α) promotes cap-dependent translation of selective mRNAs through up-regulating initiation factor eIF4E1 in breast cancer cells under hypoxia conditions. J. Biol. Chem. *288*, 18732–18742.

Young, R.M., Wang, S.J., Gordan, J.D., Ji, X., Liebhaber, S.A., and Simon, M.C. (2008). Hypoxia-mediated selective mRNA translation by an internal ribosome entry site-independent mechanism. J. Biol. Chem. *283*, 16309–16319.