Systemic Reprogramming of Translation Efficiencies on Oxygen Stimulus

Highlights
- $O_2$ stimulus reprograms protein output by altering mRNA translation efficiency
- $eIF4FH$ mediates hypoxic cap-dependent protein synthesis
- $eIF4F$ and $eIF4FH$ triage mRNAs to generate $O_2$-responsive translatomes
- Hypoxia-inducible proteins are controlled by translation efficiency, not mRNA levels

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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 $eIF4FH$. The $O_2$-regulated $eIF4F$ and $eIF4FH$ generate complex and adaptive translatomes.
Systemic Reprogramming of Translation Efficiencies on Oxygen Stimulus

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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 [O2] perturbations. Two distinct cap-dependent protein synthesis machineries select mRNAs for translation: the normoxic eIF4F and the hypoxic eIF4FH. O2-dependent remodeling of translation efficiencies enables cells to produce adaptive translatomes from preexisting mRNA pools. Differences in mRNA expression observed under different [O2] 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 [O2] 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 (Te) 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 Te 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 [O2] 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 [O2]) 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 O2 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
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 O2-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 [O2] 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 O2-regulated global remodeling of Tₜₛ, rather than changes in transcript abundance, is the principal determinant of protein output to O2 deprivation.

RESULTS

Widespread Remodeling of the Translatome by O2

We investigated the role of mRNA expression and Tₜᵦ 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% O₂) or hypoxia (1% O₂, 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\textsubscript{ss}) was defined as the total read count (RC) from sequenced fractions: R\textsubscript{ss} = P\textsubscript{RC} + MO\textsubscript{RC}. RNA-seq analysis identified approximately 46,500 and 45,000 different transcripts in normoxic (R\textsubscript{ss} \textsubscript{N}) and hypoxic (R\textsubscript{ss} \textsubscript{H}) cells, respectively (Figures S1B and S1C). R\textsubscript{ss} \textsubscript{N} and R\textsubscript{ss} \textsubscript{H} displayed a high correlation (R\textsuperscript{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\textsubscript{ss} \textsubscript{H}/R\textsubscript{ss} \textsubscript{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 (\textsuperscript{14}S\textsuperscript{N}) and hypoxic (\textsuperscript{14}S\textsuperscript{H}) cells. ~20% of proteins displayed \textsuperscript{14}S\textsuperscript{H}/\textsuperscript{14}S\textsuperscript{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, \textsuperscript{14}S\textsuperscript{H}/\textsuperscript{14}S\textsuperscript{N} displayed lower correlation (R\textsuperscript{2} = 0.24) (Figures 1D and S1F, left) than what would be predicted by R\textsubscript{ss} \textsubscript{H}/R\textsubscript{ss} \textsubscript{N}. The weak relationship between mRNA levels and protein output suggests that a switch in T\textsubscript{e} rather than changes in transcript levels, may be the primary cellular response to O\textsubscript{2} availability. To explore this possibility, we examined the T\textsubscript{e} of mRNAs identified by RNA-seq of MO and P fractions (T\textsubscript{e} = P\textsubscript{RC}/MO\textsubscript{RC}). T\textsubscript{e} \textsubscript{H}/T\textsubscript{e} \textsubscript{N} correlation (R\textsuperscript{2} = 0.34) (Figures 1E and S1G, left) was in good agreement with \textsuperscript{14}S\textsuperscript{H}/\textsuperscript{14}S\textsuperscript{N}. R\textsubscript{ss} \textsubscript{H}/R\textsubscript{ss} \textsubscript{N} had low concordance with T\textsubscript{e} \textsubscript{H}/T\textsubscript{e} \textsubscript{N}, indicating that polysome capture of transcripts cannot be simply implied by O\textsubscript{2}-regulated changes in steady-state mRNA (Figures 1F and S1H). In contrast to R\textsubscript{ss} \textsubscript{H}/R\textsubscript{ss} \textsubscript{N}, T\textsubscript{e} \textsubscript{H}/T\textsubscript{e} \textsubscript{N} displayed higher concordance with \textsuperscript{14}S\textsuperscript{H}/\textsuperscript{14}S\textsuperscript{N} (Figure 1F). As the cellular response to O\textsubscript{2} stimulus does encompass changes in mRNA steady-state levels, we measured the relationship between R\textsubscript{ss} \textsubscript{H}/R\textsubscript{ss} \textsubscript{N}, \textsuperscript{14}S\textsuperscript{H}/\textsuperscript{14}S\textsuperscript{N}, and T\textsubscript{e} \textsubscript{H}/T\textsubscript{e} \textsubscript{N} for transcripts displaying minimal variations in expression as a function of [O\textsubscript{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\textsubscript{e} \textsubscript{H}/T\textsubscript{e} \textsubscript{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\textsubscript{2}] cause a widespread remodeling of protein output that relies mostly on a systemic switch in T\textsubscript{e} and not on mRNA levels.

The eIF4F and eIF4FH Protein Synthesis Machineries Coordinate the O\textsubscript{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\textsubscript{e} \textsubscript{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\textsubscript{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\textsubscript{e} \textsubscript{H}, which we term the hypoxic eIF4F (eIF4FH) (Figure 2B). Pull-down analysis revealed that eIF4FH consists of eIF4E2, eIF4A (Unlacke et al., 2012), and eIF4G3, a functional homolog of eIF4G1 (Figure 2C and S2D). Silencing elements specific to eIF4FH 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 2E). While this prevented a T\textsubscript{e} \textsubscript{H} analysis in eIF4FH-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 eIF4FH (eIF4E2-eIF4A-eIF4G3)—that remodel T\textsubscript{e} in response to O\textsubscript{2} stimulus.

Classification of Three Major mRNA Classes Based on O\textsubscript{2}-Dependent T\textsubscript{e}s

A closer examination of T\textsubscript{e} \textsubscript{H}/T\textsubscript{e} \textsubscript{N} ratios suggests that mRNAs can be divided into three O\textsubscript{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\textsubscript{2}]. Class III mRNAs maintain or increase T\textsubscript{e} in hypoxia. Overall, classes I, II, and III represent ~25%, ~60%, and ~15% of the combined normoxic and hypoxic translomes, respectively. The presence and relative size of the three classes were maintained even for mRNAs that exhibited minimal R\textsubscript{ss} \textsubscript{H}/R\textsubscript{ss} \textsubscript{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 eIF4FH (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 eIF4FH, 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 eIF4FH (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/eIF4FH activator HIF-2\textalpha, 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 eIF4FH 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
normoxic 786-O 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 Te determinants that are hard-wired in mRNAs, which provides the basis for differential recruitment by the O2-regulated protein synthesis machineries eIF4F and eIF4FH.

**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 O2 homeostasis (Schödel et al., 2011; Wang et al., 1995). Exactly 50 canonical transcription targets of the HIF pathway were identified to exhibit an Rs H/RsN ratio ≥ 2 (Figure 1B, left), considerably less than proteins derived from class III mRNAs with minimal change in Rs H/RsN (Figure 4A). Interestingly, the majority of HIF targets showed decreased Te H/TeN (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). Te analysis revealed a substantial increase in TeH/TeN 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 eIF4FH machinery in hypoxic cells, regardless of cellular transcription competency (Figure 4H) and mRNA induction (Figure 4I), as well as in normoxic 786-O cells, where eIF4FH and eIF4E 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 Te, rather than mRNA expression, is the primary determinant of protein levels in dynamic
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 $T_e$ s. Two distinct cap-dependent protein synthesis machineries govern this phenomenon: the normoxic eIF4F and the hypoxic eIF4FH. These two translational programs remodel the cellular translome 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 elf4F or elf4F$^H$, 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 elf4F or elf4F$^H$. 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 elf4F-mediated translation (Lin et al., 2007) but facilitates elf4FH-directed hypoxic protein synthesis (Uniacke et al., 2012). It is possible that class I mRNAs encode an RNA element (or elements) that promotes elf4F activity while opposing elf4FH 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 translome on different stimuli. In support of this model, another cap-binding protein, elf4E3, 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 $T_e$s in order to generate distinct, adaptive translomes/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 \( R_{\text{H/N}} \) ratios between <2 and >0.5 (blue) and 50 proteins derived from HIF target mRNAs, with \( R_{\text{H/N}} \) ratios ≥2 (green) (see Figure 1B).

(B) Plot of change in \( R_{\text{H/N}} \) 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 \( R_{\text{H/N}} \) 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. * \( p < 0.05 \), compared to 0 hr hypoxia.

(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% CO2 humidified incubator. Cells were subjected to hypoxia (1% O2, 24 hr unless otherwise stated) at 37 °C in a 5% CO2, N2−balanced, humidified H35 HypOxystation (HypOxygen). Act. D (Arnesco) was added to cells at a final concentration of 1 μg/ml.

Polysome Fractionation and RNA-Seq
Polysome fractions 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” (R0K0) media were subjected to 1% O2 or 21% O2 pretreatment for 6 hr. Then, we replaced light media with “heavy” (R10K8) media, and cells were left to grow at 1% O2 or 21% O2 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 (Unlacek 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 (r) were calculated for RNA-seq and pSILAC analyses. Cohen’s kappa coefficients (κ) were calculated to assess concordance between changes in Tm, Rm, and fS.

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

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