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14. ABSTRACT: Inactivating mutations in the TSC1 and TSC2 tumor suppressor genes lead to the disease tuberous sclerosis complex (TSC). The TSC1/TSC2 complex integrates multiple cues to regulate protein translation and cell growth via mammalian target of rapamycin complex 1 (mTORC1). Loss of TSC functions leads to constitutive activation of mTORC1 and uncontrolled mRNA translation. In recently published data, we discovered that TSC2-deficient cells have increased protein synthesis but with reduced protein quality, leading us to hypothesize that disrupted protein homeostasis contributes to TSC pathophysiology. Consistent with this hypothesis, in unpublished data, we have found prevailing alternative translation that re-shapes proteome landscape. Our results suggest that translational re-programming can be targeted for therapeutic strategies.					
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1. INTRODUCTION

Tuberous sclerosis complex (TSC) is an autosomal dominant disease characterized by benign tumors in various tissues. The genes mutated in this disease, TSC1 and TSC2, encode tumor suppressors that are associated in a complex. The TSC1/2 complex, through its Rheb-GAP activity, is a critical negative regulator of mTORC1 under physiological conditions. Activation of mTORC1 positively stimulates cap-dependent mRNA translation via its downstream substrates S6K and 4E-BP. In our previous study, we demonstrated that TSC-mTORC1 signaling regulates the balance between cap-dependent and cap-independent translation. In this project, we aim to (1) Dissect the molecular linkage between mTORC1 and protein homeostasis; (2) Define the role of mTORC1 in ribosome dynamics and translational re-programming.

2. KEYWORDS

Tuberous sclerosis complex; mTORC1; rapamycin; mRNA; translation; ribosome; quality control; alternative translation

3. ACCOMPLISHMENTS

- **What were the major goals of the project?**

Task 1. Dissect the Molecular Linkage between mTORC1 and Protein Homeostasis

Task 2. Define the Role of mTORC1 in Ribosome Dynamics and Translational Re-programming

- **What was accomplished under these goals?**

Task 1. Dissect the Molecular Linkage between mTORC1 and Protein Homeostasis

1a. Confirm the negative role of mTORC1 signaling in the quality of newly synthesized polypeptides

The discovery that rapamycin extends the lifespan of diverse organisms has triggered many studies aimed at identifying the underlying molecular mechanisms. It has been suggested that the mammalian target of rapamycin complex 1 (mTORC1) regulates growth and aging by controlling mRNA translation. However, how a decrease in protein synthesis can extend lifespan remains an unresolved issue. Here we showed that constitutively active mTORC1 signaling increased general protein synthesis but unexpectedly reduced the quality of newly synthesized polypeptides. We demonstrated that unrestrained mTORC1 decreased translation fidelity by altering ribosome dynamics during elongation. Conversely, rapamycin treatment restored the quality of newly synthesized polypeptides mainly by slowing down ribosomal elongation. We also found distinct roles for mTORC1 downstream targets in maintaining protein homeostasis. Loss of S6 kinases, but not 4E-BP family proteins, attenuated the effects of rapamycin on the quality of translational products. Our results reveal a mechanistic connection between mTORC1 and protein homeostasis, highlighting the central role of nutrient signaling in TSC pathology.

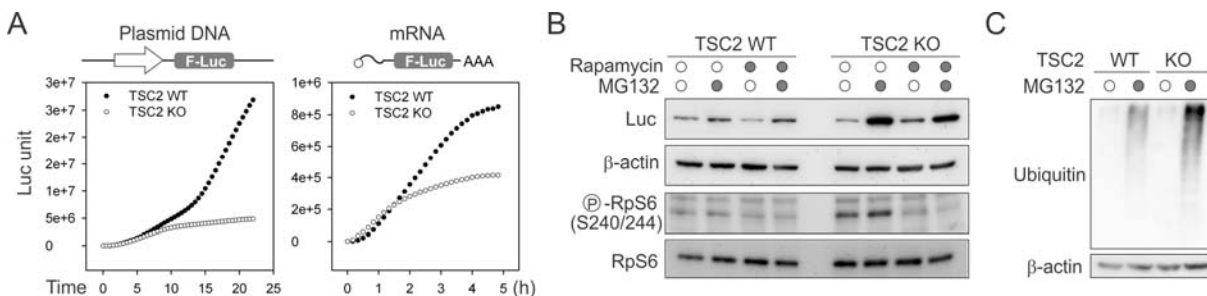


Fig 1. Hyperactive mTORC1 signaling reduces the quality of newly synthesized proteins. **(A)** TSC2 WT and TSC2 KO cells were transfected with either Luc plasmid or mRNA. Real time Luc activity was recorded immediately after transfection. **(B)** Luc-transfected TSC2 WT and TSC2 KO cells were treated with MG132 or DMSO followed by immunoblotting analysis using antibodies indicated. **(C)** Whole cell lysates of TSC2 WT and TSC2 KO cells were immunoblotted to detect ubiquitin-conjugated species.

1b. Define the role of mTORC1 downstream targets in the quality of newly synthesized polypeptides

Translation fidelity can be thought of as a competition between the cognate and near-cognate tRNAs for a given codon. It is conceivable that the increased translation speed under

hyperactive mTORC1 signaling generates more error proteins via misreading of genetic codons. We analyzed two different aspects of translation fidelity: nonsense suppression and aa-tRNA selection. To evaluate nonsense suppression we constructed a pGL3-Luc vector with a stop codon at the coding region of Luc (Fig. 2). To evaluate the aa-tRNA selection we made a vector in which the AGA codon of Luc at aa218 was mutated to the near-cognate AGC codon. Measuring the Luc activity level of R218A mutant allows us to evaluate the rate of amino acid misincorporation at this position. As expected, both Fluc(Stop) and Fluc(R218A) mutants showed less than 1% of enzymatic activities of the wild type Fluc. Despite such low expression levels, TSC2 KO cells showed an increase in Fluc activity for both Fluc(Stop) and Fluc(R218A) when compared to the wild type cells (2 fold and 1.5 fold increase, respectively). These results suggest that the ribosomes in cells with hyperactive mTORC1 signaling have a higher rate of altered aa-tRNA selection.

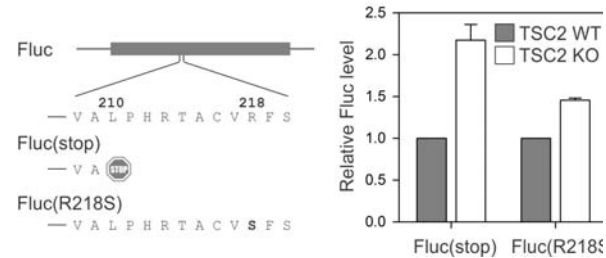


Fig 2. TSC2 WT and TSC2 KO cells were transfected with plasmids encoding Fluc mutants shown in the left panel. After 24 hr of transfection, cells were treated with 20nM rapamycin for 15 hr followed by measurement of Fluc activities (right panel). Relative Fluc activities were normalized using wild type Fluc (mean $\pm$ SEM; n=3).

1c. Determine the ribosome elongation speed controlled by mTORC1

A reliable measure of the translation of cellular mRNA is the degree of its association with ribosomes. Ribosome profiling, based on deep sequencing of ribosome-protected mRNA fragments (RPFs), has proven to be powerful in defining ribosome positions across the entire transcriptome. To investigate the ribosome dynamics during mRNA translation, we have established a modified ribosome profiling technique (Ribo-seq) adapted from the previously published protocol (Fig. 3A). Direct comparison of RPFs from both monosome and polysome fractions afforded us an opportunity to dissect the transition between initiation and elongation.

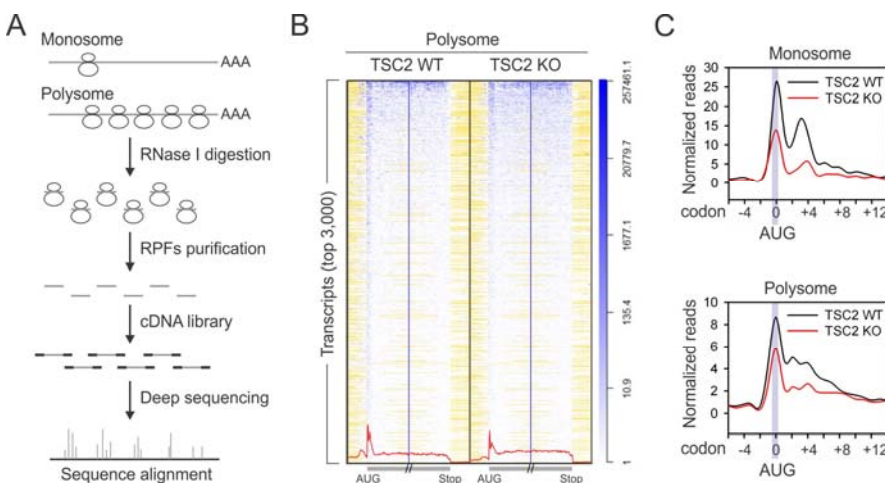


Fig. 3 Visualization of ribosome dynamics at codon resolution. **(A)** Schematic for genome-wide ribosome foot-printing technology. **(B)** Heatmap of RPF density over the entire transcriptome of TSC2 WT and TSC2 KO cells. **(C)** Normalized RPF density over the entire length of whole transcriptome from monosome (top panel) and polysome (bottom panel).

We built a heat map of RPF density over the entire transcriptome for both TSC2 WT and TSC2 KO cells (Fig. 3B). In addition to the clear enrichment of RPFs at the start of transcripts, a significant portion of footprints were also located at several downstream codons, with another prominent peak of reads positioned at the +4 codon position (Fig. 3C). These results strongly suggest the existence of a post-initiation pausing after the assembly of 80S ribosome at the

initiator codon. Interestingly, TSC2 KO cells showed a substantial decrease of RPF density around the initiation codon as compared to the wild type (Fig. 3B, 3C). In particular, the second pause peak at the +4 codon position was nearly diminished in cells lacking TSC2 (Fig. 3C). Ribosome pausing has long been attributed to the presence of specific sequence features, such as rare codons or RNA secondary structures. Our results suggest that the ribosome pausing could also be subject to regulation by nutrient signaling. It appears that mTORC1 not only promotes the ribosome loading via cap-dependent mechanism, but also reduces the initiation pausing of 80S ribosomes, resulting in a faster transition between initiation and elongation.

1d. Does mTORC1 signaling affect the integrity of ribosome?

An important prerequisite for the regulation of global protein synthesis is the continuous supply of translation machinery. The fact that mTORC1 signaling controls ribosome biogenesis at many levels underscores the involvement of mTOR in linking nutrient availability to protein synthesis. However, it is not clear why higher translation capacity jeopardizes the translation fidelity. We observed in TSC2 KO cells the formation of ribosomal half-mers that are visible by a shoulder in the polysome peaks (Fig. 4). Ribosomal half-mers represent ribosome species with an uncomplexed 40S portion. Such species are frequently observed in cells with defects in ribosome biogenesis, causing an impaired balance of 60S and 40S ribosomal subunits. The presence of ribosomal half-mers in cells with hyperactive mTORC1 signaling is consistent with the theory of error catastrophe, i.e., accumulated errors in proteins which themselves function in protein synthesis (such as ribosomal proteins) cause additional errors in translational products. This feedback loop points toward an inescapable decay of translational fidelity. Notably, treating TSC2 KO cells with rapamycin largely suppressed the formation of ribosomal half-mers (Fig. 4). Therefore, reducing mTORC1 signaling improves the ribosome assembly, which may contribute to the increased translation accuracy.

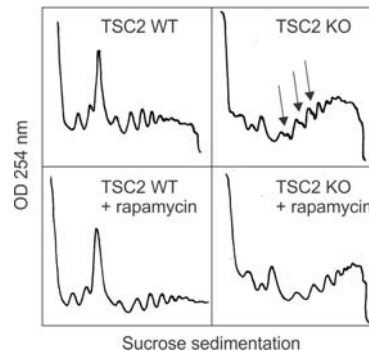


Fig 4. Ribosome profiling of TSC2 WT and TSC2 KO cells in the absence (top panel) and presence of 20 nM rapamycin (bottom panel). Arrow indicates the ribosomal halfmer.

1e. What happens to the ubiquitin/proteasome pathway?

We have established a HEK293 cell line stably expressing a proteasome reporter pZsProSensor-1, which encodes the gene for ZsGreen fused to the mouse ornithine decarboxylase (MODC) degradation domain (amino acids 410-461). In parallel, we evaluated the proteasome function using two different in vitro assays. First, the chymotrypsin-like peptidase activity of 26S proteasome will be assessed using the fluorogenic substrate Suc-LLVY-AMC. Second, the functionality of 26S proteasome will be tested using a well-characterized natural substrate, cyclin B containing the destruction box necessary for its proper ubiquitination (cyclin N100). Despite much efforts, we found little difference in the ubiquitin/proteasome pathway under altered mTORC1 signaling. These results suggest a robust adaptation mechanism for cells in response to differential nutrient signaling.

Task 2. Define the Role of mTORC1 in Ribosome Dynamics and Translational Re-programming

2a. Define molecular determinants of post-initiation pausing.

Eukaryotic translation initiation typically begins with the recruitment of a 40S ribosome subunit to the 5' untranslated region (5'UTR) of an mRNA via the cap-recognition complex eIF4F. The ribosome then scans along the 5'UTR until an initiation codon is encountered. Successful engagement of the scanning ribosome to a start codon is marked by the joining of a 60S subunit. It is generally believed that, once the 80S ribosome is assembled at the start codon, elongation proceeds to begin polypeptide synthesis. Very little is known about the ribosome dynamics at start codons prior to the commitment of elongation. Using genome-wide quantitative profiling of initiating ribosomes (QTI-seq) developed in my laboratory, we characterized the behavior of ribosomes at the start codon.

Unlike regular ribosome profiling that measures the density of ribosome footprints across the entire coding region (CDS) [8, 9], QTI-seq captures initiating ribosomes in a quantitative manner. As such, it permits global mapping of translation initiation sites (TIS) and measurement of the ribosome density at individual start codons (**Fig. 5A**). For certain transcripts, however, QTI-seq does not capture the initiating ribosomes effectively, despite the fact that some of these messages are abundant and well-translated. As exemplified by *EEF1G* and *PARK7* (**Fig. 5B**), the wide range of ribosome density at the annotated initiation sites (aTIS) suggests a previously unappreciated phenomenon: the 80S ribosome “lingers” at the start codon prior to productive elongation. To measure how long an initiating ribosome resides at the start codon before the elongation commitment, we computed aTIS pausing index (PI) that represents, on a relative scale, the dwell time of initiating ribosomes at individual start codons. It is conceivable that a strong initiation pausing limits the translational output by acting as a barrier for productive elongation. Supporting this notion, a negative correlation is evident between the aTIS PI and the CDS ribosome occupancy ($R = -0.22$) (**Fig. 5C**).

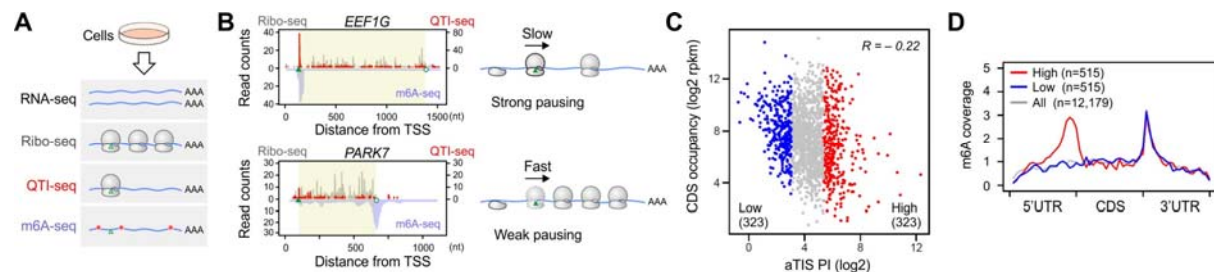


Fig. 5. (A) Schematic of RNA-seq, Ribo-seq, QTI-seq, and m⁶A-seq. (B) Examples of transcripts with high (*EEF1G*) and low (*PARK7*) values of aTIS PI. Color-coded are reads from Ribo-seq (grey), QTI-seq (red), and m⁶A-seq (light blue). (C) Scatter plot shows the negative correlation between the aTIS PI and the CDS ribosome occupancy of individual transcripts. (D) Metagene profile of m⁶A distribution across transcripts stratified based on aTIS PI values.

We next searched for common biological themes among transcripts with relatively high aTIS PI using gene ontology (GO) analysis. Genes involved in cell growth and proliferation, such as translation and DNA packaging, were overrepresented among mRNAs exhibiting strong initiation pausing. By contrast, genes responsible for RNA processing were enriched in the group with low aTIS PI. An immediate outstanding question is what, if any, sequence features could contribute to varied ribosome pausing during the transition from initiation to elongation. However, structural features in the vicinity of the start codon minimally contribute to the pausing of initiating ribosomes (data not shown). Therefore, additional regulatory factors must be in place to influence the behavior of initiating ribosomes at the start codon.

2b. Determine the principles underlying alternative TIS selection

Proper selection of the translation initiation site (TIS) is crucial for the production of desired proteins. It is commonly assumed that the first AUG codon that the scanning ribosome encounters serves as the start site for translation. However, one or more potential TIS positions could exist upstream or downstream of the main start codon. Recent ribosome profiling studies, including ours, have uncovered a surprising variety of translation initiation sites across the transcriptome. Intriguingly, many non-AUG triplets have been reported to act as alternative start codons for translation initiation. A great caution must be taken when interpreting profiling results because ribosome binding to mRNA does not necessarily represent true translation. Although proteomic approaches using mass spectrometry have identified some of these non-canonical translational products, *the frequency and efficiency of non-canonical initiation have remained open to much speculation.*

The molecular mechanism responsible for the choice of alternative start codons is complex. The stringency of TIS selection can be influenced by the start codon identity (AUG vs. non-AUG) and the sequence context surrounding the start codon. The presence of mRNA secondary structures or modifications at or near the start codon also affects the recognition efficiency. Additionally, the fidelity of TIS selection can be affected by altered levels of initiation factors like eIF1 and eIF5. Despite the crucial role of these *cis*-elements and *trans*-factors in TIS selection, *the fundamental principles of start codon recognition remains incompletely understood.*

To systematically determine the optimal TIS sequences inside cells, we developed a non-biased high-throughput approach called TIS-Selex (Fig. 6A). Unlike ribosome profiling that uses the ribosome occupancy as a proxy for protein production rate, TIS-Selex measures real translational products. We fused a library of randomized 12 nt sequence to a reporter encoding the tracer peptide SIINFEKL. Depending on the optimality of TIS sites nested in the random sequence, the tracer peptide is translated at different levels. The peptides are subsequently processed and loaded onto major histocompatibility complex class I (MHC I) K^b molecules on the cell surface. By using fluorescence-activated cell sorting (FACS), the amount of tracer peptides can be measured at single cell levels by a monoclonal antibody 25D1 [34]. As an additional control, we inserted a GFP coding sequence downstream of the tracer peptide. When a TIS upstream of the tracer peptide is suboptimal, leaky scanning occurs to allow GFP translation. Thus, the TIS optimality is positively correlated with the 25D1 signal, but negatively correlated with GFP levels (Fig. 6B).

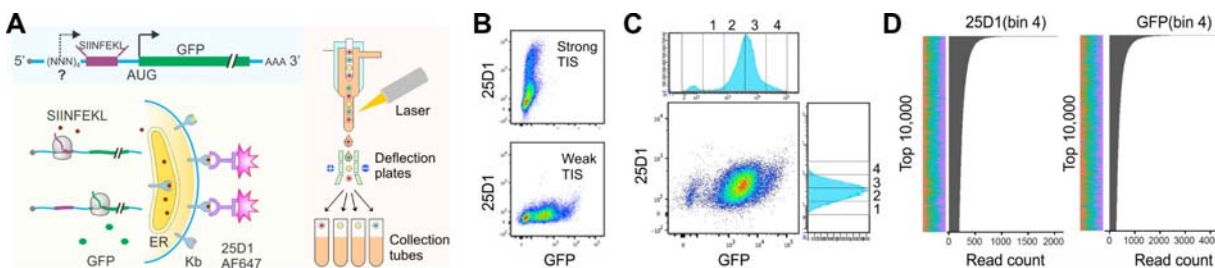


Fig. 6. (A) Schematic of TIS-Selex to identify optimal TIS sequences. A pool of transcripts encoding the tracer peptide are transfected into HEK293-Kb cells. Following 25D1 staining, cells are subjected to FACS. **(B)** Flow cytometry of examples of tracer reporters bearing strong or weak context for AUG start codon. **(C)** Enrichment of optimal TIS sequences by cell sorting and random sequence amplification. **(D)** Top optimal TIS sequences identified by TIS-Selex.

To enrich sequences with different TIS optimality, we sorted the resulting pools of transfected cells into 5 bins with FACS on the basis of 25D1 signals and GFP levels (Fig. 6C). Next, we

used deep sequencing to compute an optimality score for each random sequence based on the ratio of 25D1/GFP (**Fig. 6D**). To independently validate these results, we amplified the random sequence from the sorted cells with high 25D1 signals. Sequences with high TIS optimality can be further enriched in an exponential manner via sequential amplification. In parallel, QTI-seq will be conducted to determine the start codon identity and the sequence context.

From our preliminary results, TIS-Selex proved to be a sensitive and robust system to identify optimal TIS sequences recognized by the scanning ribosomes under the normal growth condition (**Fig. 6D**). We will apply TIS-Selex to cells under different growth conditions such as amino acid starvation to evaluate the altered stringency of start codon selection. Additionally, TIS-Selex has the potential to assess contributions of eukaryotic initiation factors (eIFs) to the initiation fidelity. For instance, eIF1 has been shown to orchestrate alternative translation by stabilizing “open” conformation of the scanning ribosome, thereby preventing the selection of suboptimal start codons [35]. We have established a stable HEK293-K^b cell line with eIF1 knockdown. In comparison to the scramble control cells, TIS-Selex is expected to elucidate the global TIS fidelity influenced by eIF1. Finally, certain ribosomal proteins have been reported to contribute to the stringency of start codon selection and their mutations have been linked to the pathogenesis of Diamond-Blackfan anemia. We have created a lentiviral RNAi library targeting individual ribosomal proteins. We anticipate that the combination of TIS-Selex and QTI-seq yields timely and much-needed information on how initiation fidelity is maintained and regulated inside cells.

2c. Examine cross-species conservation of alternative TIS codons

To analyze conservation of individual alternative TIS position on each transcript, we chose a total of 12,949 human-mouse orthologous mRNA pairs. We analyzed separately the 5'UTR and CDS regions in order to measure the conservation of uTIS and dTIS positions, respectively (**Fig. 7A**). Each group was classified into two subgroups based on their sequence similarity. For genes with high sequence similarity, 85% of the uTIS and 60% of dTIS positions were conserved between human and mouse cells. Some of these alternative TIS codons were located at the same positions on the aligned sequences. As an example, RNF10 in HEK293 cells showed three uTIS positions, which were also found in MEF cells at the identical positions on the aligned 5'UTR sequence of the mouse homolog (**Fig. 7B**). Remarkably, genes with low sequence similarity also displayed high TIS conservation across the two species (**Fig. 7A**). For instance, the 5'UTR of CTTN gene has low sequence identity between human and mouse homologs (alignment score = 40.3) (**Fig. 7C**). However, a clear uTIS was identified in both cells at the same position on the aligned region. Notably, the majority of alternative ORFs conserved between human and mouse cells were of the same type, i.e., either separated from or overlapped with the main ORF (**Fig. 7A**). The evolutionary conservation of those TIS positions

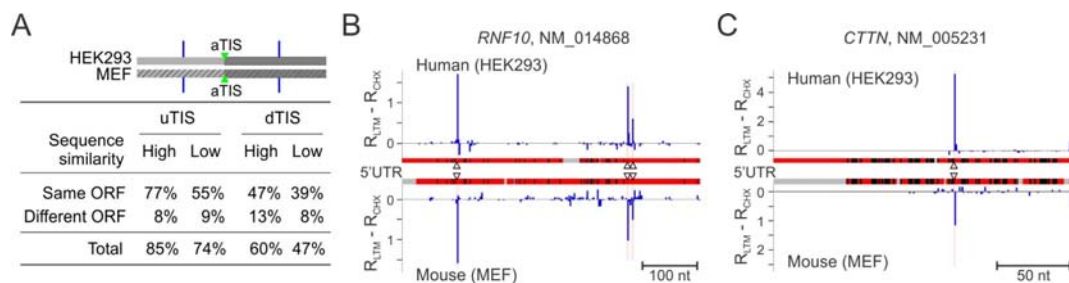


Fig. 7. (A) Evolutionary conservation of alternative TIS positions identified by GTI-seq in HEK293 and MEF cells. **(B)** Conservation of uTIS positions on the RNF10 transcript with high sequence similarity of 5'UTR between HEK293 and MEF cells. **(C)** Conservation of uTIS positions on the CTTN transcript with low sequence similarity of 5'UTR between HEK293 and MEF cells.

and the associated ORFs is a strong indication of functional significance of alternative translation in the regulation of gene expression.

2d. Elucidate how nutrient starvation affects alternative translation initiation

The integrated stress response (ISR) facilitates cellular adaptation to a variety of stress conditions via phosphorylation of the common target eIF2 α . During ISR, the selective translation of stress-related mRNAs often relies on alternative mechanisms, such as leaky scanning or reinitiation, but the underlying mechanism remains incompletely understood. Here we report that, in response to amino acid starvation, the reinitiation of ATF4 is not only governed by the eIF2 α signaling pathway, but is also subjected to regulation by mRNA methylation in the form of *N*⁶-methyladenosine (m⁶A). While depleting m⁶A demethylases represses ATF4 reinitiation, knocking down m⁶A methyltransferases promotes ATF4 translation. We demonstrate that m⁶A in the 5' untranslated region (5'UTR) controls ribosome scanning and subsequent start codon selection. Global profiling of initiating ribosomes reveals widespread alternative translation events influenced by dynamic mRNA methylation. Consistently, *Fto*-transgenic mice manifest enhanced ATF4 expression, highlighting the critical role of 5'UTR methylation in translational regulation of ISR at cellular and organismal levels.

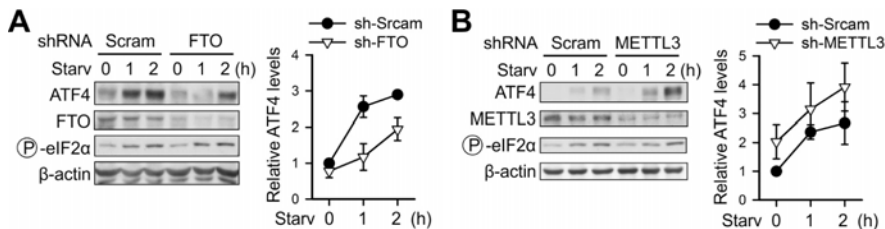


Fig 8. MEF cells with or without FTO knockdown (A) or METTL3 knockdown (B) were subject to amino acid starvation followed by immunoblotting. The right panel shows the relative ATF4 levels quantified by densitometry and normalized to β -actin.

2e. Determine translational re-programming in cells with altered mTORC1 signaling

In eukaryotic cells, protein synthesis typically begins with the binding of eIF4F to the 7-methylguanylate (m7G) cap found on the 5' end of the majority of mRNAs. Surprisingly, overall translational output remains robust under eIF4F inhibition. The broad spectrum of eIF4F-resistant translational products is incompatible with cap-independent translation mediated by internal ribosome entry sites (IRES). Here, we report that *N*⁶-methyladenosine (m⁶A) facilitates mRNA translation that is resistant to eIF4F inactivation. Depletion of the methyltransferase METTL3 selectively inhibits translation of mRNAs bearing 5'UTR methylation, but not mRNAs with 5' terminal oligopyrimidine (TOP) elements. We identify ABCF1 as a critical mediator of m⁶A-promoted translation under both stress and physiological conditions. Supporting the role of ABCF1 in m⁶A-facilitated mRNA translation, ABCF1-sensitive transcripts largely overlap with METTL3-dependent mRNA targets. By illustrating the scope and mechanism of eIF4F-independent mRNA translation, these findings reshape our current perceptions of cellular translational pathways.

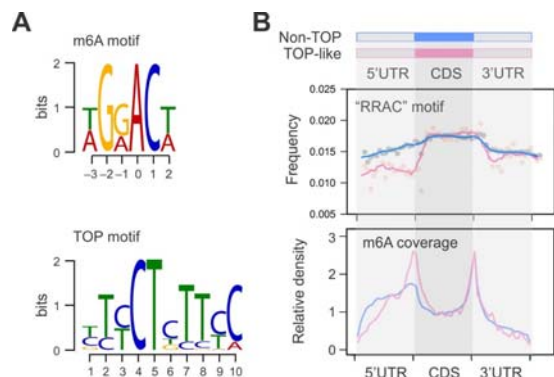


Fig 9. (A) Sequence logo representing the consensus motif relative to m⁶A (upper panel) and TOP elements (lower panel). (B) The top panel shows the frequency of m⁶A motif across TOP-like (pink line) and non-TOP (blue line) mRNAs. The lower panel shows the distribution of m⁶A.

- **What opportunities for training and professional development has the project provided?**
 1. My graduate student Robert Swanda has been awarded to receive a Training Grant support from Cornell Chemistry/Biology Interface. The title of his proposal is: Defining the sulfur amino acid response.
 2. My graduate student Xin Erica Shu has been awarded to receive a NIH Predoctoral Individual Fellowship (F31). The title of her proposal is: Dynamic O-GlcNAcylation of eIF3A in translational control of the integrated stress response.
- **How were the results disseminated to communities of interest?**

Invited and Selected Presentations

1. Invited speaker, *Translational control at the start codon*. **MD Anderson Cancer Center**, Blaffer Lecture Series, Houston, TX. September 2017
2. Invited speaker, *Translational control at the start codon*. **UT Southwestern**, Department of Physiology, Dallas, TX. September 2017
3. Invited speaker, *Translational control at the start codon*. **Fred Hutchinson Cancer Research Center**, Seattle, WA. June 2017
4. Invited speaker, *Translational control of stress response*. **Case Western Reserve University**, Department of Genetics, Cleveland, OH. May 2017
5. Invited speaker, *Translational control of heat shock response*. **Gordon Research Conference: Translation Machinery in Health and Disease**. Galveston, TX, March, 2017
6. Invited speaker, *Translational control in stress response: from ribosomes to mRNA*. **University of Rochester**, Rochester, NY. October 2016
7. Invited speaker, *m6A-mediated cap-independent translation: scope and mechanism*. **RNA modifications and epi-transcriptomics conference**. University of Chicago. Chicago, IL, September, 2016
8. Selected speaker, *m6A-mediated cap-independent translation: scope and mechanism*. **Cold Spring Harbor Laboratory Meeting: Translational Control**. Cold Spring Harbor, NY, September, 2016
9. Invited speaker, *Translational control in stress response: from ribosomes to mRNA*. **University of Georgia**, Athens, GA. April 2016
10. Invited speaker, *Translational control in stress response: from ribosomes to mRNA*. **Indiana University School of Medicine**, Indianapolis, IN. April 2016
11. Invited speaker, *Translational control in stress response revealed by ribosome profiling*. **National Institutes of Health, NICHD**, Bethesda, MD. May 2015
12. Invited speaker, *Regulation of gene expression by alternative translation*. *Translational Control: From Basics to Cancer*. **Génopolys**, Montpellier, France, April 2015
13. Invited speaker, *Decoding translational control by ribosome profiling*. **Zhejiang University Medical School**, Hangzhou, Zhejiang, P. P. China, April 2015
14. Invited speaker, *Translational control in stress response revealed by ribosome profiling*. Department of Cell Biology Seminar, **Duke University**, Durham, NC, September, 2014

15. Selected speaker, *Quantitative profiling of initiating ribosomes in vivo*. **Cold Spring Harbor Laboratory Meeting**: Translational Control. Cold Spring Harbor, NY, September, 2014
16. Invited speaker, *Decipher alternative translation by quantitative profiling of initiating ribosomes*. **EMBO Workshop**: Recoding: Reprogramming genetic decoding, Killarney, Ireland, May, 2014
17. Selected speaker, *Translational control of chaperone biosynthesis via stress ribosomes*. **Cold Spring Harbor Laboratory Meeting**: Molecular Chaperones & Stress Responses. Cold Spring Harbor, NY, May, 2014
18. Invited speaker, *Monitoring translational control using real-time ribosome profiling*. Department of Cell Biology Seminar, **Yale University**, New Haven, CT, April, 2014

4. IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**

In a post-genomic era, the importance of translational control in gene expression has been increasingly appreciated. Enabling swift regulation of gene expression, translational reprogramming can be quantitative (all-or-none vs. graded), qualitative (enabling a single mRNA to produce several different proteins), or selective (activating subsets of mRNAs for translation). By permitting rapid and selective changes in the landscape of proteome, translational reprogramming plays a crucial role in cell growth, differentiation, stress response, as well as organismal development. However, much remains to be learned about the mechanisms underlying translational reprogramming. It remains to be a formidable task to achieve programmable mRNA translation. By integrating biochemical, genetic, genomic, and computational approaches, we are actively pursuing alternative pathways of translation initiation, principles of start codon selection, and dynamic interplay between mRNA and the translation machinery.

Qualitative and quantitative characterization of gene expression is indispensable to understand dynamic phenotypes of eukaryotic cells. While our knowledge of translational regulation is steadily increasing, it is clear that the combination of emerging technologies will uncover novel aspects of this paramount cellular process. Our approach to studying initiating ribosomes (e.g., QTI-seq) is unique and crucial for comprehensive understanding of translational reprogramming. Further characterization of the dynamic interplay between mRNA modification and the translation machinery has the potential to reveal translational signatures associated with stress and disease conditions. Programmable start codon selection by Cas9-based editing has enormous implications for fundamental biology as well as therapeutic application.

- **What was the impact on other disciplines?**

Nothing to report

- **What was the impact on technology transfer?**

Nothing to report

- **What was the impact on society beyond science and technology?**

Nothing to report

5. CHANGES / PROBLEMS

Nothing to report

6. PRODUCTS

- **Publications, conference papers, and presentations**

Journal publications (* Corresponding author)

1. Zhou J, Wan J, Xin ES, Mao Y, Liu XM, Xin Y, Zhang X, Martin EH, Jens CB, and Qian SB*. 6-Methyladenosine Guides mRNA Alternative Translation during Integrated Stress Response. **Mol Cell** 2018; (in press).
2. Li X, Xiong X, Chen Y, Zhang M, Wang K, Zhou J, Mao Y, Yi D, Chen X-W, Wang C, Qian SB, and Yi C*. Single-nucleotide resolution mapping reveals distinct classes of *N*¹-methyladenosine methylome in nuclear- and mitochondrial-encoded transcripts. **Mol Cell** 2017; 68(5):993-1005.
3. Coots RA, Liu XM, Mao Y, Dong L, Zhou J, Wan J, Zhang X, Qian SB*. m⁶A facilitates eIF4F-independent mRNA translation. **Mol Cell** 2017; 68(3):504-514.
4. Tang L, Morris J, Wan J, Moore C, Fujita Y, Gilaspie S, Aube E, Nanda J, Marques M, Jangal M, Anderson A, Cox C, Hiraishi H, Dong L, Saito H, Singh CR, Witcher M, Topisirovic I, Qian SB, and Asano K*. Competition between translation initiation factor eIF5 and its mimic protein 5MP determines non-AUG initiation rate genome-wide. **Nucleic Acids Res** 2017; 45(20):11941-11953
5. Saikia M*, Wang X, Mao Y, Wan J, Pan T and Qian SB*. Codon optimality controls differential mRNA translation during amino acid starvation. **RNA** 2016; 22(11):1719-1727
6. Liu B and Qian SB*. Characterizing inactive ribosomes in translational profiling. **Translation** 2016 4(1):e1138018
7. Qian SB. Step back for seminal translation. **Nat Struct Mol Biol** 2016; 23(2):362-3
8. Zhou J, Rode KA, and Qian SB*. m⁶A: A novel hallmark of translation. **Cell Cycle** 2015; 10:1-2
9. Meyer KD, Patil DP, Zhou J, Zinoviev A, Skabkin MA, Elemento O, Pestova TV, Qian SB and Jaffrey SR. 5' UTR m⁶A promotes cap-independent translation. **Cell** 2015; 163(4):999-1010
10. Zhou J, Wan J, Gao X, Zhang X and Qian SB*. Dynamic m⁶A mRNA methylation directs translational regulation of heat shock response. **Nature** 2015; 526(7574):591-4
 - Highlighted in **Nat Chem Biol**
 - Recommended by **Faculty 1000**
11. Gao X, Wan J, and Qian SB*. Genome-wide profiling of alternative translation initiation sites. **Methods Mol Biol** 2016; 1358:303-16
12. Wei S and Qian SB*. Ribosome profiling: principles and variations. **eLS** 2015 John Wiley & Sons, Ltd: Chichester. DOI: 10.1002/9780470015902.a0025984
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- Featured on News and Views of **Nat Struct Mol Biol**
- Highlighted in **Science**

14. Bettencourt C1, de Yébenes JG, López-Sendón JL, Shomroni O, Zhang X, Qian SB, Bakker IM, Heetveld S, Ros R, Quintáns B, Sobrido MJ, Bevova MR, Jain S, Bugiani M, Heutink P, Rizzu P. Clinical and neuropathological features of spastic ataxia in a Spanish family with novel compound heterozygous mutations in STUB1. **Cerebellum** 2015; 14(3):378-81
15. Gao X, Wan J, Liu B, Ma M, Shen B, and Qian SB*. Quantitative profiling of initiating ribosomes in vivo. **Nat Methods** 2015; 12(2):147-53. PMCID: PMC4344187

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- **Other products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name: Shu-Bing Qian, PhD
Project Role: PI
Nearest person month worked: 1
Contribution to Project: Dr. Qian is responsible for the overall administration and direction of the project, including designing experimental protocols, interpreting results, writing manuscripts and supervising research assistants.

Name: Jun Zhou, PhD
Project Role: Postdoc
Nearest person month worked: 12
Contribution to Project: Dr. Zhou will be responsible for mTOR and mRNA modification experiments (Aim 1, Aim 2).

Name: Xiangwei Gao, PhD
Project Role: Postdoc
Nearest person month worked: 12
Contribution to Project: Dr. Gao will be responsible for ribosome profiling experiments (Aim 1, Aim 2).

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report

- **What other organizations were involved as partners?**

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

N/A

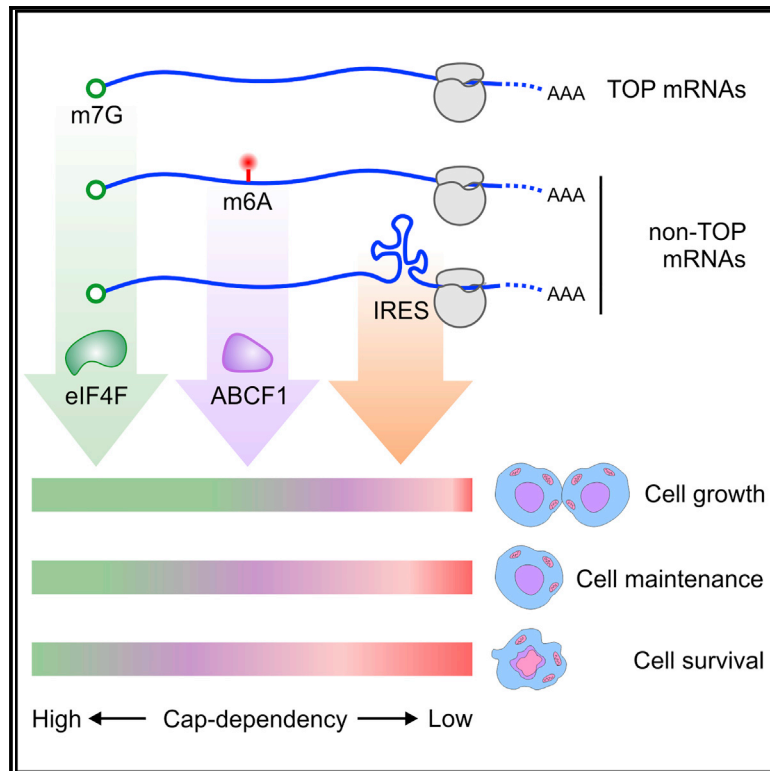
9. APPENDICES

Copies of journal articles

Molecular Cell

m⁶A Facilitates eIF4F-Independent mRNA Translation

Graphical Abstract



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In Brief

Coots et al. show that eIF4F inhibition partially represses global protein synthesis. m⁶A in the 5' UTR facilitates eIF4F-independent mRNA translation, and ABCF1 appears to be critical for m⁶A-facilitated mRNA translation. These differential translation modes are coordinated in response to environmental perturbations.

Highlights

- eIF4F inhibition partially represses global protein synthesis
- Translation of non-TOP mRNAs depends on 5' UTR m⁶-methyadenosine
- ABCF1 is critical for eIF4F-independent mRNA translation
- ABCF1 coordinates with METTL3 in m⁶A-facilitated mRNA translation

m⁶A Facilitates eIF4F-Independent mRNA Translation

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SUMMARY

In eukaryotic cells, protein synthesis typically begins with the binding of eIF4F to the 7-methylguanylate (m⁷G) cap found on the 5' end of the majority of mRNAs. Surprisingly, overall translational output remains robust under eIF4F inhibition. The broad spectrum of eIF4F-resistant translomes is incompatible with cap-independent translation mediated by internal ribosome entry sites (IRESs). Here, we report that N⁶-methyladenosine (m⁶A) facilitates mRNA translation that is resistant to eIF4F inactivation. Depletion of the methyltransferase METTL3 selectively inhibits translation of mRNAs bearing 5' UTR methylation, but not mRNAs with 5' terminal oligopyrimidine (TOP) elements. We identify ABCF1 as a critical mediator of m⁶A-promoted translation under both stress and physiological conditions. Supporting the role of ABCF1 in m⁶A-facilitated mRNA translation, ABCF1-sensitive transcripts largely overlap with METTL3-dependent mRNA targets. By illustrating the scope and mechanism of eIF4F-independent mRNA translation, these findings reshape our current perceptions of cellular translational pathways.

INTRODUCTION

Eukaryotic cells primarily employ a cap-dependent mechanism to initiate translation for the majority of mRNAs (Gebauer and Hentze, 2004; Hinnebusch, 2014; Jackson et al., 2010). The 5' end of eukaryotic mRNAs is modified with a m⁷G cap structure, which is recognized by an eukaryotic initiation factor 4E (eIF4E). eIF4E forms the eIF4F complex by binding to eIF4G (a scaffold protein) and eIF4A (a helicase) (Gross et al., 2003; Marintchev et al., 2009; Schütz et al., 2008). The cap recognition determines which mRNAs are to be translated and is subject to regulation by eIF4E-binding proteins (4E-BPs). When hypophosphorylated, 4E-BPs outcompete eIF4G for a binding site on eIF4E and prevent eIF4F assembly at the 5' end of transcripts (Pause et al., 1994). One major signaling pathway that phosphorylates 4E-BPs is the mammalian target of rapamycin complex

1 (mTORC1) (Ma and Blenis, 2009; Zoncu et al., 2011). By sensing extracellular signals as well as the intracellular energy status, activated mTORC1 phosphorylates 4E-BPs that dissociate from eIF4E, thereby promoting eIF4F complex assembly (Sonenberg and Hinnebusch, 2009). Despite this well-established regulatory mechanism, in many cell lines, mTORC1 inhibition has only modest effects on the rate of protein synthesis (Beretta et al., 1996; Choo et al., 2008). The simplest interpretation of this conundrum is that cells rely on a cap-independent mechanism for a substantial amount of mRNA translation.

Cap-independent translation occurs during normal cellular processes (e.g., mitosis and apoptosis) or when the cap-dependent translation machinery is compromised by either stress or disease (Sonenberg and Hinnebusch, 2007). The best-characterized cap-independent initiation mechanisms involve internal ribosome entry sites (IRESs) (Hellen and Sarnow, 2001). Discovered in picornavirus mRNAs, the IRES element in the 5' untranslated region (5' UTR) forms a complex secondary structure capable of recruiting the translation machinery in the absence of some or even all initiation factors. A growing body of evidence suggests that certain cellular mRNAs may use the similar IRES mechanism for cap-independent translation initiation (Gilbert et al., 2007). Systematic approaches have been elaborated to identify putative IRES elements in human and viral genomes (Weingarten-Gabbay et al., 2016). Despite their capability of internal initiation, it is unclear whether these events truly occur within the original sequence context under physiological conditions. In fact, many cellular mRNAs that have been considered to contain IRESs failed to pass through stringent tests for internal initiation (Gilbert et al., 2007).

It has been hypothesized that some cellular mRNAs exhibit a relaxed cap dependence because of the presence of so-called cap-independent translation enhancers (CITEs) within the untranslated region (Terenin et al., 2013). CITEs are elements in the mRNA capable of recruiting key initiation factors, thereby promoting the assembly of translation initiation complexes. Despite years of speculation, the nature of CITE elements remains obscure. We recently discovered that mRNA methylation in the form of m⁶A enables cap-independent translation (Meyer et al., 2015; Zhou et al., 2015). As exemplified by selective translation of heat shock-induced Hsp70 mRNA, this finding suggests the existence of a translation initiation mechanism that is neither cap nor IRES dependent. This new mode of translation initiation offers an attractive solution to the central puzzle that many

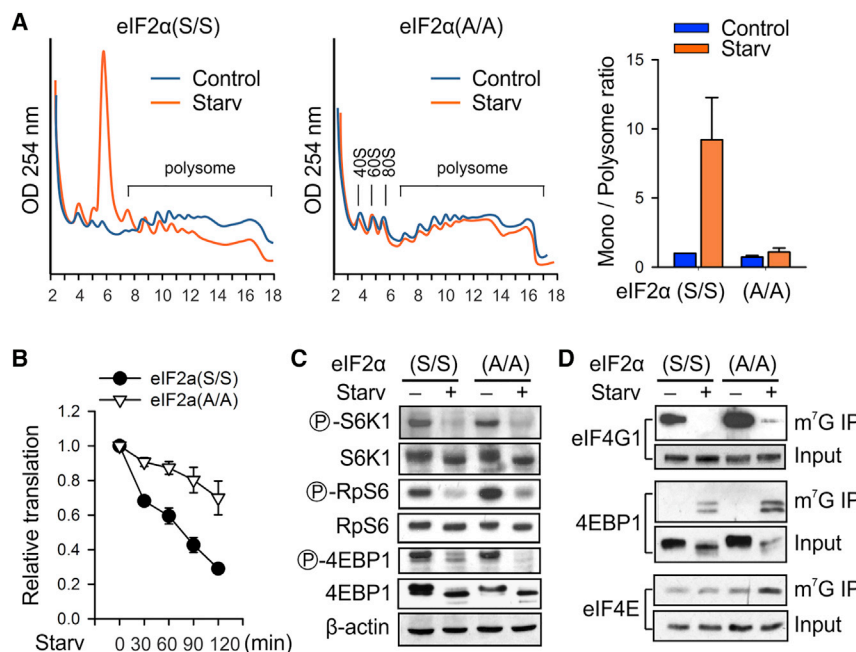


Figure 1. A Substantial Amount of Cellular Translation Is Resistant to eIF4F Inhibition

(A) Sucrose gradient-based polysome profiling of eIF2α (S/S) and eIF2α (A/A) MEF cells before and after 1 hr of amino acid starvation. The right panel shows the monosome/polysome ratio calculated using areas below the curve. Error bars, mean ± SEM; n = 3, biological replicates.

(B) Global protein synthesis in starved eIF2α(S/S) and eIF2α(A/A) MEF cells was quantified from puromycin labeling. Error bars, mean ± SEM; n = 3, biological replicates.

(C) Immunoblotting of mTORC1 downstream targets in eIF2α(S/S) and eIF2α(A/A) MEF cells before and after 1 hr of amino acid starvation.

(D) Immunoblotting of m⁷GTP pulldown assay in eIF2α(S/S) and eIF2α(A/A) MEF cells before and after 1 hr of amino acid starvation.

See also [Figures S1–S3](#).

eIF2α (A/A) cells showed few changes in the polysome pattern upon amino acid starvation. Consistently, measurement of global protein synthesis revealed much

cap-independent translation events do not follow the IRES mechanism. However, mechanistic details underlying m⁶A-mediated translation initiation are poorly understood. Several fundamental questions remain unanswered. First, since many transcripts bear 5' UTR methylation, how much does m⁶A-mediated translation contribute to cellular protein synthesis? Second, for capped mRNAs with 5' UTR methylation, are the canonical cap-dependent translation and the m⁶A-enabled cap-independent translation mutually exclusive? Third, what is the biological logic behind the selection for different modes of translation initiation? Here, we investigated the scope and mechanism of eIF4F-independent mRNA translation, which revealed a dynamic coordination between different translation modes in response to environmental and physiological stimuli.

RESULTS

The Scope of Physiological Cap-Independent Translation

In response to amino acid deprivation, global protein synthesis is suppressed via inhibition of mTORC1 and activation of the general control non-derepressible-2 kinase (GCN2) (Hinnebusch, 2005; Ma and Blenis, 2009; Wek et al., 2006; Zoncu et al., 2011). While the former regulates eIF4F-mediated 5' end cap recognition, the latter controls the formation of a ternary complex (TC) comprised of eIF2, GTP, and methionine-loaded initiator tRNA (Pisarev et al., 2007). To dissect the contribution of these two rate-limiting steps to the overall translational output, we took advantage of a mouse embryonic fibroblast (MEF) cell line harboring a non-phosphorylatable eIF2α in which the serine 51 (S/S) was mutated to an alanine (A/A) (Scheuner et al., 2001). As expected, wild-type eIF2α (S/S) cells readily responded to amino acid deprivation by showing polysome disassembly and concomitant increase of monosome (Figure 1A). To our surprise,

less repression of translation in starved eIF2α (A/A) cells than the wild-type (Figure 1B; Figure S1A). The striking resistance to amino acid limitation is also seen in cells lacking GCN2 kinase (Figure S1B). In addition, this phenomenon is highly reproducible under different types of stress, such as unfolded protein response in the endoplasmic reticulum (Figure S1C).

Amino acid deprivation is expected to inhibit mTORC1 signaling and consequently suppress eIF4F complex formation at the 5' end cap (Jewell et al., 2013). It is thus surprising to observe continuous translation in starved eIF2α (A/A) cells. Previous studies suggested that the sensitivity of mTORC1 to nutrient starvation is coupled with GCN2/eIF2α signaling (Ye et al., 2015). It is possible that, in the absence of eIF2α phosphorylation, mTORC1 remains active even under limited supply of amino acids. However, this is not the case. Similar to wild-type cells, eIF2α (A/A) cells exhibited rapid dephosphorylation of mTORC1 downstream targets S6K1 and 4E-BP1 upon amino acid deprivation (Figure 1C). Notably, the phosphorylation status of the elongation factor eEF2 was comparable between S/S and A/A cells (Figure S1D). To directly measure the cap functionality in these cells, we conducted a m⁷G cap pull-down assay before and after nutrient starvation. It is clear that, upon amino acid deprivation, the m⁷G-associated scaffold protein eIF4G1 was largely replaced by 4E-BP1 in both wild-type and eIF2α (A/A) cells (Figure 1D). Therefore, the cap-recognition machinery is inactive under amino acid starvation irrespective of eIF2α phosphorylation.

To independently assess the contribution of cap recognition to global protein synthesis, we took advantage of a chemical compound 4EGI-1 that inhibits eIF4F complex formation by destabilizing eIF4E-eIF4G interaction (Moerke et al., 2007). [³⁵S] metabolic labeling revealed approximately 30% reduction in protein synthesis in both eIF2α (S/S) and (A/A) cells (Figure S2A). Notably, the majority of cellular translation sustained even after

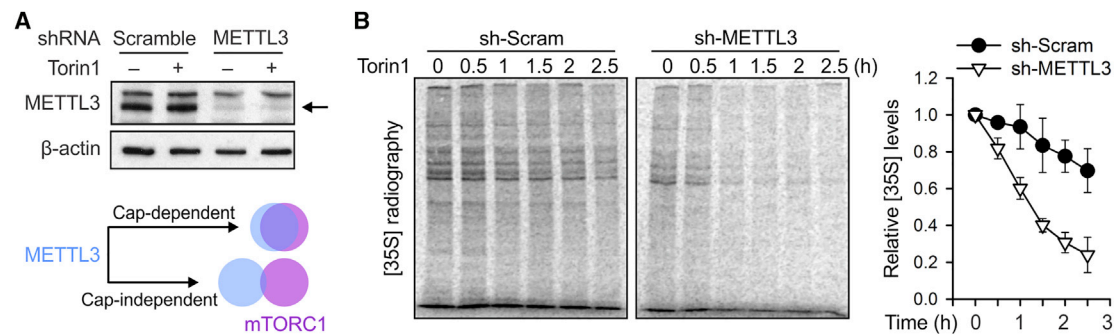


Figure 2. Physiological Cap-Independent Translation Is Dependent on m⁶A Modification

(A) Immunoblotting of MEFs with or without METTL3 knockdown. The lower panel shows a schematic diagram distinguishing cap-dependent and cap-independent translation.

(B) Global protein synthesis in MEF cells with or without METTL3 knockdown was measured after pre-treatment of 1 μ M Torin1 for various times. The right panel shows quantification of [³⁵S] autoradiograph after Torin1 treatment. Error bars, mean $\pm$ SEM; n = 3, biological replicates.

See also Figure S4.

prolonged treatment of 4EGI-1 (5 hr). We next examined the effect of Torin1, a potent active-site mTOR inhibitor (Thoreen et al., 2009). As expected, Torin1 treatment caused a rapid depletion of phosphorylation for mTORC1 downstream targets (Figure S2B). However, pre-exposure of cells to a high dose of Torin1 (1 μ M) only led to less than 50% reduction in protein synthesis (Figure S2C). Therefore, a substantial amount of translation likely follows a mechanism independent of eIF4F.

Cap-Independent Translation Follows a Non-IRES Mechanism

It is possible that the translation maintained under eIF4F inactivation relies on a cap-independent mechanism like IRES or, as recently reported, an alternative cap-recognition mechanism mediated by eIF3d (Lee et al., 2016). In both cases, only a small subset of mRNAs undergo specialized translation. However, we found similar patterns of translational products resolved on the SDS-PAGE gel before and after eIF4F inactivation (Figures S1 and S2). This result suggests that the same transcripts are capable of experiencing different modes of translation. We next examined the sensitivity of eIF4F-independent translation to hippuristanol, an eIF4A inhibitor that does not affect certain IRES-driven translation (Bordeleau et al., 2006). Nearly all of the translations were repressed in the presence of hippuristanol irrespective of eIF2 α signaling (Figure S3A). Therefore, eIF4F-independent translation still requires the scanning process. Further supporting its non-IRES feature, we observed minimal activation of IRES-driven translation in starved eIF2 α (A/A) cells (Figure S3B). These results collectively suggest that, when the eIF4F-dependent translation is inactivated, cells readily employ a different mode of translation that is neither cap nor IRES dependent.

m⁶A Mediates Translation That Is Neither Cap Nor IRES Dependent

We previously reported that m⁶A enables mRNA translation in a cap- and IRES-independent manner (Meyer et al., 2015; Zhou et al., 2015). We next investigated whether the substantial amount of translation persisted under eIF4F inactivation follows

the m⁶A-dependent mechanism. To test this possibility, we knocked down METTL3, a core subunit of methyltransferase complex, from MEF cells using shRNA. With more than 90% depletion of METTL3 (Figure 2A), we observed approximately 50% reduction of m⁶A levels on mRNAs (Figure S4A). METTL3 knockdown caused nearly 30% decrease of global protein synthesis (Figure S4B), which is consistent with the recent study that reported cytosolic function of METTL3 in translation (Lin et al., 2016). However, it is unresolved whether METTL3 plays a role in cap-dependent or cap-independent translation. We reasoned that if METTL3 mediates cap-dependent translation, the translational targets sensitive to METTL3 knockdown should overlap with mTORC1-sensitive targets. If so, METTL3 depletion is not expected to further decrease protein synthesis in the presence of mTORC1 inhibitors. In stark contrast, MEFs lacking METTL3 exhibited much greater sensitivity to Torin1 than the scramble control (Figure 2B), with nearly 80% reduction of protein synthesis after 2 hr treatment of Torin1. To substantiate this finding further, we conducted METTL14 knockdown in MEF cells. Similar to METTL3 depletion, reducing METTL14 also sensitized MEF cells to Torin1 treatment (Figure S4C). The additive effect between methyltransferase knockdown and Torin1 treatment clearly indicates that m⁶A-responsive mRNA translation differs from eIF4F-controlled protein synthesis. To ensure that it is the m⁶A modification rather than the physical METTL3 binding that mediates eIF4F-independent translation, we complemented with either wild-type METTL3 or an inactive D395A mutant to MEFs lacking endogenous METTL3. In the presence of Torin1, only the wild-type METTL3, but not the mutant, restored the global protein synthesis (Figure S4D).

m⁶A-Mediated Translation Differs from eIF4F in mRNA Targets

To elucidate the scope of m⁶A-mediated translation, we examined the translation potential of endogenous transcripts in cells with either METTL3 knockdown or eIF4F inhibition. Previous genome-wide studies revealed that the mRNA subsets highly sensitive to mTORC1 signaling consist almost entirely of transcripts with 5' terminal oligopyrimidine (TOP) elements

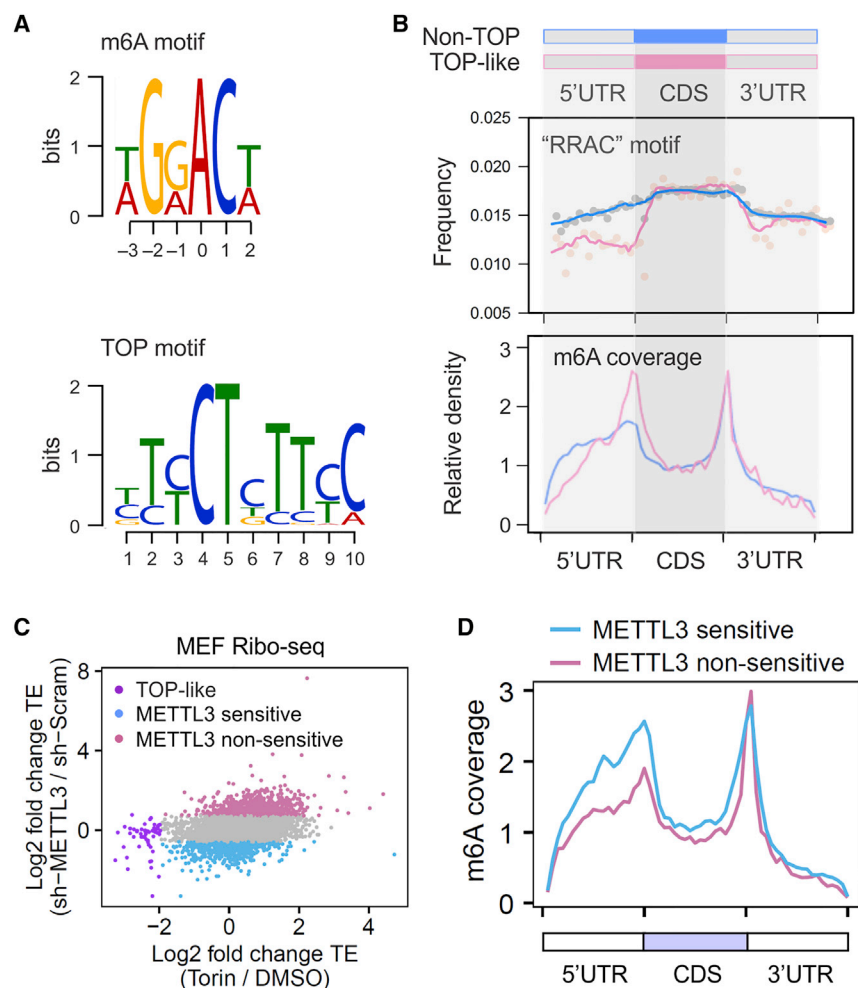


Figure 3. m⁶A Mediates eIF4F-Independent Translation

(A) Sequence logo representing the consensus motif relative to m⁶A (top) and TOP elements (bottom, derived from Thoreen et al., 2012). For m⁶A motif, "0" indicates the position of m⁶A. For TOP motif, "1" represents the 5' end position.

(B) The top panel shows the frequency of m⁶A motif across TOP-like (pink line) and non-TOP (blue line) mRNAs. The lower panel shows the distribution of m⁶A modification across TOP-like (pink line) and non-TOP (blue line) mRNAs using m⁶A-seq datasets obtained from MEFs.

(C) Scatterplot shows the TE fold change in MEF cells in response to Torin1 treatment or METTL3 knockdown. Blue dots refer to TOP-like transcripts. Both METTL3-sensitive (bottom 10%) and non-sensitive (top 10%) mRNAs are highlighted.

(D) m⁶A coverage obtained from m⁶A-seq was plotted for METTL3 non-sensitive (pink line) and METTL3 sensitive (blue line) mRNAs. Relative regions of 5' UTR, CDS, and 3' UTR are shown as the same size.

See also Figure S4.

(Hsieh et al., 2012; Thoreen et al., 2012). Supporting this notion, the translation of these TOP mRNAs was highly sensitive to Torin1 treatment as revealed by ribosome profiling (Ribo-seq) in MEF cells (Figure S4E). Given the mutually exclusive nature between the TOP motif and m⁶A sequence context (Figure 3A), it is possible that TOP mRNAs in general have less m⁶A modification in the 5' UTR and thus heavily rely on the cap-dependent mechanism for translation. Indeed, sequence survey of mouse transcriptome revealed low levels of m⁶A consensus sequence in the 5' UTR of TOP mRNAs (Figure 3B). We further compared the methylation landscape between TOP and non-TOP mRNAs using m⁶A-seq data derived from MEF cells (Geula et al., 2015). Virtually no m⁶A modification occurs at the first half of TOP 5' UTR. Since both coding region sequence (CDS) and 3' UTR regions show comparable m⁶A distribution between TOP and non-TOP mRNAs, we argue that it is the 5' UTR sequence feature that influences the relative cap dependency during translation.

To test this hypothesis, we examined ribosome profiling datasets obtained from MEF cells depleted of METTL3. Indeed, TOP mRNAs showed little response to METTL3 knockdown ($Rho = 0.11$) (Figure 3C). In coupling with m⁶A-seq data-

sets, we found that transcripts undergoing translational downregulation in the absence of METTL3 generally had higher 5' UTR methylation levels (Figure 3D). The similar observation was evident when we used different m⁶A-seq datasets derived from MEF cells (Schwartz et al., 2014) (Figure S4F). As an independent validation, we analyzed datasets obtained from HeLa cells with or without METTL3 depletion (Wang et al., 2015). Direct comparison of the translation efficiency

(TE) revealed that translation of non-TOP mRNAs was more sensitive to METTL3 depletion than that of TOP mRNAs ($p = 2.2 \times 10^{-16}$) (Figure S4G). Taken together, these data suggest that m⁶A modification in the 5' UTR renders these transcripts insensitive to eIF4F inhibition by enabling cap-independent translation.

To search for potential m⁶A readers that mediate eIF4F-independent mRNA translation, we examined YTH domain family proteins residing in the cytoplasm. Interestingly, knocking down YTHDF3, but not YTHDF1 and YTHDF2, re-sensitized MEF cells to Torin1 treatment (Figure S5A). The putative role of YTHDF3 in eIF4F-independent translation is consistent with several recent studies reporting that YTHDF3 facilitated mRNA translation, including m⁶A-mediated circular RNA translation (Li et al., 2017; Shi et al., 2017; Yang et al., 2017).

METTL3 Recognizes Internal m⁶A, but Not the m⁷G Cap

Previous studies demonstrated that exogenously overexpressed METTL3 co-immunoprecipitated with cap-binding proteins such as CBP80/20 and eIF4E (Lin et al., 2016). These results have been interpreted as fitting a model in which METTL3 promotes cap-dependent translation. To test the cap-binding capability

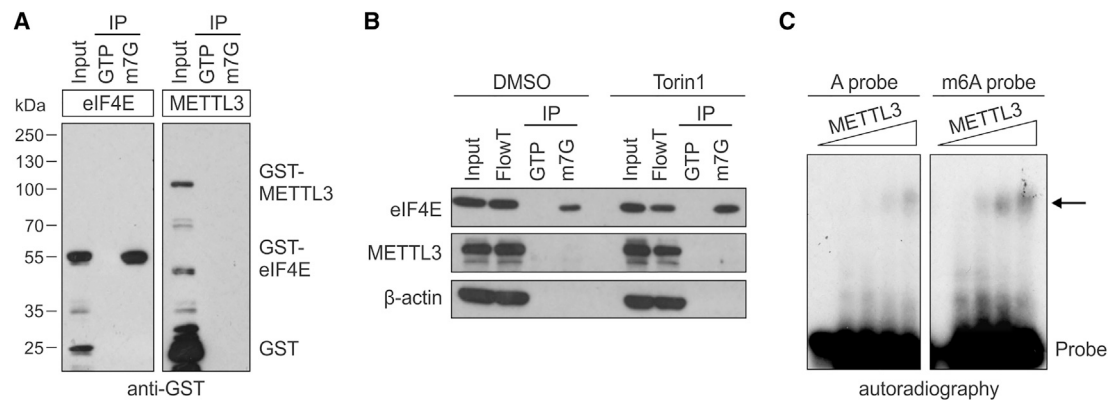


Figure 4. METTL3 Recognizes m⁶A, but Not the m⁷G, Cap Structure

(A) Recombinant eIF4E and METTL3 proteins were purified from *E. coli* as GST fusion proteins followed by incubation with immobilized beads coated with m⁷GTP. Immunoblotting was conducted using an anti-GST antibody.

(B) Whole-cell lysates from HEK293 cells with or without Torin1 treatment were incubated with immobilized beads coated with m⁷GTP followed by immunoblotting using antibodies indicated.

(C) Synthesized mRNA probes with or without single m⁶A were radiolabeled with ³²P followed by incubation with an increasing dose of recombinant METTL3 (0, 0.3, 0.6, 1.2, and 2.4 μg). The mRNA-protein complexes were resolved on a SDS-PAGE gel.

See also Figure S5.

of METTL3, we employed a direct cap-binding assay using recombinant proteins purified from *E. coli*. The immobilized m⁷G cap readily precipitated eIF4E, as expected, but failed to pull down METTL3 (Figure 4A). We next examined the cap-binding feature of endogenous proteins by incubating the m⁷G beads with whole-cell lysates. Although the m⁷G cap readily pull down a considerable amount of eIF4E, no detectable METTL3 was precipitated by the m⁷G cap (Figure 4B). Notably, the m⁷G cap failed to capture METTL3 upon mTORC1 inhibition, despite the fact that more eIF4E molecules were recovered from the same lysates. Therefore, endogenous METTL3 does not bind to the cap structure regardless of the functional status of eIF4F.

Given the inherent AdoMet binding pocket of METTL3 (Wang et al., 2016a; Wang et al., 2016b), we next examined whether METTL3 directly interacts with methylated mRNA. Using a synthesized 30 nt mRNA with or without a single m⁶A site at the consensus sequence, we conducted a gel-shifting assay. Although the non-methylated probe weakly associated with METTL3, introducing a single m⁶A site significantly promoted METTL3 binding (Figure 4C). By contrast, METTL3 showed little preference in association with the capped mRNA synthesized via *in vitro* transcription (Figure S5B). This result indicates that METTL3 directly binds to the internal m⁶A, but not the 5' end cap structure.

Cap-Independent Translation of Hsp70 Requires ABCF1

We previously demonstrated that m⁶A enables cap-independent translation, especially under stress conditions. However, it is unclear how 5' UTR methylation promotes preinitiation complex assembly without forming the canonical eIF4F complex. In an attempt to identify potential mediators responsible for m⁶A-mediated cap-independent translation, we employed quantitative proteomic analysis to compare proteins associated with the same message experiencing cap-dependent or cap-independent translation. Hsp70 mRNA undergoes translational switch

in response to heat shock stress (Zhou et al., 2015) and represents an ideal endogenous transcript to address this question. We adopted a method based on endogenous mRNA pull-down without chemical crosslinking using biotinylated probes (Figure 5A). To identify and quantify the associated protein components, we employed an unbiased quantitative approach where digested peptides were labeled with 4-plex isobaric mass tags (iTRAQ) and subjected to liquid chromatography-tandem mass spectrometry analysis (LS-MS/MS). In addition to Hsp70 mRNA, we included β-actin mRNA as a control. HeLa was chosen because of the relatively high basal level of Hsp70 mRNA prior to heat shock stress, therefore permitting direct comparison of components associated with the same transcript before and after stress.

As expected, nearly all the ribosomal proteins showed reduced enrichment after heat shock stress, forming a distinct cluster with high peptide scores (Figure 5A; Table S1). Interestingly, several translation initiation factors were selectively enriched on the Hsp70 transcript, but not the β-actin mRNA, upon heat shock stress (Figure 5A, right). Among the most prominent factors are α, β, and γ subunits of eIF2 (>9-fold). Since eIF2 controls the ternary complex formation, it is clear that the stress-induced Hsp70 mRNA is capable of recruiting the initiator tRNA even when the cap recognition machinery is inactive under heat shock stress. A close inspection of the quantitative proteomic data revealed that eIF2A, eIF5, and ABCF1 were also associated with the stress-induced Hsp70 mRNA. In particular, ABCF1 showed a similar stoichiometry as eIF2 as judged by their comparable peptide scores (Figure 5A, right).

We validated the proteomic result by conducting immunoprecipitation (IP) of various endogenous proteins from cell lysates before and after stress. The non-canonical initiation factor eIF2A failed to pull down typical initiation factors such as eIF4G1 and eIF2β (Figure 5B). By contrast, eIF5 constitutively associated with these initiation factors irrespective of the stress

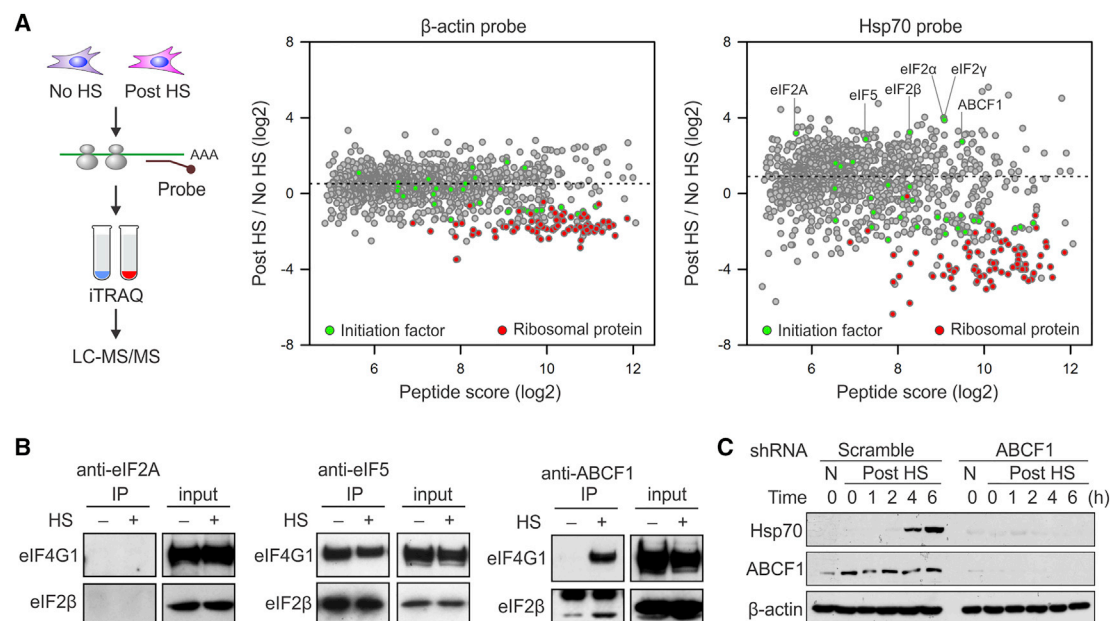


Figure 5. ABCF1 Is Essential in Cap-Independent Translation of Hsp70

(A) The left panel shows the schematic of quantitative mass spectrometry using iTRAQ. Proteins enriched on β -actin mRNA (middle) or Hsp70 mRNA (right) purified from HeLa cells after heat shock stress are presented as scatterplots. The original peptide score ($\log_2$) and stress-induced fold changes ($\log_2$) are shown in the x axis and the y axis, respectively.

(B) Whole-cell lysates from heat-shocked HeLa cells were subjected to immunoprecipitation using antibodies indicated followed by immunoblotting.

(C) MEF cells with or without ABCF1 knockdown (Scramble) were collected at indicated times after heat shock stress (43°C, 1 hr) followed by immunoblotting. N, no heat shock.

See also Figure S6.

condition. Only ABCF1 was able to precipitate eIF4G1 and eIF2 β in a stress-dependent manner (Figure 5B). These results suggest that ABCF1 facilitates initiation complex assembly on stress messages in response to heat shock stress. To resolve the physiological role of ABCF1 in stress-induced Hsp70 synthesis, we knocked down ABCF1 in MEF cells using shRNA-expressing lentiviruses. Remarkably, after heat shock stress, the Hsp70 synthesis was severely abolished in cells lacking ABCF1 (Figure 5C). The critical role of ABCF1 in Hsp70 synthesis was also seen in HeLa cells, despite the different basal levels of these proteins (Figures S6A and S6B). Notably, the cellular Hsp70 mRNA levels were even higher in cells with ABCF1 knockdown (Figure S6C), further supporting the translational deficiency of Hsp70 synthesis in the absence of ABCF1.

ABCF1 Facilitates m⁶A-Mediated Translation

Having found that a substantial amount of cellular translation follows the eIF4F-independent mechanism under the normal growth condition, we asked whether ABCF1 is also responsible for physiological cap-independent translation. ABCF1 knockdown in non-stressed MEFs resulted in about 20% reduction of global protein synthesis (Figure S6D), which is consistent with the previous report (Paytubi et al., 2009). To confirm that ABCF1-responsible translation indeed follows cap-independent initiation mode, we suppressed eIF4F-dependent translation by Torin1 treatment. Similar to the cells lacking METTL3, ABCF1 knockdown rendered MEF cells highly sensitive to Torin1 treat-

ment by showing >80% reduction of protein synthesis (Figure 6A). The increased sensitivity to mTOR1 inhibition in the absence of ABCF1 supports the notion that ABCF1-responsible translation differs from eIF4F-mediated cap-dependent translation.

Since ABCF1 resembles METTL3 in mediating eIF4F-independent translation, we predicted that the ABCF1-sensitive transcripts should overlap with METTL3-responsive mRNA targets. This is indeed the case. Ribosome profiling of MEF cells lacking either ABCF1 or METTL3 showed a strong correlation in the changes of TE ($r = 0.57$; Figure 6B). Further supporting the notion that ABCF1 facilitates m⁶A-mediated translation, transcripts experiencing decreased translation in the absence of ABCF1 have higher 5' UTR methylation than the one resistant to ABCF1 depletion (Figure 6C). Taken together with the crucial role of ABCF1 and METTL3 in the cap-independent translation of Hsp70 (Figure S6E), these results indicate a functional coordination between ABCF1 and METTL3 in m⁶A-facilitated translation.

ABCF1 Controls METTL3 Translation

ABCF1 (also termed ABC50) is an ATP-binding cassette protein that, unlike most ABC proteins, lacks membrane-spanning domains (Paytubi et al., 2009; Tyzack et al., 2000). Previous studies demonstrated that ABCF1 promotes translation initiation by interacting with eIF2 and ribosomes (Paytubi et al., 2009; Tyzack et al., 2000). Curiously, in MEF cells lacking

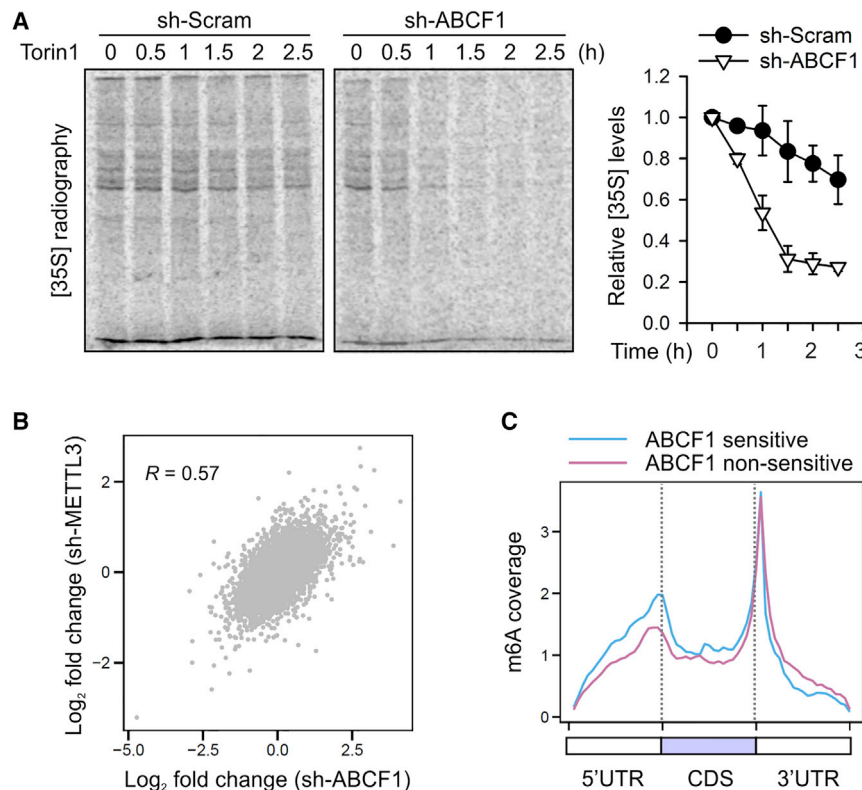


Figure 6. ABCF1 Mediates eIF4F-Independent Translation

(A) Global protein synthesis in MEF cells with or without ABCF1 knockdown was measured after pre-treatment of 1 μ M Torin1 for various times. The right panel shows quantification of [³⁵S] auto-radiograph after Torin1 treatment. Error bars, mean $\pm$ SEM; n = 3, biological replicates.

(B) Ribosome profiling data from cells with ABCF1 or METTL3 knockdown were used to determine the fold changes of TE. A scatterplot is presented to show positive correlation.

(C) m⁶A coverage obtained from m⁶A-seq was plotted for ABCF1-sensitive (blue line) and non-responsive (pink line) mRNAs. Relative regions of 5' UTR, CDS, and 3' UTR are shown as the same size.

See also Figure S6.

right). The m⁶A-facilitated translation of m⁶A “writer” METTL3 suggests a self-regulatory mechanism that offers an alternative translation mode when the cap machinery is inhibited.

DISCUSSION

For years, researchers have been fixated on the idea that eukaryotic mRNA translation relies on two mutually exclusive

mechanisms: cap-dependent ribosome scanning and cap-independent internal ribosome entry. Despite the predominant belief that eIF4F-mediated cap-dependent translation contributes to the majority of protein synthesis in eukaryotic cells, it is puzzling that inhibiting cap recognition by chemical inhibitors or genetic ablation only has modest effect on protein synthesis (Beretta et al., 1996; Choo et al., 2008; Yanagiya et al., 2012). The simplest interpretation of this conundrum is that cells rely on cap-independent initiation mechanism for a substantial amount of mRNA translation. The IRES-driven translation has become essentially synonymous with 5' cap-independent mRNA translation. However, the extent of cellular mRNAs that have been considered to contain IRESs remains controversial (Gilbert, 2010). Although certain mRNAs use the IRES mechanism to achieve the ribosome specificity (Xue et al., 2015), additional concepts are needed to explain how cells maintain robust translation during episodes of eIF4F inhibition. Here, we report that m⁶A-mediated translation initiation follows a cap- and IRES-independent mechanism. Unlike IRES-driven or eIF3d-mediated specialized translation, the m⁶A-promoted translation co-exists with eIF4F-mediated translation initiation for a great deal of transcripts. The scope of cap-independent translation is therefore much broader than previously appreciated.

METTL3 is an essential enzyme for m⁶A modification of mRNAs, which primarily occurs in the nucleus (Liu et al., 2014). A recent study adds a new twist to its functionality by demonstrating a cytosolic role of METTL3 in translation (Lin et al., 2016). It surprisingly acts as a “reader” rather than a “writer” of methylated transcripts because the m⁶A catalytic activity

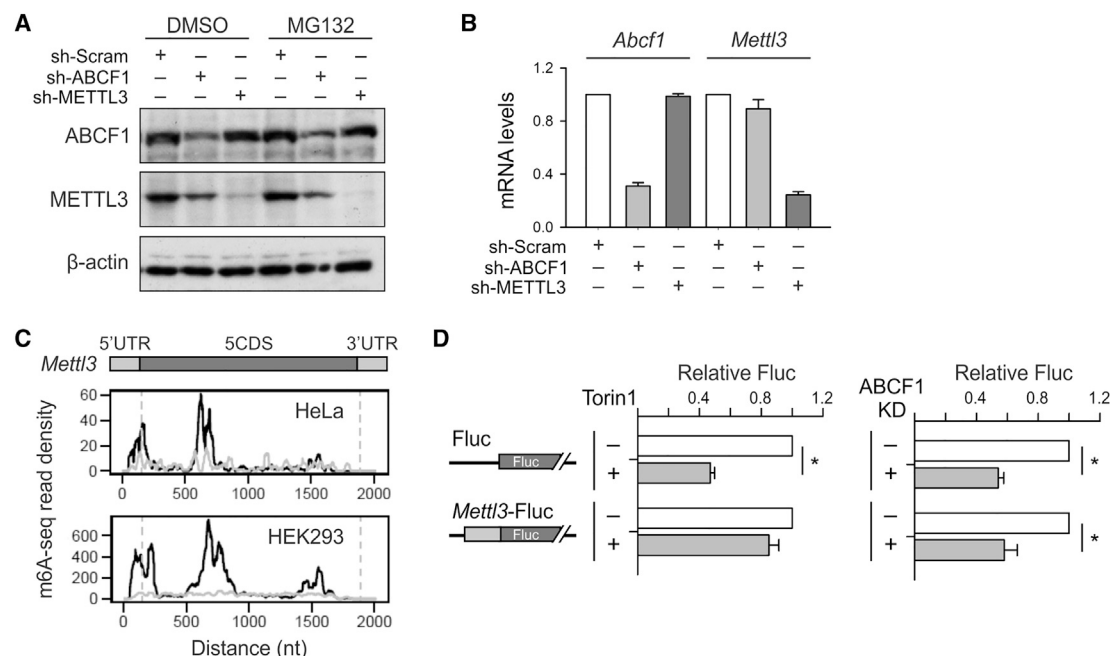


Figure 7. ABCF1 Mediates Translational Control of METTL3

(A) MEF cells with ABCF1 or METTL3 knockdown were treated with 5 μ M MG132 for 16 hr. Whole-cell lysates were collected for immunoblotting using the antibodies indicated.

(B) Total RNAs were purified from MEF cells with ABCF1 or METTL3 knockdown followed by qPCR. Error bars, mean $\pm$ SEM; n = 3, biological replicates.

(C) m⁶A coverage of METTL3 mRNA using m⁶A-seq datasets obtained from HeLa and HEK293 cells. The transcript architecture is shown above.

(D) MEF cells were transfected with Fluc plasmids shown in the left and Fluc levels were recorded by real-time luminometry. Error bars, mean $\pm$ SEM; n = 3, biological replicates. *p < 0.05 (t test).

See also Figure S7.

is dispensable in METTL3-promoted translation (Lin et al., 2016). However, it is unclear why only a subset of mRNAs is subjected to translational regulation by METTL3 in this manner. Although METTL3 appears to promote translation in cancer cells, it is not essential in embryonic stem cells (Geula et al., 2015). These puzzling observations call into question the exact role of METTL3 in translational regulation. One of the key questions is whether METTL3-promoted translation follows cap-dependent or cap-independent mechanisms. We provided evidence that METTL3, in fact, primarily facilitates translation independent of eIF4F. In addition, METTL3 does not seem to have the m⁷G cap-binding capacity, although it readily associates with internal m⁶A sites. The finding that m⁶A-mediated translation occurs on fully capped mRNAs suggests that the same transcript undergoes multiple translational modes, which explains the incomplete inhibition of translation by eIF4F inactivation. Indeed, only under the inactivation of mTORC1 signaling does the remaining translation become highly sensitive to METTL3 depletion. Intriguingly, a recent study reported that m⁶Am at the 5' end of mRNAs stabilizes mRNAs and likely promotes translation (Mauer et al., 2017). Since m⁶Am is part of the 5' cap structure, it is of importance to demonstrate whether m⁶Am functions as internal m⁶A or coordinates with the m⁷G cap in translational control.

What advantage might m⁶A-mediated translation confer when the cap machinery is fully functional inside cells? The answer to this question has two distinct, but interwoven, parts. The first lies

in the selectivity of mRNA translation and the second lies in the redistribution of cellular resources. Although mTORC1 primarily controls cap-dependent mRNA translation, it preferentially regulates the translation of TOP mRNAs via poorly understood mechanisms (Hamilton et al., 2006; Thoreen et al., 2012). Notably, the TOP mRNAs are among the most abundant messages in the cell, comprising up to 30% of cellular mRNAs during rapid growth in rich media (Warner, 1999). It is not surprising that the translation of TOP mRNAs must be quickly attenuated in response to limited supply of amino acids. It is conceivable that a diverse group of mRNAs must maintain their translation irrespective of the nutrient signaling. The m⁶A-mediated translation permits translation of some "privileged" mRNAs to produce proteins important for cell maintenance as well as cell survival. We propose that different modes of translation are coordinated to produce adaptive translational responses in response to environmental and physiological stimuli.

Under stress conditions, like amino acid starvation, the amount of ternary complex becomes limited as a result of GCN2-triggered eIF2 α phosphorylation (Liu and Qian, 2014; Wek et al., 2006). How does m⁶A-mediated translation initiation acquire the ternary complex to ensure productive translation? We found that ABCF1 serves as an alternative recruiter for the ternary complex during non-canonical translation. ABCF1 is a close relative of the yeast protein GCN20, which is presumed to cooperate with GCN1 in starvation-induced translational

response (Marton et al., 1997). Although GCN20 and ABCF1 are similar in their ABC domains, they differ markedly in their N termini. Intriguingly, it is the N-terminal region of ABCF1 that interacts with eIF2 in mammalian cells (Paytubi et al., 2008). In cells lacking ABCF1, heat shock-induced Hsp70 translation was severely impaired. Importantly, ABCF1 also plays a role in m⁶A-facilitated translation under the normal growth condition. Not surprisingly, cells with ABCF1 knockdown exhibits similar translational phenotypes as cells lacking METTL3. Perhaps the most interesting finding is the self-regulation of METTL3 translation that is not only dependent on m⁶A, but also subjected to ABCF1 regulation. This positive feedback loop provides a mechanism by which cells activate m⁶A-mediated translation upon inhibition of cap-dependent translation. Since the cap machinery evolved at a late stage during eukaryogenesis after the emergence of the nucleus and mRNA cap structure (Hernández, 2009), it is conceivable that a cap-independent initiation mechanism exists for capped mRNAs in early eukaryotes. The dynamic coordination between different translation modes encourages us to reconsider our traditional view of eIF4F as the primary driver of protein synthesis.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one table and can be found with this article online at <https://doi.org/10.1016/j.molcel.2017.10.002>.

AUTHOR CONTRIBUTIONS

R.A.C. and S.-B.Q. conceived the project and designed the study. R.A.C. performed most of the experiments. X.-M.L. performed cap binding and protein interaction assays as well as Ribo-seq and RNA-seq. Y.M. and J.W. analyzed the sequencing data. J.Z. conducted m⁶A-seq. L.D. assisted Ribo-seq. X.Z. conducted MS experiment. S.-B.Q. wrote the manuscript. All authors discussed results and edited the manuscript.

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Codon optimality controls differential mRNA translation during amino acid starvation

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ABSTRACT

It is common wisdom that codon usage bias has evolved in the selection for efficient translation, in which highly expressed genes are encoded predominantly by optimal codons. However, a growing body of evidence suggests regulatory roles for non-optimal codons in translation dynamics. Here we report that in mammalian cells, non-optimal codons play a critical role in promoting selective mRNA translation during amino acid starvation. During starvation, in contrast to genes encoding ribosomal proteins whose translation is highly sensitive to amino acid deprivation, translation of genes involved in the cellular protein degradation pathways remains unaffected. We found that these two gene groups bear different codon composition, with non-optimal codons being highly enriched in genes encoding the ubiquitin–proteasome system. Supporting the selective tRNA charging model originally proposed in *Escherichia coli*, we demonstrated that tRNA isoacceptors decoding rare codons are maintained in translating ribosomes under amino acid starvation. Finally, using luciferase reporters fused with endogenous gene-derived sequences, we show that codon optimality contributes to differential mRNA translation in response to amino acid starvation. These results highlight the physiological significance of codon usage bias in cellular adaptation to stress.

Keywords: translation; genetic code; codon bias; tRNA; starvation; adaptation

INTRODUCTION

In a post-genomic era, the importance of translational control in gene expression has been increasingly appreciated (Schwanhaussner et al. 2011). Under adverse conditions, many stress-signaling pathways converge on key translational factors, thereby attenuating global protein synthesis (Sonenberg and Hinnebusch 2009; Spriggs et al. 2010). However, subsets of mRNAs undergo selective translation to produce proteins that are vital for cell survival and stress recovery (Liu and Qian 2014). This stress adaptation is particularly important under nutrient deprivation. In response to scarcity of essential nutrients like amino acids, cells undergo reprogramming to up-regulate cellular degradation pathways including the ubiquitin–proteasome pathway (UPP) and autophagy (He and Klionsky 2009). As a result, efficient degradation of cellular proteins ensures recycling of amino acids when the external supply is lost. Amino acids are building blocks for protein synthesis. It is not surprising that mRNA translation is tightly coupled to nutrient sensing systems such as the mammalian target of rapamycin complex 1 (mTORC1) and the general control nonderepressible 2 (GCN2) kinases (Wek et al. 2006; Ma and Blenis 2009). mTORC1 phosphorylates multiple targets that concertedly

regulate protein metabolism. For instance, through phosphorylation of 4E-binding protein (4E-BP), mTORC1 increases cap-dependent translation (Ma and Blenis 2009; Zoncu et al. 2011). Conversely, through inhibition of Unc51-like kinases 1/2 (Ulk1/2), mTORC1 suppresses autophagy (Mizushima 2010). Upon nutrient deprivation, suppressed mTORC1 signaling not only reduces global protein synthesis but also triggers activation of the autophagy pathway (He and Klionsky 2009). Additionally, phosphorylation of the serine amino acid residue 51 (Ser-51) in the eukaryotic initiation factor 2 α by GCN2 mediates translation control during nutrient deprivation (Dever et al. 1992; Berglanger et al. 1999; Sood et al. 2000). Despite the progress in our understanding of these signaling pathways, it remains puzzling how cells maintain continuous synthesis of proteins essential for the degradation pathway under the limited supply of amino acids. Neither the specific translation-promoting features of these messages nor the regulatory mechanism has been clearly defined.

During translation, ribosomes rely on aminoacylated (charged) tRNAs to read codons in mRNAs. Since there are

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61 sense codons for only 20 amino acids, each amino acid is often encoded by multiple synonymous codons. Notably, synonymous codons are not present in equal frequencies and such codon usage bias is pervasive across species, genomes, as well as individual genes (Plotkin and Kudla 2011). Similarly, tRNA isoacceptors carrying the same amino acid are not uniformly expressed in cells (Bulmer 1987). Recent studies reported differential tRNA expression levels between cellular proliferation and differentiation (Gingold et al. 2014), highlighting the critical role of tRNA concentrations in fundamental biological processes. The concept of codon optimality reflects the balance between the supply of charged tRNAs in the cytosolic pool and the demand of tRNA usage by translating ribosomes. It is generally believed that optimal codons are decoded faster and more accurately by the ribosome than non-optimal codons (Drummond and Wilke 2009). Consistent with this notion, there is a predominant use of optimal codons in highly expressed genes (Sharp and Li 1986; Hershberg and Petrov 2008; Tuller et al. 2010). In contrast, non-optimal codons are postulated to slow down translation elongation. By tuning the elongation rate, codon usage bias has been reported to influence the behavior of nascent chains, including cotranslational folding as well as interaction with the signal recognition particle (SRP) (Pechmann et al. 2014; Yu et al. 2015).

Despite several reported effects, it is not clearly established whether synonymous codon choices imbue certain messages with different translational potential, and whether this can influence their expression during stress conditions, especially during starvation. Intriguingly, amino acid starvation induces selective charging of rare tRNA isoacceptors in *Escherichia coli*, as initially predicted by a theoretical model (Elf et al. 2003) and later confirmed by experimental evidence (Dittmar et al. 2005). Hence it seems likely that the codon composition in eukaryotic genomes could contribute to selective mRNA translation in response to amino acid deprivation. In this study, we have addressed this important question in the context of messages encoding the UPP system. We demonstrate that codon optimality has a broad and powerful contribution to differential translation of these genes in response to amino acid starvation in mammalian cells. To gain mechanistic insights into starvation adaptation in mammalian cells, we took advantage of genome-wide ribosome profiling, global codon usage analysis, quantitative tRNA arrays, and luciferase reporter assays. Our data reveal a previously unappreciated mechanism underlying cellular adaptation to amino acid shortage at the level of mRNA translation.

RESULTS

Differential mRNA translation during amino acid starvation

To determine the global effect of amino acid starvation on mRNA translation, we performed ribosome profiling

(Ribo-seq) on HEK293 cells grown either in complete media or in amino acid-depleted media for 1 h (Gao et al. 2015). Ribo-seq provides a snapshot of ribosome positions on the mRNA at a given time. We computed the relative translation efficiency (TE) of individual transcripts by normalizing Ribo-seq data to mRNA levels measured by RNA-seq. While a large group of genes underwent translational attenuation upon starvation (486 genes with a decrease of more than threefold), a considerable number of genes exhibited translational up-regulation (735 genes with an increase of more than threefold) (Fig. 1A). In line with previous studies (Ingolia et al. 2009; Gao et al. 2015), genes with substantial translational suppression are enriched with ribosome biogenesis and translation as revealed by gene ontology (GO) analysis (Supplemental Fig. S1A). Wilcoxon test showed that the fold change of translation genes is significantly lower than the genome average (0.33 of translation vs. 1.42 of genome, $P < 2.2 \times 10^{-16}$). Genes showing resistance to translational attenuation demonstrated a rather broad category and none of the GO terms were significantly enriched (Supplemental Fig. S1A). However, despite the lack of a significant difference with respect to the genome (Wilcoxon test value 1.40 of UPP genes vs. 1.42 of genome, $P = 0.85$), nearly all the genes encoding UPP and autophagy pathways maintained or up-regulated their translation efficiency during amino acid deprivation (Fig. 1B).

Eukaryotic 26S proteasome is an ATP-dependent protease that works in tandem with the ubiquitin system to break down proteins in the cell (Adams 2003; Murata et al. 2009). The proteasome is a multi-subunit protein, where three subunits have hydrolytic activity as threonine proteases for the cleavage of peptide bonds. These activities are caspase-like activity (or peptidylglutamyl-peptide hydrolyzing activity), trypsin-like activity, and chymotrypsin-like activity, respectively (Murata et al. 2009). Direct measurement of proteasome function showed a rapid increase of chymotrypsin activities in cells upon amino acid withdrawal (Supplemental Fig. S1B). Given the critical role of degradation pathways in recycling internal amino acids, it makes intuitive sense that the up-regulation of UPP and autophagy-related proteins is essential for cell survival during starvation.

To validate the ribosome profiling results, we directly measured the distribution of several UPP transcripts between monosome and polysome fractions in cells with or without starvation (Fig. 1C). A recent study by Heyer and Moore has shown that a certain subset of mRNAs in *Saccharomyces cerevisiae* can be translated by monosomes (Heyer and Moore 2016). However, the study did not show that UPP genes are translated by monosomes. Since it is generally believed that most actively translated mRNAs are enriched in polysomes, whereas inactive mRNAs are associated with monosomes (Arava et al. 2003), we used the polysome to monosome ratio to help us determine whether a particular mRNA is efficiently or poorly translated. As expected, during starvation genes encoding ubiquitin-activating enzymes (UBA1,

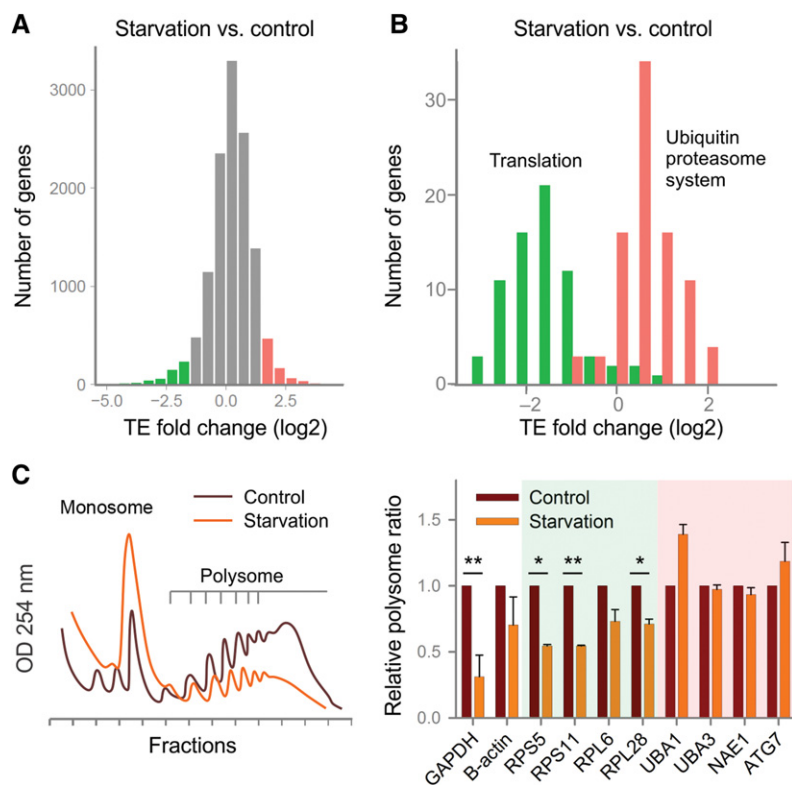


FIGURE 1. Differential mRNA translation during amino acid starvation. (A) HEK293 cells with or without amino acid starvation (1 h) were subjected to Ribo-seq and RNA-seq followed by computation of relative translation efficiency (TE). Distribution of TE fold change upon starvation is presented with up-regulated transcripts ($n = 735$) shown in red and down-regulated transcripts ($n = 486$) shown in green. The TE fold change threshold is set at threefold, which is shown in log₂ scale. (B) TE fold change of transcripts encoding translation (green) and the ubiquitin-proteasome system (red). The genes involved in the ubiquitin-proteasome system and translation pathways were identified based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Supplemental Tables S2, S3). (C) Polysome profile for HEK293 cells with or without amino acid starvation was subjected to sucrose gradient sedimentation. Equal volumes of fractions were analyzed for the distribution of various mRNAs using RT-qPCR. The ratio of polyribosome-associated mRNA relative to the monosome-associated is presented for each gene. Data shown here are representative of three biological replicates. Error bar, $\pm$ SD; (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$, $n = 3$.

UBA3), NEDD8-activating enzyme (NAE1), and autophagy-related gene (ATG7) showed little changes or even increased mRNA levels in the polysome (Fig. 1C; Supplemental Fig. S2). In contrast, genes encoding housekeeping proteins, and ribosomal proteins RPS5, RPS11, RPL6, and RPL28 showed evident reduction of their transcripts in polysome fractions upon amino acid starvation (Fig. 1C; Supplemental Fig. S2). Therefore, translation of UPP mRNA subsets is clearly resistant to amino acid starvation.

Codon optimality contributes to differential mRNA translation during amino acid starvation

Under adverse conditions like amino acid deprivation, translational response mainly occurs at the stage of initiation (Sonenberg and Hinnebusch 2009; Spriggs et al. 2010). However, for mRNAs undergoing selective translation, we

lack a clear understanding of how elongation proceeds under the shortage of charged tRNAs. Having found the differential translation of mRNAs encoding the UPP genes, in contrast to ribosomal genes in response to amino acid deprivation, we considered whether the codon composition of these two gene groups might be different. We examined the relative synonymous codon usage (RSCU) profiles of UPP genes (up-regulated TE) and ribosomal genes (downregulated TE) (Fig. 2A). Using the codon usage frequency in the human genome as a reference, we separated rare codons from common codons. Although common codons exhibit no distinct patterns between the two gene groups, rare codons are more enriched in UPP genes. Some rare codons such as ATA (isoleucine), TTA, and CTA (leucine) are abundant in the majority of UPP genes, but absent in many genes encoding ribosomal proteins. We further confirmed this finding using the tRNA adaptation index (tAI) (dos Reis et al. 2004), an alternative measure of codon usage that is measured by taking into consideration the relative copy number of tRNA isoacceptors (Fig. 2B). The frequency of non-optimal codons is significantly higher in UPP genes than in ribosomal genes (Mann Whitney test, $P < 0.0001$). The frequency of non-optimal codons in UPP genes was also higher than the genome average (Fig. 2B).

Next, we decided to question whether the differential response of UPP vs. ribosomal protein-related genes could be attributed to distinct codon adaptation in response to amino acid limitation. We found that, for both gene groups, starvation-induced changes of translation efficiency are negatively correlated with the codon adaptation index (CAI) ($r = -0.6316$) (Fig. 2C). The CAI value of a gene is the cumulative score of the synonymous codon usage bias of the codons that make up that gene. A higher CAI value indicates a higher frequency of common codons and a higher probability of gene expression (Sharp and Li 1987). A negative correlation between CAI values and starvation-induced TE changes suggested that in both these gene families common-codon enrichment leads to higher sensitivity to starvation (as evident in ribosomal genes). In contrast, rare codon-enriched genes become more resistant to amino acid shortage, thereby achieving selective mRNA translation (as evident in UPP genes). The correlation between starvation-induced translation efficiency and CAI does not hold

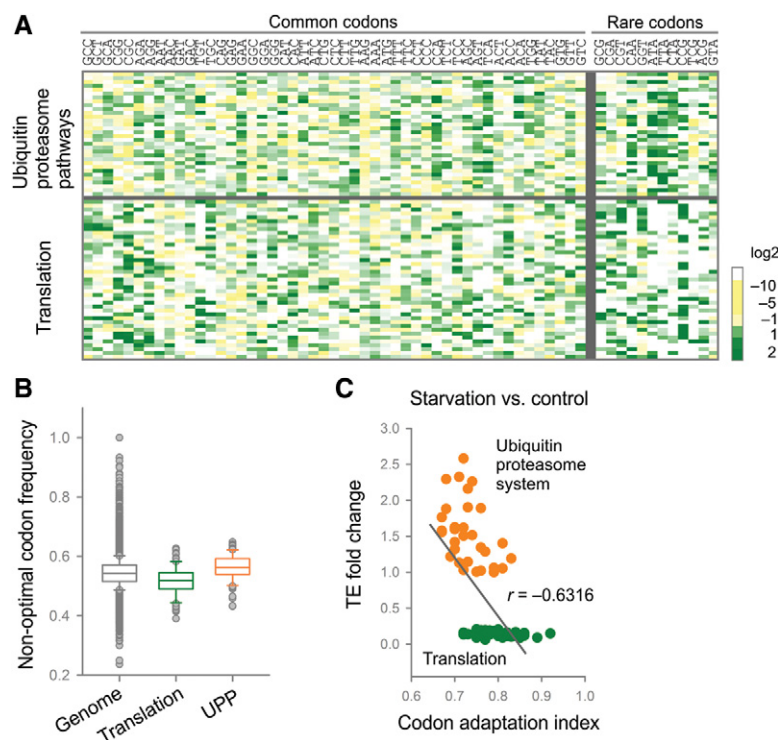


FIGURE 2. Codon composition contributes to differential mRNA translation during amino acid starvation. (A) The relative synonymous codon usage (RSCU) profile for UPP and translation genes is presented as a heatmap. Relative codon usage frequency for each codon in a gene was calculated by dividing the frequency of that particular codon by the total number of synonymous codons under the assumption of equal usage of the synonymous codons for an amino acid. The codons are divided into common and rare categories based on the human codon usage frequency. The absence of a codon is denoted by white. The color change from yellow to green denotes a lower to higher enrichment of a particular codon. (B) Boxplot showing the frequency of non-optimal codons found in the whole genome (whole human transcriptome), UPP genes, and translation genes. The codons are divided into optimal and non-optimal categories based on their tRNA adaptation index (tAI) values. tAI value for the genes was calculated using a previously published algorithm (dos Reis et al. 2004). (C) Scatterplot showing the correlation between codon adaptation index (CAI) and TE fold change upon starvation for UPP and translation genes. The Pearson correlation coefficient is shown in the plot. TE fold change for each gene was generated from Ribo-seq data. CAI values were calculated using Biopython package.

for the entire genome (Supplemental Fig. S3). This is not surprising because synonymous codon usage patterns are created in the genome to reflect the action of natural selection (Plotkin and Kudla 2011). The relationship between TE and codon bias observed in UPP and ribosomal genes is perhaps an adaptive response to starvation acquired through evolution.

Rare tRNAs are enriched in elongating ribosomes during amino acid starvation

How does the presence of rare codons promote mRNA translation during amino acid starvation? We wondered if it is possible that the messages containing more non-optimal codons are less sensitive to the limited supply of charged tRNA molecules. According to the “selective tRNA charging” model, proposed and validated for *E. coli* (Elf et al. 2003), a gen-

eral prediction is that the charged levels of tRNA isoacceptors depend on the ratios between their total concentrations and the frequencies at which their cognate codons appear in the transcriptome. When the supply of amino acids becomes rate limiting, the charged levels of certain tRNA isoacceptors decoding common codons will approach zero, but not the isoacceptors for rare codons. To provide direct evidence that this theory also holds in mammalian cells, we examined ribosome-associated tRNA levels using a microarray method. Similar to ribosome profiling, we collected RNase I-digested polyribosome samples followed by isolation of tRNA species occupying E, P, and A sites of the ribosome (Supplemental Fig. S4A). In parallel, we purified total tRNA molecules from whole cell lysates as control. Hybridization of radio-labeled tRNA samples to specific probes revealed relative ratios of charged tRNA molecules in translating ribosomes. While cell lysates contained both nuclear and mitochondrial tRNA species, ribosome samples were highly enriched with nuclear-encoded tRNAs (Supplemental Fig. S4B). Therefore, ribosome-associated tRNA species represent the pool of tRNA isoacceptors actively engaging in translation. To further validate that the tRNA microarrays are an accurate depiction of tRNAs associated with A/P/E ribosome sites, we performed the microarray experiment on RNA obtained from total cell lysate (total RNA), polysome fractions of cells treated with

cycloheximide (CHX), and monosome fractions of cells treated with lactimidomycin (LTM). LTM treatment freezes ribosomes in the initiation step of translation (Lee et al. 2012). The microarray clearly shows an enrichment of initiator-tRNA^{Met} in the LTM-treated sample (Supplemental Fig. S5).

Next we used the microarrays to compare the landscape of ribosome-associated tRNAs from cells with and without amino acid starvation (Fig. 3A). Although there was no clear tRNA pattern clustered by amino acids, we observed remarkable differences of tRNA isoacceptors decoding the same amino acid (Fig. 3B). For instance, rare tRNAs leucine isoacceptors, tRNA^{Leu} (UAA1/2), showed increased signals in starvation samples, whereas the most abundant leucine isoacceptor, tRNA^{Leu} (CAG), exhibited a concomitant decrease. The similar trend holds true for isoacceptors of tRNA^{Ser}, tRNA^{Thr}, and tRNA^{Val}. In particular, there is a strong inverse

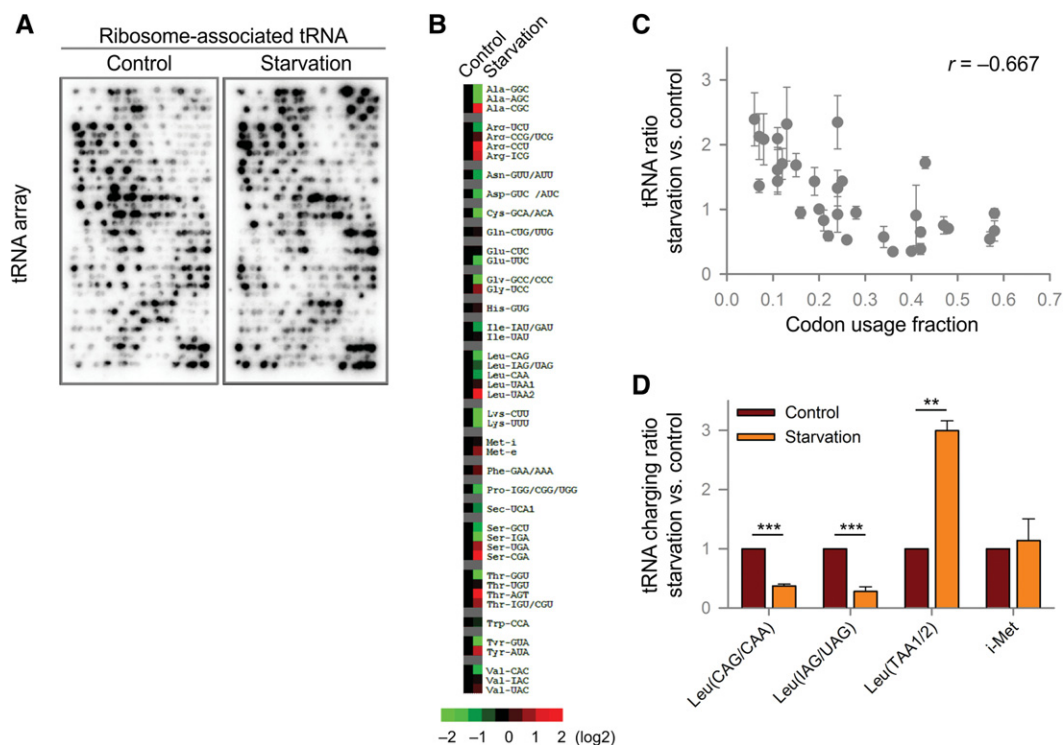


FIGURE 3. tRNA isoacceptors decoding rare codons are selectively enriched in translating ribosomes during amino acid starvation. (A) Representative tRNA microarray results obtained from HEK293 cells with or without amino acid starvation. (B) Heatmap showing enrichment of specific tRNAs associated with translating ribosomes during amino acid starvation. The isoacceptors for each tRNA are arranged in the decreasing order of their frequency from *top to bottom*. The signal obtained for each tRNA during starvation is normalized to the control. The heatmap plots the $\log_2$ of normalized tRNA enrichment values. The color change from green to red signifies an increased association with the ribosome. This heatmap is representative of two biological replicates (Supplemental Fig. S4C). (C) Scatter plot showing the correlation between tRNA isoacceptor signals after starvation and the codon usage fraction. tRNAs without isoacceptors are not included in this analysis. The Pearson correlation coefficient is shown in the plot. (D) tRNA charging levels of tRNA isoacceptors in control and starvation conditions. The assay used for this qRT-PCR-based analysis was adapted from Loayza-Puch et al. (2016). Primers used for qPCR are described in Materials and Methods. Error bar, $\pm$ SD; (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$.

correlation between the changes of tRNA isoacceptors and their codon usage fractions (Fig. 3C). Therefore, the abundant tRNA isoacceptors are more likely depleted from translating ribosomes than the rare tRNAs under limited amino acid supply. This result is consistent with the observation in *E. coli* where tRNA isoacceptors that read rare codons retain high charging levels during starvation (Dittmar et al. 2005). As an additional test we measured the charging level of various tRNA^{Leu} isoacceptors in control and amino acid starved cells using a qRT-PCR-based method (Loayza-Puch et al. 2016). Our results show that the charging level of all the abundant isoacceptors decreased while the charging level of rare isoacceptors tRNA^{Leu}(UAA1/2) increased during amino acid starvation (Fig. 3D). The level of charged initiator-methionine tRNA (i-Met) remained the same upon starvation, as observed in our array data (Fig. 3B,D). The tRNA charging data further validate our hypothesis that during starvation rare tRNA isoacceptors are enriched in translating ribosomes, and this causes the translation of messages containing rare codons to be relatively resistant to amino acid deprivation.

Codon composition influences translation efficiency during amino acid starvation

To further demonstrate the correlation between codon usage and translational potential during amino acid starvation, we constructed firefly luciferase (Fluc) reporters by fusing an extra 15-codon sequence at the NH₂ terminus (Fig. 4A). Sequences containing rare codons were chosen from UPP genes (NAE1 and UBA3), whereas sequences enriched with common codons were derived from genes encoding ribosomal proteins (RPL41 and RPL28). Consistent with the behavior of endogenous genes, chimeric reporters bearing either NAE1 or UBA3 sequences maintained Fluc levels in transfected cells exposed to amino acid starvation (Fig. 4B). In contrast, reporters bearing the RPL41 or RPL28 sequences showed reduced Fluc levels during amino acid deprivation. Therefore, addition of sequences with different codon usage leads to a distinct translational response to amino acid shortage.

We next swapped the synonymous codons in these reporters to validate the role of codon composition in starvation-induced translational regulation. Without changing

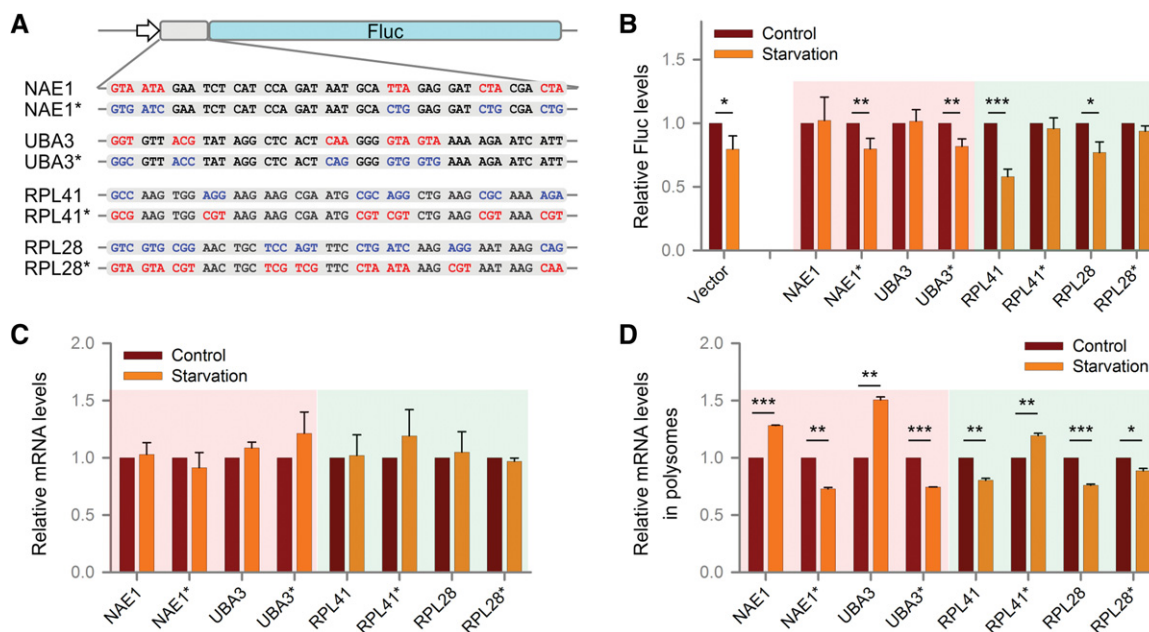


FIGURE 4. Rare codons contribute to differential mRNA translation during amino acid starvation. (A) Fluc reporters were constructed by fusing a 15-codon sequence derived from UPP (UBA3 and NAE1) or ribosomal protein (RPL41, RPL28) genes in the amino termini. Certain rare codons in the UPP genes were changed to common codons while certain common codons in the ribosomal proteins were changed to rare codons without changing the encoded amino acid (mutants denoted by asterisk). The codon features are color-coded with red for rare codons and blue for common codons. (B) HEK293 cells were transfected with plasmids encoding the Fluc reporters illustrated in A followed by amino acid starvation. The relative Fluc activities under starvation are normalized by control. (C) The relative mRNA level of all the Fluc reporters was determined using qRT-PCR during control and amino acid starvation. The forward and reverse primers used for PCR covered a region of the inserted gene sequence and a region of the luciferase sequence, respectively. The primer sequences are included in Supplemental Table S1. (D) Polysome profile distribution was determined for each Fluc reporter mRNA during control and amino acid starvation. Equal volumes of fractions were analyzed for the distribution of the target mRNAs using RT-qPCR. The ratio of polyribosome-associated mRNA fraction relative to the monosome-associated fraction is presented for each gene. The primers used are the same as described in C. (B, C, D) Error bar, $\pm$ SD; (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$; $n = 3$.

the encoded amino acid, we replaced the rare codons with the common codons in reporters containing sequences derived from UPP genes (Fig. 4A). Remarkably, these mutant reporters regained the translational response after amino acid starvation by showing reduced Fluc levels in transfected cells (Fig. 4B). Furthermore, when the common codons of RPL41 or RPL28 were changed to the corresponding rare codons, these reporters maintained their translation efficiency in transfected cells upon amino acid deprivation (Fig. 4B). Additionally, we tested the total mRNA levels of all four reporters in control and starvation conditions; our data showed that the total mRNA levels for all of them remain unaltered during starvation (Fig. 4C). The polysome distribution of the reporter mRNAs during control and amino acid starvation exhibited the same pattern as observed for the native mRNAs (Fig. 4D; Supplemental Fig. S6). Together, these results firmly establish the critical role of codon optimality in translational adaptation in response to starvation.

DISCUSSION

Codon usage bias is a universal feature of all genomes and has been proposed to regulate translation efficiency, accuracy, mRNA stability, and protein folding (Plotkin and Kudla

2011; Presnyak et al. 2015). Although it is widely believed that codons adapted to tRNA pools might be preferentially used in highly expressed genes, the precise nature of fitness gain associated with translational adaptation remains a topic of active debate. Despite the fact that “codon optimization” might lead to enhanced protein production, maintaining rare codons in genomes could serve beneficial purposes in gene expression. Two recent studies using *Neurospora* and *Synechococcus elongate* reported that rare codons present in circadian genes play a critical role in maintaining the circadian rhythm (Xu et al. 2013; Zhou et al. 2013). In yeast cells, genes encoding membrane and secretory proteins are enriched with rare codons at positions critical for SRP recognition (Pechmann et al. 2014). All these phenomena rely on rare codon-mediated translation slowdown that potentially influences co-translational folding (Yu et al. 2015). Our present work demonstrates a surprising role for non-optimal codons in promoting mRNA translation during amino acid starvation. Our findings not only expand the functional role of codon usage but also uncover an important cellular adaptation mechanism in response to amino acid deprivation.

Amino acids are essential cellular nutrients, hence it is imperative for cells to adapt to amino acid deprivation via

multiple mechanisms. Up-regulation of the UPP pathway ensures efficient protein degradation and recycling of amino acids when the external supply is limited (He and Klionsky 2009). We have previously reported that UPP mRNAs maintained their translation potential during amino acid starvation (Gao et al. 2015), although the underlying mechanism remained elusive. It has been suggested that by sensing the presence of nutrients, activated mammalian TOR complex 1 (mTORC1) signals to various components of the translation initiation machinery to regulate cap-dependent translation (Gingras et al. 2004; Hay and Sonenberg 2004). mTOR-dependent translation reprogramming in vivo has been studied by using ribosome profiling (Hsieh et al. 2012). Treatment of pancreatic cancer cells with mTOR inhibitor PP242 and rapamycin affected the translation efficiency of several target mRNAs. UPP mRNAs were not identified in this group of mTOR responsive genes. It is possible, however, that the decreased polysome concentration and reduced TE of the ribosomal genes could be the result of diminished mTOR signaling. However, our data strongly indicate that the nature of codons present in the mRNAs can also play an important role in the translation of these mRNAs, especially during amino acid starvation.

Additionally, computational simulation studies suggest that initiation is rate-limiting under normal growth conditions but elongation becomes the limiting step under severe amino acid starvation (Firczuk et al. 2013; Racle et al. 2013; Shah et al. 2013). In *E. coli*, genes encoding amino acid biosynthetic enzymes preferentially use codons that are poorly adapted to the typical pool of charged tRNAs, but are well adapted to starvation-induced tRNA pools (Dittmar et al. 2005). We, for the first time, demonstrate the presence of a similar mechanism in mammalian cells, by which UPP mRNAs are selectively translated during amino acid starvation. Our results reveal a coordinated regulation between amino acid availability, tRNA charging, and selective mRNA translation. It highlights the physiological significance of codon usage bias in cellular adaptation and survival.

MATERIALS AND METHODS

Cell culture

Human embryonic kidney 293 (HEK293) cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. For amino acid starvation treatments, the cells were grown in Hank's balanced salt solution (HBSS) supplemented with 10% dialyzed fetal bovine serum. Cells were treated with 100 μ g/mL cycloheximide (CHX) for 3 min prior to ribosome profiling (Ribo-seq) experiments.

Ribosome profiling and data analysis

Ribo-seq and RNA-seq were performed based on the reported protocol (Gao et al. 2015). Briefly, cells were treated with CHX (100 μ g/mL) for 3 min at 37°C and then lysed in polysome buffer

(pH 7.4, 10 mM HEPES, 100 mM KCl, 5 mM MgCl₂, and 100 μ g/mL CHX) by vortexing for 20 sec using Lysing Matrix-D (Fisher) for five times with an interval of 40 sec on ice. The cleared lysates were separated by sedimentation through sucrose gradients (15%–45% w/v). Collected polysome fractions were digested with RNase I and the ribosome protected fragments (RPFs) were size selected and purified by gel extraction. After library construction, deep sequencing was performed using Illumina HiSeq2000. The trimmed RPF reads were first mapped by Tophat to transcriptome (Ensembl release 70). Non-uniquely mapped reads were disregarded for further analysis due to ambiguity. The 13th position (12 nt offset from the 5' end) of the uniquely mapped read was defined as the ribosome "P-site" position. The quantification of P-site was completed by mapping uniquely mapped reads to each individual mRNA transcript according to the NCBI Refseq gene annotation. Thirty-one and 39 million Ribo-seq reads were obtained for control and amino acid (AA) starvation, respectively, similarly 10 and 16 million reads were obtained for RNA-seq. These uniquely mapped reads were counted to calculate the mRNA and ribosome footprint RPKM values for all transcripts. For each transcript, the translation efficiency (TE) was calculated by dividing ribosome footprint RPKM by the corresponding mRNA RPKM. The fold change of translation efficiency was calculated by the ratio of TE in control vs. the TE in AA starvation. The genes with TE fold change >2 and <0.5 were used for Gene Ontology analysis (GO analysis). GO analyses were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID). GO term enrichment was summarized and visualized with REVIGO.

Global codon usage calculation and other data analysis

Structures of Refseq protein-coding genes were downloaded from the UCSC Genome Browser. To create the relative synonymous codon usage (RSCU) profile for individual translation and UPP-related genes, the transcript isoform with the longest coding sequence (CDS) length was selected as the representative transcript. Each of the 61 sense codons was counted, respectively, on individual mRNA CDS, and the individual codon count was later divided by the total number of codons of CDS to obtain relative codon usage. RSCU value for every codon was calculated under the assumption of equal usage of the synonymous codons for an amino acid. tRNA adaptation index (tAI) calculations were performed according to previously published protocol (dos Reis et al. 2004). CAI values for the genes were calculated using the Biopython package. The statistical analysis throughout the manuscript was performed using Statistical Package R, and all other computation analysis was performed using in-house programming.

Isolation and labeling of tRNA from RPFs

HEK293 cells grown in control and starvation media were treated with CHX (100 μ g/mL for 3 min) and ribosome-protected fragments (RPFs) were isolated as described in Ribo-seq experiments. Total RNA was obtained from RPFs using TRIzol (Invitrogen) according to the manufacturer's protocol. Total RNA (5 μ g) was labeled with radioactive γ -³²P-ATP and separated on 10% TBE-urea acrylamide gels. The tRNA band on the gel was visualized with a phosphor-imager and excised based on the expected size. The tRNA was then eluted in 450 μ L of crush and soak buffer (50 mM KOAc, 200 mM KCl, pH 7.5) with rocking at 4°C overnight. After

centrifugation, ethanol precipitation was performed to obtain the clean tRNA samples.

Hybridization and microarray analysis

Hybridization and microarray analysis was performed based on previously reported protocols (Saikia et al. 2012). Briefly, the labeled tRNA sample was first dissolved in microarray hybridization buffer (Sigma-Aldrich) containing 20 µg of salmon sperm DNA and 10 µg of poly(A). This mixture was then applied to the hybridization chamber of the GeneTAC Hyb4 station (Genomic Solutions). The following program was used for hybridization: 75°C (2 min), 60°C (probe introduction), 90°C (5 min), and 60°C hybridization for 16 h. Following hybridization, the slides were washed on the Hyb4 station twice with 2× SSC, 0.1% (w/v) SDS at 50°C, twice with 0.1× SSC, 0.1% (w/v) SDS at 42°C, and twice with 0.1× SSC at 42°C. Slides were then removed from the station, rinsed with 0.1× SSC, and dried by centrifugation. The slides were then exposed to the phosphor-imager to visualize the signals deriving from the hybridized tRNAs. Microarray slides were imaged using a FUJI BAS scanner. ³²P intensities were quantified and corrected for background noise using Fuji BAS software. The median values for eight replicate spots were obtained for each tRNA and divided by the median value of the two added tRNA standards (*E. coli* tRNA^{Lys} and yeast tRNA^{Phe}) to obtain the normalized signal for each tRNA in a particular sample.

Luciferase assay

Cells were transfected with the luciferase reporter for 24 h followed by splitting equally in two wells of a six-well plate for another 24 h incubation. The cells were then subjected to either complete (control) or amino acid depleted media (starvation) for 1 h. Cell pellets were lysed in reporter lysis buffer (Promega) followed by centrifugation to clear the lysates. Luminescence reactions were initiated with Promega DLR (100 µL; Promega) added to the lysates (30 µL). Luciferase activities were measured using a Synergy 2 Luminescence Plate Reader (Biotek).

Proteasome activity assay

HEK293 cells were plated equally (10,000 cells per well) in 96-well white-walled plates. The wells were subjected to amino acid starvation conditions for various time points (0, 0.5, 1, 2, and 3 h, respectively). Proteasome-Glo Cell-Based Reagent (Promega, catalog # G8660) was prepared as per manufacturer's protocol and an equal volume was added to each well. The content of the plate was mixed at 700 RPM for 2 min and then incubated at room temperature for 10 min. Luminescence was read using a Synergy 2 Luminescence Plate Reader (Biotek).

tRNA-charging assay

The tRNA-charging assay protocol was adapted from a recent publication (Loayza-Puch et al. 2016). Briefly, RNA was isolated using acetate-saturated phenol/CHCl₃ (pH 4.8). Precipitated RNA was resuspended in 10 mM NaOAc/HOAc (pH 4.8). Samples were split in two, one half (5 µg) was oxidized with 50 mM NaIO₄ for 30 min at room temperature and the other half (5 µg) was incubated in 50 mM

NaCl. Samples were quenched with 100 mM glucose for 5 min at room temperature, purified in G25 columns (GE Healthcare), and then ethanol precipitated. tRNAs were deacylated in 50 mM Tris-HCl (pH 9) for 30 min at 37°C. RNA was precipitated and then ligated to the 3' adaptor (5'-5rApp/TGGAATTCTCGGGTGCCAA GG/3ddC/-3') using T4 RNA ligase 2 (NEB) for 4 h at 37°C. Relative aminoacylation levels were calculated by qRT-PCR using tRNA-specific primers. Due to similar sequences in some cases, multiple tRNA isoacceptors were detected using the same primer. Primer sequences are as follows: reverse primer, GCCTTGGC ACCCGAGAATTCCA; tRNA Leu(CAG/CAA) primer, GTCAGGA TGGCCGAGCGGTC; tRNA Leu(IAG/UAG), GGTAGCGTGGCC GAGCGGTC; tRNA Leu(TAA1/2), ACCAGGATGGCCGAGTGG T; i-Met, AGCAGAGTGGCGCAGCG.

Additional methods

Total RNAs from fractions of polyribosome analysis were prepared using TRIzol (Invitrogen). mRNA levels were monitored by RT-qPCR using Power SYBR Green PCR Master Mix (Applied Biosystems). The primer sets used for qPCR are included in Supplemental Table S1.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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