Translational Tolerance of Mitochondrial Genes to Metabolic Energy Stress Involves TISU and eIF1-eIF4G1 Cooperation in Start Codon Selection

Graphical Abstract

Highlights

- TISU, translation initiator of short 5' UTR, is enriched among mitochondrial genes
- TISU-mediated initiation involves eIF1-eIF4G1 cooperation and cap complex eviction
- Low energy induces a stress response that includes inhibition of global translation
- TISU-directed translation enables continuous translation under energy stress

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In Brief

The regulatory element TISU is a translation initiator of short 5' UTR mRNAs. Sinvani et al. report that TISU is enriched in mitochondrial genes and confers resistance to translation inhibition mediated by AMPK and energy deprivation, allowing cells to cope with the stress. TISU-mediated initiation involves cap complex eviction and eIF1-eIF4G1 cooperation.

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TRANSLATIONAL TOLERANCE OF MITOCHONDRIAL GENES TO METABOLIC ENERGY STRESS INVOLVES TISU AND eIF1-eIF4GI COOPERATION IN START CODON SELECTION

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SUMMARY

Protein synthesis is a major energy-consuming process, which is rapidly repressed upon energy stress by AMPK. How energy deficiency affects translation of mRNAs that cope with the stress response is poorly understood. We found that mitochondrial genes remain translationally active upon energy deprivation. Surprisingly, inhibition of translation is partially retained in AMPKα1/AMPKα2 knockout cells. Mitochondrial mRNAs are enriched with TISU, a translation initiator of short 5′ UTR, which confers resistance specifically to energy stress. Purified 48S preinitiation complex is sufficient for initiation via TISU AUG, when preceded by a short 5′ UTR. eIF1 stimulates TISU but inhibits non-TISU-directed initiation. Remarkably, eIF4GI shares this activity and also interacts with eIF1. Furthermore, eIF4F is released upon 48S formation on TISU. These findings describe a specialized translation tolerance mechanism enabling continuous translation of TISU genes under energy stress and reveal that a key step in start codon selection of short 5′ UTR is eIF4F release.

INTRODUCTION

Intracellular energy status is critical for most biological activities and therefore is under tight control. Perturbations in energy metabolism are associated with disease including cancer and the metabolic syndrome (Dazert and Hall, 2011; Laplante and Sabatini, 2012). Protein synthesis is one of the most energy-consuming cellular processes and metabolic stresses that reduce intracellular energy availability induce rapid inhibition of cap-dependent translation in order to save energy consumption. This response is mediated by the AMP-activated protein kinase (AMPK), a highly conserved sensor of the cellular energy status and an important target for drugs used against type 2 diabetes (Zhang et al., 2009). AMPK stimulates ATP production pathways, mostly by enhancing mitochondrial biogenesis (Cantó et al., 2010; Jäger et al., 2007; Lin et al., 2005; O’Neill et al., 2011; Winder et al., 2000). In parallel, AMPK switches off anabolic pathways that consume ATP such as protein synthesis by inhibiting cap-dependent translation (Bolster et al., 2002; Dubbelhuis and Meijer, 2002; Krause et al., 2002; Reiter et al., 2005). In contrast, translation of mRNAs of proteins that are required for the energy stress response must be maintained. Presently, the features of cellular mRNAs and the mechanism underlying translational resistance to energy stress are largely unknown.

An important element that controls translation initiation efficiency is the AUG context (Hinnebusch, 2011). Also, translation initiation is often regulated by the 5′ UTR, in particular the length (Kozak, 1991a, 1991b, 1991c), the presence of stem-loop structures (Kozak, 1986; Pelletier and Sonenberg, 1989), and the existence of AUG(s) upstream of the main ORF (uAUGs) (Calvo et al., 2009; Kozak, 1984). Efficient recognition of the AUG initiation codon requires 5′ UTR length of at least 20 nucleotides (Kozak, 1991b). Shorter 5′ UTRs generally exhibit leaky translation initiation (Kozak, 1991a, 1991b; Sedman et al., 1990). Previously we identified and characterized an element called TISU (translational initiator of short 5′ UTR), located between the transcription start site up to position +30, which, remarkably, controls both transcription and translation (Elfakess and Dikstein, 2008). TISU is present in ~4% of all genes, preferentially in those with “housekeeping” functions. TISU controls transcription and is bound by the YY1 transcription factor. The core of TISU consists of an invariable ATG sequence, which serves also as the exclusive translation initiation codon in 80% of TISU genes. In the rest of TISU genes the AUG of the element initiates an uORF. This initiating AUG is preceded by an unusually short 5′ UTR with a median length of 12 nucleotides (Elfakess and Dikstein, 2008). Detailed analysis of TISU established it as an element optimized to direct efficient translation initiation from mRNAs with a 5′ UTR as short as 5 nucleotides (Elfakess and Dikstein, 2008; Elfakess et al., 2011). Besides the −3A and the +4G, additional flanking sequences, including positions −2 and −1 and the nucleotides in positions +5 to +8, are unique to TISU and cooperate to direct accurate and efficient translation initiation from very short 5′ UTRs. Importantly, TISU activity is cap dependent (Elfakess et al., 2011). These findings suggest a translation initiation mode that is cap dependent but independent of scanning (Elfakess et al., 2011). Considering its unique features, it is
The process of translation initiation occurs in several mechanistic steps (for review, see Hinnebusch, 2011; Jackson et al., 2010; Sonenberg and Hinnebusch, 2009) that include the formation of a 43S preinitiation complex comprising the 40S subunit, eIF1, eIF1A, eIF2-GTP-Met-tRNAmeti, and probably eIF5. This complex is then recruited to the mRNA through the cap-bound eIF4F. Subsequently the 43S complex scans the 5’ UTR as an “open” complex until an initiation codon is encountered. Initiation complex formation involves a switch of the scanning conformation to a “closed” one followed by eIF5-mediated release of eIF2-bound P1 along with eIF1 (Cheung et al., 2007; Maag et al., 2005; Martin-Marcos et al., 2014). Initiation is then completed by the joining of the 60S subunit to the 48S complex and concomitant displacement of eIF2-GDP and other factors (eIF3, eIF4B, eIF4F, and eIF5). Studies in yeast unraveled the critical roles that eIF1 plays in ternary complex recruitment and in discrimination against near-cognate AUG start codons recognition (Cheung et al., 2007; Martin-Marcos et al., 2011, 2013; Mitchell and Lorsch, 2008; Nanda et al., 2009). Consistent with the high conservation of eIF1, it was shown to inhibit initiation from suboptimal AUG contexts in higher eukaryotes as well (Elfakess et al., 2011; Ivanov et al., 2010; Pestova and Kolupaeva, 2002). Moreover, canonical cap-dependent translation imposes a seemingly strong constraint on TISU-mediated translation by positioning eIF4F and the AUG-48S initiation complex in an overlapping location. To what extent the mechanistic events associated with the initiation process directed by TISU resemble or differ those of the canonical pathway is presently unknown.

Here we investigated the molecular mechanism and the physiological importance of TISU-mediated translation initiation. Our findings revealed that eIF1 and eIF4GI cooperate to facilitate TISU-mediated translation initiation. We also demonstrate that a key step in start codon selection of short 5’ UTR mRNAs is eIF4F release. The unique mechanism directed by TISU operates in energy-producing genes and confers resistance to inhibition of cap-dependent translation in response to energy stress.

RESULTS

Sequence-Specific Recognition of TISU by the Basal Translation Apparatus

When an initiator AUG is preceded by a very short 5’ UTR, it is frequently bypassed by the 48S ribosome, resulting in leaky translation initiation (Elfakess and Dikstein, 2008; Kozak, 1991b). An exceptional paradigm is an AUG in the context of the TISU sequence (Elfakess and Dikstein, 2008; Elfakess et al., 2011). We analyzed translation initiation from an AUG in either a canonical Kozak context or the TISU element, preceded by a very short 5’ UTR. Several cell lines were transfected with in vitro synthesized GFP mRNA (Figures 1A and S1) in which translation from the upstream AUG yields a 30 kDa protein, while leaky translation initiation from an in-frame downstream AUG yields a 27 kDa protein. Translation from the canonical upstream AUG varied significantly between different cell types, constituting approximately 45%, 20%, and 15% of total translation in 293T, HeLa, and MEFs cells, respectively, while being undetectable in HepG2 cells (Figure 1A, right lanes and graph). In stark contrast, TISU-mediated translation started almost exclusively from the upstream AUG in all the cell lines (Figure 1A, left lanes and graph). These findings suggest that leaky translation initiation is highly variable and has a potential to generate alternative protein products in a cell-type-dependent manner. On the other hand, accurate translation initiation directed by TISU is robust and is unaffected by differences in the cellular milieu.

The ability of TISU to confer translation initiation fidelity in different cellular environments raises the question of whether the basal translation machinery has intrinsic capability to initiate from an AUG in a TISU context. To address this issue, we examined whether TISU-mediated initiation can be reconstituted in vitro only with basal components of the translation apparatus. We used a translation initiation system containing initiator Met-tRNAi; purified 40S ribosomal subunit; purified eIFs that include eIF1, eIF1A, eIF2, eIF3, eIF4F, and ATP; and GMP-PNP, a non-hydrolysable GTP analog that stalls the 48S ribosomal complex on the initiation site. The formation of the 48S complex at the initiator AUG was detected by inhibition of primer extension with reverse transcriptase due to ribosome binding (toe-printing) (Kozak, 1998). In the presence of all the components, the short 5’ UTR mRNA bearing the TISU element generated a strong toe-print ~15 nt downstream of the AUG, which corresponds to the position of the leading edge of the small ribosomal subunit (Figure 1B, compare lane 1 to lane 2). In contrast, ribosome binding directed by the canonical AUG element was very weak and could be observed only after a prolonged exposure (Figure 1B, lanes 7 and 8). This result demonstrates that the TISU sequence is sufficient for efficient AUG recognition by the translation machinery and that no additional cellular factor is required for the basal activity of TISU.

To determine the minimal set of translation initiation factors, which are required for TISU-containing mRNAs, we performed toe-print reactions in the absence of individual factors (Figure 1B). As expected, TISU-mediated translation initiation is stimulated by eIF2 (lane 16), initiator tRNA (lane 17), eIF3 (lane 15), and eIF4F (lane 3). Reduction of the 48S complex formation by eIF4F and eIF3 omission was not complete, most probably due to cross-contamination of these factors (Méthot et al., 1996). Interestingly, removal of eIF1 decreased initiation from the TISU AUG, which was unexpected, considering the previous studies demonstrating that eIF1 inhibits translation from an AUG preceded by a short 5’ UTR (Cheung et al., 2007; Mitchell and Lorsch, 2008; Nanda et al., 2009; Pestova et al., 1998; Pestova and Kolupaeva, 2002). Exclusion of both eIF1 and eIF1A resulted in loss of translation initiation (Figure 1B, lanes 6 and 12). This is consistent with a previous report on the redundant activities of the two factors in vitro (Pestova et al., 1998). Interestingly, omission of both eIF4F and eIF1 had much greater effect on 48S formation than omission of eIF1A and eIF4F (Figure 1C).

eIF1 and eIF4GI Cooperate to Control Translation Initiation Fidelity and Ribosomal Scanning

The differential effects of eIFs on TISU and non-TISU AUG in vitro prompted us to examine the relevance of these findings to translation in vivo. We first knocked down eIF1 using siRNA, and 48 hr later cells were transfected with GFP reporter constructs containing either a TISU or a canonical AUG context preceded by a short 5’ UTR (US AUG) followed by an in-frame downstream
AUG (DS AUG), or by a long unstructured 5’ UTR as shown schematically in Figure S2 (available online). With a knockdown (KD) efficiency of ~60% of elf1 protein, TISU US-AUG level was reduced by ~35%, consistent with the in vitro results, while the canonical US-AUG was slightly increased (~20%) (Figure S2B). Translation of the mRNA containing the long 5’ UTR was marginally affected, which is unexpected, considering that elf1 promotes ribosomal scanning (Hinnebusch, 2011; Mitchell and Lorsch, 2008). We found in the human genome database (http://genome.ucsc.edu) that elf1 has a highly similar paralog called elf1B that is expressed as abundantly as elf1. We therefore knocked down either elf1B alone or the two human paralogs of elf1 together, and 48 hr later expressed the GFP reporter genes described above. With the elf1B siRNA treatment, elf1 levels were barely changed and this was nevertheless associated with a modest differential effect on TISU and canonical AUG (Figure S2C), as seen with the KD of elf1. Upon elf1 and elf1B double siRNA transfection, the KD efficiency was increased and led to a clear and more dramatic differential effect the role of elf1 in scanning (Pestova and Kolupaeva, 2002). In contrast to the canonical US-AUG, TISU US-AUG levels were greatly diminished, without concomitant increase of leaky translation. These findings bolster the idea that elf1 facilitates TISU-mediated translation and is a major player in its potency.

We also examined the roles of elf4GI and elf3c, subunits of elf4F and elf3, respectively, on translation initiation. Strikingly, elf4GI depletion caused the same phenotype as elf1 KD with respect to the different AUG contexts and 5’ UTR lengths (Figure 2B). Specifically, in elf4GI-deficient cells, the relative translation of the canonical US-AUG to the DS-AUG was increased while TISU US-AUG was almost abolished. Similar to elf1, elf4GI depletion caused the reduction of translation from the unstructured long 5’ UTR, indicating its role in promoting ribosomal scanning. To examine whether the similarity of the KD effects of elf4GI and elf1 is associated with indirect effect on elf1 expression, we analyzed elf1 levels in elf4GI KD cells and found that its expression was unaffected (Figure 2B). Downregulation of elf3c, on the other hand, diminished translation irrespective of AUG (Figure 2A). The ratio of the canonical US-AUG to the DS-AUG was increased, suggesting that leaky scanning was inhibited. In addition, translation from the long 5’ UTR which is dependent on ribosomal scanning was reduced, consistent with...
context and 5’ UTR length (Figure S2D). These findings raise the possibility that eIF1 and eIF4GI functionally cooperate to facilitate translation initiation fidelity of TISU and non-TISU mRNAs as well as ribosomal scanning. This possibility is supported by the observation that omission of both eIF4F and eIF1 cooperate for efficient TISU-mediated translation in vitro (Figure 1C).

To assess the effects of eIF1/eIF1B, eIF4GI, and eIF3c KD on global translation, we subjected cell lysates from siRNA-treated cells to polysome profiling. While eIF1/eIF1B double KD did not cause visible effect on global translation, depletion of either eIF4GI or eIF3c resulted in accumulation of 80S monoribosome and a decrease in polysomes, indicating for global inhibition of translation initiation (Figure S3). To examine the influence of initiation factor depletion on translation of specific genes, we pooled the fractions from the free, the light, and the heavy polysomes; extracted their RNA; and performed RT-qPCR analysis of six non-TISU and eight TISU genes. KD of eIF1/eIF1B and eIF3c caused modest reduction of translation of most TISU and non-TISU genes (Figure S3). The mild effects of eIF1 and eIF3c depletion may be a consequence of insufficient KD or compensation by other factors with redundant activities such as eIF1A (see Figure 1B). Downregulation of eIF4GI had a more notable effect on translation of all the examined genes, consistent with its general role in translation (Figure S3). To investigate the underlying basis for the shared activities of eIF1 and eIF4GI on initiation site selection, we examined whether they can interact with each other as shown for the yeast homologs (Asano et al., 2000; He et al., 2003; Valásek et al., 2002). Initial coimmunoprecipitation (coIP) experiments with endogenous proteins failed to reveal an association between the two proteins. Considering that such an interaction may be transient, we carried out coIP following overexpression of HA-tagged eIF1 or, as a control, HA-eIF1A. The results revealed efficient coprecipitation of endogenous eIF4GI with HA-eIF1, but not with HA-eIF1A or control antibodies (Figure 2C).
To determine whether the eIF1-eIF4GI interaction is direct, we carried out GST pull-down assays, in which eIF1 and eIF1A were fused to GST, expressed in bacteria, purified, and subjected to in vitro binding reaction with recombinant His-tagged eIF4GI fragments (Figure S4A). The results revealed an interaction between eIF1 and the middle domain of eIF4GI (amino acids 674–1,129) and a weak interaction with the most C-terminal region (amino acids 1,130–1,599) (Figure S4A, left). These interactions are highly specific, as similar amounts of GST or GST-eIF1A (Figure S4A, right) failed to interact with any of the eIF4GI fragments. We also carried the reciprocal GST pull-down assay (Figure S4B), which confirmed that the central region, but not to the C-terminal domain, is the major eIF1-binding site. These results suggest that mammalian eIF4GI specifically interacts with eIF1.

**Release of eIF4F Following Initiation Complex Assembly on TISU**

Considering the short length of the 5′ UTR of TISU genes, both the cap and the 5′ UTR nucleotides are expected to occupy the exit channel of the ribosome. On the other hand, translation directed by TISU is highly eIF4F and cap dependent, suggesting that the positions of eIF4F and the ribosome on the mRNA are overlapping. To elucidate this incompatibility, we considered the previous observation that in yeast eIF1 is released upon AUG recognition (Cheung et al., 2007; Martin-Marcos et al., 2011, 2013; Mitchell and Lorsch, 2008; Nanda et al., 2009) and raised the hypothesis that upon assembly of the ribosome on TISU’s AUG the cap complex dissociates from the mRNA together with eIF1 to allow the positioning of the short 5′ UTR in the ribosomal exit channel. To test this possibility, we analyzed the 48S and 80S complexes with two mRNAs, one bearing a 5 nt long 5′ UTR under the control of TISU and the other with a long and unstructured 5′ UTR with a canonical AUG context. These mRNAs were generated without poly(A) to prevent retention of eIFs on the mRNA through PABP. Ribosomal-mRNA complexes were formed with rabbit reticulocyte lysates (RRLs) in the presence of either nonhydrolysable GTP analog (GMP-PNP) or cycloheximide, which cause accumulation of either 48S or 80S ribosomal complexes, respectively. The complexes were then resolved on sucrose gradients, and the collected fractions were monitored and then analyzed by western blot. With both mRNAs formation of the 48S complex is evident from the pattern of the gradient and the presence of the small ribosomal subunit (Rps5) in the peak fractions 5 and 6 (Figure 3A, left). Likewise, eIF2α is associated with the 48S fractions 5 and 6 in both mRNAs. Interestingly, with TISU mRNA the patterns of eIF4GI and eIF4E are highly similar, showing a sharp reduction in the 48S peak fraction 6 and enrichment in the top fraction 2. Likewise, eIF1 is diminished from 48S...
fraction 6 and is detected in top fraction 2 with TISU mRNA. With the long 5′ UTR mRNA, the amounts of eIF4GI, eIF4E, and eIF1 in the top fraction 2 are much lower. For both mRNAs eIF3d is concentrated in 48S fractions 5–7 (Figure 3A, left). A similar analysis of a short 5′ UTR mRNA with a canonical AUG context resulted in eIF4F release, reminiscent of TISU (Figure S5A), supporting the notion that initiation from an AUG close to the 5′ end of an mRNA is associated with ejection of the cap complex (Figure 3B). The analysis of the 80S complex (Figure 3A, right) revealed the expected presence of the small and large ribosomal subunits in the peak fractions 6–8. On the other hand, for both mRNAs, all the initiation factors are shifted to the top of the gradient, suggesting their release upon 80S formation (Figures 3A and 3B).

TISU Confers Resistance to Translation Inhibition Imposed by AMPK Agonists

The mechanistic studies of TISU revealed a unique mode of translation initiation directed by the basal machinery that is strictly dependent on the AUG context. We next wished to explore the possible physiological importance of this mechanism. As a first step, we analyzed the functional classes associated with the TISU genes in which their ORF begins from the element’s AUG. One of the most enriched functional classes is the mitochondrion (Efakess and Dikstein, 2008). Within this class we found a significant enrichment of genes involved in mitochondrial activities and energy metabolism (Table S1, Table S2, and Table S3). As translation of exogenous and endogenous genes governed by TISU is highly eIF4GI dependent (Figures 2 and S3), it is expected that eIF4GI depletion would affect energy homeostasis. To test this possibility, we measured cellular ATP levels in cells treated with control or eIF4GI siRNA, using luminometric assay. The data, after normalization to cell number, revealed a significant reduction in endogenous ATP levels upon eIF4GI KD (Figure 4A). With eIF1/eIF1B KD no change in ATP levels was observed (data not shown), which is in line with its modest effect on translation of endogenous TISU genes (Figure S3). A possible explanation is the functional redundance of eIF1 and eIF1A as shown in Figure 1B.

Among TISU genes are the two catalytic subunits of AMPK, a highly conserved sensor of the cellular energy status (Zhang et al., 2009). AMPK stimulates ATP production pathways, mostly by enhancing oxidative metabolism and mitochondrial biogenesis (Cantó et al., 2010; Jäger et al., 2007; Lin et al., 2005; O’Neill et al., 2011; Winder et al., 2000), while switching off anabolic pathways that consume ATP, in particular protein synthesis (Elfakess et al., 2011), an ATP-consuming process, we examined TISU activity under conditions of low energy availability. As a first step we treated HepG2 cells with the AMPK agonist 5-aminomidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) and then analyzed global translation using polysome profiling. The results confirmed that the energy stress mimic exerted by AICAR induced AMPK phosphorylation and phosphorylation of its downstream substrate acetyl-CoA carboxylase (ACC) (Figure 4B) and inhibited global translation, as evident from the reduction in the relative amount of polysomes and the increase of the 80S monoribosome (Figures 4C and S6B). To examine the impact of AICAR on translation of specific genes, fractions from the free, the light, and the heavy polysomes were pooled; RNA was prepared and subjected to RT-qPCR analysis of six non-TISU and 19 TISU genes (Figure 4D). Consistent with the global inhibitory effect of AICAR on translation, we found that translation of most non-TISU mRNAs was inhibited, as the amount of mRNA was shifted from the polysomal fractions to the free fractions. In contrast, the free and the polysomal association of all tested TISU mRNAs, including the AMPK catalytic subunits, were unaffected by AICAR. We examined the effect of AICAR on genes involved in fatty acid oxidation (FAO) in the mitochondria, which are also involved in energy production but are non-TISU. We found that their translation is unaffected as well (Figure 4D).

The resistance to AICAR treatment observed for TISU mRNAs can be mediated either by the TISU element or by another unknown feature present in the endogenous TISU mRNAs tested above. To examine the importance of the TISU sequence itself in the translational activity under conditions of induced AMPK, we transfected into HepG2 cells in vitro synthesized GFP mRNA with a 5 nt long 5′ UTR under the control of TISU (Figure 5A). Cells were treated with two AMPK agonists AICAR and metformin and the AMPK antagonist Compound C. As a mRNA with 5 nt long 5′ UTR followed by canonical AUG context was inactive in these cells (Figure 1A), we used as a control a GFP reporter under the control of the native Rpl18 sequences, which also have short 5′ UTR and a canonical AUG context. TISU-directed translation was either enhanced or unchanged by AICAR and metformin, respectively, while Compound C diminished TISU activity in the presence or absence of these drugs (Figure 5A). In contrast, both AICAR and metformin decreased translation directed by Rpl18 sequences while Compound C partially restored AICAR-mediated inhibition (Figure 5A). We validated that both AICAR and metformin induced the phosphorylation of AMPK (Figure 5B), thus causing its activation. We also confirmed that the translational activity of TISU under these conditions remains cap dependent by transfecting into cells TISU mRNAs containing an unmethylated cap analog, which was inactive in directing translation in the absence or presence of AICAR (Figure 5C).

TISU mRNAs Remain Translationally Active under Metabolic Energy Deficiency

Having shown that TISU-mediated translation initiation is resistant to the inhibition conferred by AMPK inducers, we next wished to investigate the direct effect of the cellular energy status on its activity. For these experiments we used differentiated mouse C2 myotubes that represent muscle cells (Figure S6) and mouse embryonic fibroblasts (MEFs) (Figure 6). Cells were grown in the presence or absence of glucose and then subjected to analysis of global translation using polysome profiling. The results confirmed that low availability of metabolic energy induced AMPK and ACC phosphorylation (Figures 6C and S6C) and significantly reduced the relative amount of polysomes while increasing the 80S monoribosomes (Figures 6A and S6B). We next examined the effect of this stress on a subset of non-TISU
and TISU mouse genes by RT-qPCR as described above. With the non-TISU mRNAs (Rps18, Rpl18, Gapdh, and Eef2) we observed an increase in the free fractions and a concomitant decrease in the polysomal fractions upon glucose starvation in the two cell types (Figures 6D and S6D). Remarkably, translation of all the 15 analyzed TISU mRNAs was unaffected by the glucose starvation, both in the myotubes (Figure S6D) and in MEFs (Figure 6D), indicating that their resistance to this stress is not cell type specific. Likewise, translation of fatty acid oxidation genes, which are non-TISU, was unchanged by this stress (Figure S6E).

Inhibition of translation upon energy stress is thought to be mediated by AMPK (Bolster et al., 2002; Dubbelhuis and Meijer, 2002; Krause et al., 2002; Reiter et al., 2005), which is activated by this stress (Figures 6C and S6C) and induces 4E-BP dephosphorylation (Figure 7E). We therefore examined the direct involvement of AMPK by performing a similar experiment in MEFs derived from a mouse deficient for the two catalytic subunits of AMPK (AMPKα1 and AMPKα2, AMPK DKO). Surprisingly, we found that glucose deprivation halted translation in the DKO cells as well (Figure 6B). Furthermore, even in the absence of AMPK, the effect on 4EBP dephosphorylation was retained (Figure 7E). We determined the level of inhibition by calculating the ratio between the RNA in the polysomal and the free fractions (P/F). While the energy stress decreased this ratio by 27.5% ± 0.5% in the WT MEFs, the decrease in the AMPK DKO MEFs is 8% ± 1.7%. These findings suggest that the contribution of AMPK to inhibition of translation by energy stress is significant, but part of the translational response to this stress is clearly AMPK independent. Analysis of translation of specific TISU and non-TISU genes revealed that the effect of the glucose deprivation is quite similar to the WT MEFs, and TISU genes are refractory to this stress in the AMPK DKO cells as well (Figure S7).

The resistance of all tested endogenous TISU genes to the strong translational inhibition caused by energy stress may involve either the TISU element or other regulatory elements associated with these genes. To examine the role of TISU more directly, we used GFP reporters, either as mRNAs that were in vitro synthesized or a plasmid. In these reporters the
GFP is driven by different 5' UTRs and AUG contexts as schematically shown (Figure 7A). These include a long and unstructured 5' UTR (mRNA), a short 5' UTR and AUG of the Rpl18 gene (plasmid), and a 5 nt long 5' UTR that is followed by an AUG either in canonical or TISU contexts (mRNAs) which are followed by an in-frame downstream AUG. The reporter mRNAs or plasmid was transfected into the MEFs, which were then subjected to glucose starvation. Translation directed by the long 5' UTR, the short 5' UTR with the Rpl18 sequences, and the canonical US-AUG was inhibited by reduced energy availability in the WT MEFs (Figure 7B). In contrast, the reporter driven by TISU continued to be translated in spite of the energy deficiency. This result indicates that the TISU AUG context rather than the 5' UTR length confers the energy stress tolerance. The effect of glucose starvation on translation is largely mediated by the energy sensor AMPK (Figures 6A and 6B), which is activated by this stress (Figure 6C). Therefore we carried out similar experiments in AMPK DKO MEFs. In these cells the inhibitory effect of energy stress on translation of all the reporters was reduced (Figure 7C), indicating that AMPK mediates the translational inhibition of the different reporters. We examined the effect of the energy stress on 4EBP dephosphorylation in the WT and the AMPK DKO MEFs and found that the effect exerted by glucose starvation of 4EBP is comparable, suggesting that the stronger translational inhibition observed in the WT MEFs involves an additional, unknown component.

We also determined the effect of rapamycin on translation directed by the different reporters and found that it inhibited translation directed by all short 5' UTR reporters including Rpl18, canonical AUG, and TISU (Figure 7D). The reporter

Figure 5. TISU-Mediated Initiation Is Refractory to AMPK-Mediated Translation Inhibition

(A) HepG2 cells were transfected with in vitro-synthesized mRNA containing TISU or a construct bearing the Rpl18 sequences (promoter, 5' UTR, and initiating AUG) upstream to GFP. Cells were then incubated with 2 mM AICAR or 2 mM metformin (MTF) with or without 20 μM Compound C (CC) for 6 hr. Translation of GFP was analyzed by western blot. Quantified results of four to six experiments (average ± SEM) and representative blots are shown. *p < 0.05, ***p < 0.0005.

(B) Western blot analysis of phosphorylated and total AMPK.

(C) mRNAs bearing TISU were in vitro transcribed in the presence of m7GpppG cap analog or the unmethylated cap analog ApppG. The mRNAs were transfected into HepG2 cells and then treated with AICAR as described. Translation of GFP was analyzed by western blot using GFP antibodies.
driven by the long and unstructured 5' UTR was refractory to 20 nM rapamycin (Figure 7D) as well as 100 nM (data not shown). Interestingly, the effect of rapamycin on 4EBP dephosphorylation is more pronounced than that conferred by the energy stress (Figure 7E). These findings suggest that short 5' UTR mRNAs are highly sensitive to severe mTOR inhibition. On the other hand, unstructured 5' UTR mRNAs are resistant to mTOR inhibition. Taken together, the results with the different reporters unravel the importance of the 5' UTR length and AUG contexts for the differential translational response to various stresses.

**DISCUSSION**

Translation is one of the most energy-consuming cellular processes and therefore is a major target for inhibition in response...
Figure 7. The Differential Translational Response to Energy Stress and Rapamycin Involves AUG Context and 5' UTR Length, Respectively

(A) A scheme of the reporter mRNAs used for transfection.
(B and C) Wild-type MEFs (B) or AMPKα1/2 DKO-MEFs (C) were transfected with each of the mRNAs shown in (A) and incubated overnight with (CT) or without glucose (GS). Translation of GFP was analyzed by western blot. Quantified results of five to six experiments (average ± SEM) and representative blots are shown.
(D) WT MEFs were transfected with each of the constructs shown in (A) and incubated overnight with 20 nM rapamycin. Quantified results of four experiments (average ± SEM) and representative blots are shown. In all graphs, *p < 0.05, **p < 0.005, and ***p < 0.0005.
(E) Western blot analysis of 4EBP under glucose starvation (GS) and rapamycin in WT MEFs and GS in AMPK DKO MEFs.
to various cellular stresses (Sonenberg and Hinnebusch, 2009). However, under conditions in which global protein synthesis is impaired, some proteins must continue to be synthesized as part of the adaptation to the stress. In many cases the molecular details underlying the mechanism used by cellular mRNAs to bypass the inhibition of translation imposed by various stresses are largely unknown. In the present study we discover a tolerance mechanism that selectively operates under the stress caused by reduced energy levels. Our findings reveal that genes and pathways that are specifically needed for the cellular response to energy stress such as mitochondrial enzymes, factors involved in ATP production and fatty acid metabolism, remain translationally active when global translation is inhibited. The strategy adopted by a subset of these genes to counteract the energy-stress-mediated inhibition involves their translational control by the TISU element. We demonstrate that initiation directed by this sequence is sufficient to confer resistance to inhibition of translation mediated by AMPK and energy stress. This tolerance mechanism is specific to this stress, as the inhibition by other stresses such as rapamycin and amino acid deprivation is maintained (this study and Elfakess et al. [2011]). Consistent with that, we also examined the data from a genome-wide analysis of translational response to metformin, an inducer of AMPK (Larsson et al., 2012), and found that the vast majority of TISU genes were unaffected by this drug.

There are several mechanisms for suppression of the general translation initiation during stress. These include the well-characterized impairment of initiation factors eIF2 and eIF4E. While eIF2 does not respond to energy deprivation (data not shown), eIF4E activity is suppressed through 4EBP activation in a manner dependent and independent of AMPK (Figure 7E), in accordance with previous studies (Inoki et al., 2003; Kalender et al., 1995; Korneeva et al., 2000; Mader et al., 1995; Marcotrigiano et al., 2001; Villa et al., 2013; Yanagida et al., 2009). By the use of a set of reporters with different 5′ UTR lengths and AUG contexts along with eIF4GI KD experiments, we found that eIF4GI promotes leaky translation initiation when the AUG is too close to the cap, while facilitating TISU-mediated translation as well as ribosomal scanning. All these features are common to those attributed to eIF1. Importantly, these factors can directly interact with each other, suggesting that their physical association facilitates translation initiation fidelity.

How does eIF1-eIF4GI interaction promote initiation by TISU? Our previous findings, as well as the data presented here, strongly argue for the cap-dependent nature of initiation directed by TISU. Taking into account the short length of the 5′ UTR of TISU mRNAs, it is expected that both the cap and the 5′ UTR nucleotides will be within the ribosomal exit channel upon 80S formation. This situation is incompatible with continued association of eIF4F with the mRNA through the cap. The analysis of the fate of translation initiation factors in the 48S and 80S TISU ribosomal complexes reported here supports a model (Figure 3B) in which the cap-binding complex is evicted from the mRNA 5′ end upon 48S initiation complex formation. We propose that eIF4GI is dissociated along with a fraction of eIF1, as a consequence of their physical association allowing proper initiation (Figure 3B).

In addition to its role in translation TISU is also a critical element for transcription (Elfakess and Dikstein, 2008) raising the possibility that the transcriptional and the translational activities of TISU are coordinated. TISU binds the YY1 transcription factor (Elfakess and Dikstein, 2008). Our attempts to address the role of YY1 for mitochondrial gene expression in HepG2 cells and in MEFs were unsuccessful. However, it was recently reported that targeted knockout of YY1 in skeletal muscle resulted in reduced expression of mitochondrial genes including many TISU genes (Blättler et al., 2012). In addition, YY1 is required for the inhibition of these genes by rapamycin at the mRNA level, through disruption of its interaction with the coactivator PGC-1α (Blättler et al., 2012). As PGC-1α is specifically expressed in cells and tissues with high mitochondrial content, it is possible that the activity of YY1 through TISU may be particularly important in PGC-1α-expressing cells while in other cell types YY1 activity through TISU in transcription is redundant.

While mitochondrial and energy-producing genes are highly enriched with the TISU element, there are other genes in these classes that are not regulated by TISU. An example is the set of genes involved in fatty acid oxidation in the mitochondria, which we found to be also resistant translation inhibition caused by metabolic energy stress. These genes most likely utilize other, yet-unknown mechanisms to maintain their translation ongoing during energy stress. Additional studies
are required to elucidate other tolerance mechanism and whether and how these are deregulated in disease states. Another unexpected discovery emerging from this study is that cells harbor an additional, AMPK-independent mechanism of inhibition of translation initiation in response to energy deprivation. The molecular basis of this mechanism awaits further investigation.

**EXPERIMENTAL PROCEDURES**

**Preparation of mRNA for In Vitro and In Vivo Translation Assays**

All constructs used for mRNA preparation were described previously (Elfakess and Dikstein, 2008; Elfakess et al., 2011). For synthesis of capped mRNA, the constructs containing the T7 promoter were linearized and used with the RibomAXTM Large Scale RNA Production Systems T7 (Promega) supplemented with Ribo m7G Cap Analog (New England Biolabs). The reaction was stopped by the addition of RQ1 RNase-Free DNaseI (Promega) and the mRNA extracted with phenol:chloroform and precipitated with ethanol. The capped mRNAs were denatured at 65°C for 10 min and then placed on ice for 2 min. The concentration of the synthesized mRNAs was determined, and their integrity was confirmed by agarose gel electrophoresis.

**Cells, In Vivo Translation Assays, and Antibodies**

HepG2, HeLa, and HEK293T cell lines were maintained in DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. MEFs were maintained as above in addition to 1 mM pyruvate. C2 myoblasts were maintained as above but with 20% serum. To differentiate the C2 cells to myotubes, when the cells reached 80% confluency they were grown in DMEM supplemented with 2% horse serum for 48 hr (Yaffe and Saxel, 1977). AICAR and metformin treatments of HepG2 cells were done in the absence of serum. For the in vivo translation assays, 0.5–1 µg of the in vitro-transcribed mRNA was transfected into cells that had been previously seeded on 12-well plates, using DharmafECTI transfection reagent (Dharmacon, Thermo Scientific). Two (HepG2) or four (MEFs) hours after transfection, the cells were treated with the different drugs or media as indicated in the figure legends. Cells were harvested and cell lysates were subjected to 12% SDS-polyacrylamide gel electrophoresis. The levels of GFP protein were determined using the commercial mouse monoclonal-anti-GFP antibody (Abcam). Antibodies against AMPK, phospho AMPK, 4EBP, and ACC, and phospho ACC are from Cell Signaling. RPS5 (ab58345) and eIF4G (ab74649) antibodies are from Abcam. eIF3c (sc74507) and eIF3d (sc28856) are from SantaCruz. eIF1 antibodies are a kind gift from Ariel Stanhill (Technion, Haifa), and anti-His are from QIAGEN.

**Assembly of Ribosomal Complexes and Toe-Printing Assays**

The reconstitution of 48S ribosomal complexes from purified components was performed as described earlier (Pisarev et al., 2007). Primer extension inhibition (toe-printing) analysis of 48S initiation ribosomal complex formation was performed with the use of AMV reverse transcriptase and the primer 5′-TGAACCTTGGCCGCAATCCT-3′. cDNA was analyzed by denaturing 6% PAGE alongside sequencing reactions. Assembly of 48S and 80S complexes was performed as described (Pisarev et al., 2007). Briefly, 50 µl rabbit reticulocyte lysate (Promega, Rabbit Reticulocyte Lysate System L4960), 1.4 µl amino acid mix, 4 mM GMP-PNP (Sigma) or 2 mg/ml cycloheximide (Sigma), 50 units RNase inhibitor (Promega), and 8 µg capped mRNA were mixed and incubated for 10 min at 30°C (total volume of 70 µl). For 48S complex, the mixture was preincubated for 5 min at 30°C before adding the mRNA. Samples were chilled on ice and loaded onto 8%–32% sucrose gradient made with buffer containing 20 mM TRIS (pH 8), 100 mM KAc, 2 mM DTT, 2.5 mM MgAc (for 48S), or 5 mM MgCl2 and 0.1 mg/ml cycloheximide (for 80S). Gradients were centrifuged at 40,000 RPM in a SW41 rotor for 3.5 hr (for 48S) or 3 hr (for 80S) at 4°C. Gradients were fractionated and the optical density at 254 nm was continuously recorded using ISCO absorbance detector UA-6. Proteins from each fraction were precipitated by TCA and subjected to western blot.

Knockdown of eIFs, measurement of ATP levels, plasmid construction, coIP, GST pull-down assay, and global and gene-specific translation analysis are described in the Supplemental Experimental Procedures.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures, three tables, and Supplemental Experimental Procedures and can be found with this article at http://dx.doi.org/10.1016/j.cmet.2015.02.010.

**AUTHOR CONTRIBUTIONS**

R.D., H.S., and O.H. conceived and designed the study, analyzed the data, and wrote the paper. H.S. and O.H. carried out most of the experiments. The toe-printing assays following 48S assembly from purified factors were carried out by Y.S. and H.S. in N.S.’s lab. A.T.-B.-H. conducted the bioinformatics analyses. B.V. contributed the AMPKα1/AMPKα2 knockout cells.

**ACKNOWLEDGMENTS**

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Supplemental Information

Translational Tolerance of Mitochondrial Genes to Metabolic Energy Stress Involves TISU and eIF1-eIF4GI Cooperation in Start Codon Selection

Hadar Sinvani, Ora Haimov, Yuri Svitkin, Nahum Sonenberg, Ana Tamarkin-Ben-Harush, Benoit Viollet, and Rivka Dikstein
Figure S1, related to Figure 1A

A.

TISU  Canonical

B.

GFP RNA levels after transfection

- TISU Canonical
- TISU Canonical
- TISU Canonical
- TISU Canonical
- TISU Canonical

HepG2  HeLa  293T  MEF
Figure S2, related to Figure 2

A. TISU/ Canonical
   CMV-P  AUG  US  GFP
   CMV-P  AUG  DS  GFP

B. TISU  Canonical  Long 5'UTR
   Si-C  Si-eIF1  Si-C  Si-eIF1  Si-C  Si-eIF1  Si-C  Si-eIF1
   US-DS-  α-GFP  α-GFP  α-GFP

C. TISU  Canonical  Long 5'UTR
   Si-C  Si-eIF1B  Si-C  Si-eIF1B  Si-C  Si-eIF1B  Si-C  Si-eIF1B
   US-DS-  α-GFP  α-GFP  α-GFP

D. TISU  Canonical  Long 5'UTR
   Si-C  Si-eIF3c  Si-C  Si-eIF3c  Si-C  Si-eIF3c
   US-DS-  α-GFP  α-GFP  α-GFP

Relative translation

- US AUG
- DS AUG
Figure S4, related to Figure 2

A.

1. PABP  eIF4E  eIF3/eIF4A  eIF4A  1599

84  197

4GI  84-197

4GI  198-674

4GI  675-1129

4GI  1130-1599

B.

Input  GST-4GI  GST-4GI  GST-4GI

1130-1599  675-1129  4GI  1599

Coomassie staining

Coomassie staining

eIF1

Input  GST  GST-eIF1A

1130-1599  675-1129  4GI  1599
Figure S5, related to Figure 3

A.

Sucrose density (Top→ bottom)

1 2 3 4 5 6 7 8 9 10

OD 254nM

48S

TISU

Canonical

TISU

Canonical

TISU

Canonical

TISU

Canonical

TISU

Canonical

B.

CT

AICAR

CT

AICAR

RPS 5

RPS 5

RPL 11

RPL 11

F 40s 60s 80s

Polysomal fractions
Figure S6, related to Figure 6

A. Myoblasts vs Myotubes

B. OD 254nm

C. Sucrose density (10%-50%)

D. mRNA level

E. mRNA level

ARFGAP2, ATP5O, CAPZA2, CT, GS, DOLPP1, DPFI2, EEF2, (NON-TISU), GAPDH, (NON-TISU), MRPL35, NDUFA9, NDUFS6, SIRT1, SUCGL2, ABCF3, ALG1, ATP6V1B2, CPT1A (FAO), ACSL5 (FAO), ACAA2 (FAO), ACAA1 (FAO), CPT1A (FAO)
Figure S7, related to Figure 6
Supplementary figure legends

**Figure S1:** A. Representative image of an agarose gel that was used to estimate the integrity of the *in vitro* transcribed mRNAs used for *in vivo* and *in vitro* translation assays. B. Analysis of the relative stability of *in vivo* translated mRNAs. *In vitro* transcribed mRNAs were transfected into the indicated cell lines and total RNA was prepared 24 hours after transfection. GFP mRNA was analyzed by real-time RT-qPCR.

**Figure S2:** The effects of eIF1, eIF1B and or eIF3c KD on translation efficiency and accuracy. HEK293T cells were transfected with siRNA against eIF1 (B), eIF1B (C), eIF3c (D) or with a non-targeting siRNA as a negative control as indicated in each section. After 48h cells were transfected with the GFP reporter genes shown schematically (A). Cells were harvested 24h after the second transfection and analyzed for GFP expression by Western blot as described in Fig. 2. The graphs represent average ± SE of 3-4 independent experiments.

**Figure S3:** The effects of eIF1/eIF1B, eIF4GI and eIF3c KD on translation. HEK293T cells were transfected with siRNA against the indicated translation initiation factors and 72h later subjected to ribosomal profiling and western blot with the indicated antibodies (upper panels). The black and the red lines show the profile of the control (CT) and the siRNA treated cells, respectively. The bottom panel shows real-time qPCR analysis of the levels of the indicated non-TISU and TISU mRNAs in the polysome-free fractions (F), light polysomes fractions (L) and in the heavy polysomes fractions (H).
**Figure S4:** eIF4GI interacts directly with eIF1. **A.** Upper panel: a scheme showing eIF4GI domains and eIF4GI protein fragments used for the *in vitro* binding assays with eIF1 and eIF1A. The black boxes denote protein interaction sites. Lower panel, GST and GST-eIF1 (left) or GST and GST-eIF1A (right) were coupled to glutathione sepharose beads and incubated with four different fragments of eIF4GI, each with a C-terminal 6XHis tag. The pulled-down complexes were washed and then run on SDS-PAGE followed by western blot with anti-6XHis antibody. Inputs represent 1% of the lysate used for the binding. The arrows indicate the position of the eIF4G fragments in the input lanes. The GST and the GST fusion proteins used for the binding reactions are shown in the Coomassie blue stained gel shown on the left side of each panel. **B.** A reciprocal binding assay in which GST-4GI-675-1129 and GST-4GI-1129-1599 fragments coupled to glutathione sepharose beads were incubated with bacterially expressed His-eIF1. Input represents 5% of the amount used for binding.

**Figure S5:** **A.** The fate of eIFs following 48S complex assembly on short 5’UTR mRNAs. Capped mRNAs, bearing a short 5’UTR with an AUG in either TISU or canonical AUG contexts, were incubated with RRL in the presence of GMP-PNP to stall the 48S. The canonical AUG mRNA has also an in-frame downstream AUG as similar to the TISU used in Fig. 3A. The TISU mRNA used in this experiment lacks a downstream AUG. Ribosomal-mRNA complexes were then subjected to 8-32% sucrose gradient sedimentation and the collected fractions were recorded (top panel) and analyzed by western blot for the distribution of eIFs and ribosomal subunits as indicated (bottom panels). **B.** Analysis of ribosomal proteins along the sucrose gradients following energy stress. Western blot analysis of the distribution of RPS5
and RPL11 in the 11 fractions of the sucrose gradients in control and AICAR treated HepG2 cells.

**Figure S6:** Translational response of C2 myotubes to energy stress. A. A picture taken from C2 myoblasts (left) and C2 cells that were differentiated into myotubes (right). B. C2 myotubes were glucose deprived for 8 hours and then subjected to ribosomal profiling. The black and the red lines show the profile of the control (CT) and glucose starved (GS) cells, respectively. C. Western blot analysis of phosphorylated and total AMPK and ACC under glucose starvation (GS). D. Real-Time qPCR analysis of the levels of the indicated non-TISU and TISU mRNAs in the polysome-free fractions (F) of the gradient, light polysomes fractions (L) and in the heavy polysomes fractions (H). E. The effect of energy stress on translation of fatty acid oxidation genes (FAO). MEFs were glucose deprived for 8 hours and then subjected to ribosomal profiling (see Fig. 6A). Real-Time qPCR analysis of the levels of the indicated FAO mRNAs in the polysome-free fractions (F), light polysomes fractions (L) and in the heavy polysomes fractions (H).

**Figure S7:** The translational response of non-TISU and TISU genes to energy stress in AMPK deficient cells. AMPK DKO MEFs were glucose deprived for 8 hours and then subjected to ribosomal profiling (see Fig. 6B). Real-Time qPCR analysis of the levels of the indicated non-TISU and TISU mRNAs in the polysome-free fractions (F), light polysomes fractions (L) and in the heavy polysomes fractions (H).
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Tables

**Table S1:** Functional classification of the TISU genes in which the AUG of TISU constitutes the beginning of their ORF, using the Database for Annotation, Visualization and Integrated Discovery (DAVID). The table shows the functional groups with the highest enrichment score, their enrichment fold, P-value and the false discovery rate (FDR).

**Table S2:** List of human genes bearing TISU element with up to 2 mismatches. The file also contains a venn diagram showing the number of genes that common in human and mouse.

**Table S3:** List of mouse genes bearing TISU element with up to 2 mismatches.
Supplemental Experimental Procedures

Knockdown of eIFs

HEK293T cells were seeded on a 6-well plate and transfected with 50 nM Dharmacon siGENOME SMART pool siRNA purchased from Thermo Scientific (eIF1 M-015804-01-0010; eIF1B M-019996-00, eIF4GI M-019474-01 and eIF3 M-009036-01) using DharmaFECT1 transfection reagent. The Dharmacon ON-TARGETplus Non-targeting siRNA #3 was used as a negative control. 48h after the initial transfection, cells were transfected with the GFP reporter plasmids using the standard CaPO4 method. Cells were harvested 24h after the second transfection.

Measurement of ATP levels

HEK293T cells in 96-well plate (7,000 cells/well) were transfected with control or eIF4GI siRNA as described above. Then, 72h later cells were subjected to ATP levels measurement using CellTiter-Glo Luminescent Assay (promega).

Plasmid construction

Four different fragments of Homo sapiens eIF4GI (4GI 84-197, 4GI 198-674, 4GI 675-1129, 4GI 1130-1599) were amplified by PCR and cloned into pET28a expression vector in frame to its C-terminal 6XHis tag sequence. GST-eIF1 and GST-eIF1A were constructed by cloning PCR fragments encoding eIF1 and eIF1A into pGEX6p1 expression plasmid in frame to its N-terminal GST sequence using the T-PCR cloning method (Erijman et al., 2011).

Co-immunoprecipitation and GST pull down assay
HEK293T cells were transfected with plasmids directing expression of either HA-tagged eIF1 or eIF1A. 24h later cells were lysed using IP buffer (20mM Tris pH8, 125 mM NaCl, 10% Glycerol, 0.5% NP-4, 0.2mM EDTA) to which fresh protease inhibitor cocktail (Sigma, 1:100) and PMSF 200µM (1:100) were added. Protein extract was subjected to immunoprecipitation assay using either HA antibody (Sigma) or control antibody in IP buffer, at 4°C for 16h. Each reaction was then washed three times with IP buffer. After the washes 30µl of protein sample buffer were added to each sample. 2% of the input and 30% of each IP sample were then subjected to 8% and 15% SDS-PAGE followed by western blot.

GST-eIF1, GST-eIF1A and various 4GI protein fragments were expressed in E. Coli BL-21 DE3 strain. GST fusion proteins were then purified using glutathione sepharose beads (GE healthcare). GST pull-down reactions were performed in HEMG buffer (20mM HEPES, 12.5mM MgCl2, 0.5mM EDTA, 0.1% NP-40, 10% Glycerol and 100 mM KCl), at 4°C for 2h. Each reaction was then washed three times, after which 30µl of protein sample buffer was added. 1% or 5% input amount and 30% of each pull-down sample were then subjected to 12% SDS-PAGE followed by western blot. In addition, 30% of each pull-down assay sample were also subjected to another SDS-PAGE followed by coomassie blue staining. His-eIF1 was purified as previously described (Elfakess et al., 2011).

**Global and gene specific translation analysis**

HepG2 and MEFs and C2 cells were cultured in 15 cm plates and the following day were treated with 2mM AICAR for 3 hours in the absence of serum (HepG2) or were incubated in glucose free DMEM supplemented with 10% dialyzed FBS or 20nM rapamycin for 8 hours (MEFs) or 2% dialyzed horse serum (C2 myotubes). The cells
were then incubated with 100 µg/ml Cycloheximide for 5 minutes and then washed twice with cold buffer containing 20mM Tris pH 8, 140mM KCl, 5mM MgCl2 and 100 µg/ml Cycloheximide. The cells were collected and lyzed with 500 µl of same buffer that also contains 0.5% Triton, 0.5% DOC, 1.5mM DTT, 150 units RNase inhibitor and 5µl of protease inhibitor (Sigma). The lyzed samples were centrifuged at 12,000g at 4°C for 5 minutes. The cleared lysates were loaded onto 10-50% sucrose gradient and centrifuged at 41,000 RPM in a SW41 rotor for 90 minutes at 4°C. Gradients were fractionated and the optical density at 254 nm was continuously recorded using ISCO absorbance detector UA-6. RNA was isolated using Trizol and Direc-Zol RNA mini-prep kits (Zymo Research). cDNA was prepared from 500ng RNA using High-capacity cDNA reverse transcription kit (Applied Biosystems). Real-Time PCR was done with Power-SYBR green master mix (Applied Biosystems) in 7300 Real-Time PCR System. Primers sequences can be provided upon request.