elf4GI links nutrient sensing by mTOR to cell proliferation and inhibition of autophagy

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Introduction

Translational control is an important regulatory point that affects diverse processes such as development, differentiation, apoptosis, cellular growth, responses to cellular stresses, and tumorigenesis. Translation of most mRNAs is controlled at the rate-limiting step of initiation, the assembly of a translationally competent 80S ribosome. The eukaryotic 5′ end 7-methyl-GTP (m7GTP) cap-tags mRNAs for translation by recruiting the eukaryotic initiation factor (eIF)4F complex, which is composed of three proteins: cap-binding protein eIF4E, scaffolding protein eIF4G, and ATP-dependent RNA helicase eIF4A. The multi-domain adaptor proteins elf4GI and related family member elf4GII are key initiation factors in this process. For instance, absence of elf4GI from in vitro translation systems prevents initiation complex formation and protein synthesis (Ali et al., 2001). Moreover, apoptosis and picornavirus infection both result in general translational repression, which is associated, in part, with proteolytic cleavage of elf4G (Marissen and Lloyd, 1998; Connolly et al., 2006; Constantinou and Clemens, 2007).

An important regulatory signaling pathway for translation is the AKT/mTOR protein kinase network, which responds to growth factors, stress, and the nutritional state of the cell (Holcik and Sonenberg, 2005). When activated, mTOR phosphorylates and inactivates key translation regulatory proteins known as elf4E binding proteins (4E-BPs) (Gingras et al., 1998). Stresses such as lack of growth factors, nutrient deprivation, or hypoxia inhibit mTOR, generating hypo-phosphorylated (active) forms of the 4E-BPs that sequester eIF4E, preventing elf4F complex formation and blocking cap-dependent mRNA translation (Gingras et al., 1998). mTOR may also phosphorylate elf4GI, possibly indirectly (Raught et al., 2000). Although a role for elf4GI phosphorylation in translation is not established, it may be required for efficient elf4F formation in some tissues (Vary et al., 2007).

Deregulation of the mTOR pathway, and of mRNA translation from negative feedback responses, is associated with increased transformation and oncogenesis (Cully et al., 2006). In this regard, overexpression of elf4GI is also associated with malignancy in some human cancers (Schneider and Sonenberg, 2007), particularly breast cancers (Braunstein et al., 2007). Engineered overexpression of elf4GI in immortalized fibroblasts also results in malignant transformation (Fukuchi-Shimogori et al., 1997). In this paper, we demonstrate that the elf4GI member of the elf4G family selectively promotes translation of mRNAs involved in cellular bioenergetics and mitochondrial activity, the majority of which are of low abundance and contain multiple upstream open reading frames (ORFs). Reduction of...
eIF4GI abundance, in turn, is shown to partially but distinctly phenocopy depletion of mTOR, impairing nutrient sensing and inducing autophagy.

Results
Silencing of eIF4GI and DAP5, but not other eIF4G factors, only modestly reduces global protein synthesis rates
To address the physiological functions of eIF4G, levels of eIF4GII were reduced by siRNA silencing in MCF10A cells, an immortalized breast epithelial cell line that preserves translational control pathways that are often uncoupled with frank transformation (Connolly et al., 2006). Transfection of siRNA to eIF4GII rapidly reduced eIF4GII protein levels by ~10-fold for more than 4 d (Fig. 1 A; Fig. S1, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200710215/DC1). Indirect immunofluorescence analysis revealed that eIF4GII was down-regulated in almost all cells (Fig. S1 C), in both the nucleus and cytoplasm (Fig. S1 D). Unexpectedly, protein synthesis rates in eIF4GII-silenced cells were decreased by only 20% compared with control cells (measured by [35S]-methionine incorporation rate) (Fig. 1 A, right), despite a 90% reduction in eIF4GII protein levels (Fig. 1 A; Fig. S1, A and B). Results were unchanged by normalization to equal cell number rather than equal protein content, and there was no evidence for increased [35S]-methionine uptake in eIF4GII-silenced cells (unpublished data). Identical results were also obtained in other cell types including primary human neonatal dermal fibroblasts and BT-474 breast cancer cells (Fig. S2). The siRNA transfection itself had no effect on protein synthesis, as mock-transfected and control siRNA-transfected cells behaved identically (unpublished data).

eIF4GII is the major eIF4G isoform in most mammalian cells (>85%) (Bovee et al., 1998). In the budding yeast, which also possesses two homologues of eIF4G, single knockout of either eIF4G causes minor growth defects, but the combined knockout is lethal (Goyer et al., 1993). We therefore investigated whether eIF4GII maintains translation in eIF4GII-silenced cells. Efficient silencing of both eIF4GII and II did not decrease protein synthesis more than depletion of eIF4GII alone (Fig. 1 B). In contrast, modest silencing of initiation factor eIF3 S1 and S8 expression impaired protein synthesis by more than 50% (Fig. 1 B), as did moderate silencing of eIF3 S8 alone (~50%) (unpublished data). Importantly, silencing of eIF4G largely blocked assembly of eIF4F cap-initiation complexes, shown by recovery of complexes using mGTP-cap chromatography and visualization by immunoblot analysis (Fig. S2 E). eIF4GII, but not eIF4GII-silenced cells, also showed decreased levels of eIF4E (Fig. 1 C; Fig. S4 A), consistent with a previous report (Svitkin et al., 2005), and phosphorylation of eIF4E at serine 209 was abolished. eIF4E phosphorylation requires assembly of eIF4E with eIF4G and the kinase Mnk1 (Pyronnet et al., 1999). It is therefore unlikely that an occult pool of functional eIF4G remains in silenced cells.

Inhibition of cap-dependent translation by treatment of eIF4GII or eIF4GII-silenced cells with the cap-dependent mRNA translation inhibitors rapamycin (targeting mTOR) and LY294002 (targeting PI3K) blocked cap-dependent mRNA translation and activated 4E-BP1 (shown by its dephosphorylation) (Fig. 1 D, right). Nevertheless, after drug removal, both control and silenced cells fully and rapidly recovered protein synthesis. Similar results were obtained when cells were challenged with other physiological stresses that also down-regulate cap-dependent mRNA translation such as hypertonic and hypoxic stress (Fig. S2, C and D). Inhibition of translation elongation mediated by puromycin treatment was also readily reversed with drug wash-out in eIF4GII-silenced cells (Fig. 1 E). Rates of de novo mRNA translation, as tested by transfection of a bicistronic reporter after eIF4GII silencing, were similar in both eIF4GII-depleted and control cells (Fig. S2 F). eIF4GII silencing also had no impact on translation elongation rates. eIF2 phosphorylation was unaltered and ribosome half-transit times were similar in control and eIF4GII-depleted cells (71 and 64 s, respectively, P = 0.3; Fig. S3, and unpublished data).

The eIF4G family member DAP5 has recently been shown to participate in the maintenance of overall protein synthesis (Lee and McCormick, 2006; Nousch et al., 2007). Independent silencing of eIF4GII or DAP5 reduced overall protein synthesis by 20–30%, whereas co-depletion blocked it by ~60% (Fig. 1 F). In contrast, double depletion of eIF4GII and eIF4GII family member Paip1, reportedly a global translation-enhancing factor, did not further decrease overall protein synthesis compared with eIF4GII depletion alone (Fig. 1 F; Fig. S4 A and B) (Craig et al., 1998). Thus, both eIF4GII and DAP5 together are required to maintain high rates of global protein synthesis.

Proliferative and bioenergetic alterations in eIF4GII-silenced cells phenocopy mTOR pathway inhibition
Despite a very limited reduction of protein synthesis, eIF4GII-silenced cells were morphologically quite distinct compared with control or DAP5-silenced cells, displaying a spindly and vacuolated appearance with membrane roughing (Fig. 2 A). Forward scatter flow cytometry analysis showed a ~15% decrease in overall cell size in eIF4GII-silenced compared with control cells (Fig. 2 B), or eIF4GII or DAP5-silenced cells (unpublished data). The doubling time of eIF4GII-depleted cells was also increased 40% compared with control cells, beginning 48 h after eIF4GII silencing (Fig. 2 C), and involved decreased S phase accumulation and pronounced G1 phase restriction (Fig. 2 D). eIF4GII-silenced cells showed no alterations in cell cycle (Fig. 2, C and D). In agreement with our results, Dap5 silencing has been shown to decrease proliferation (Lee and McCormick, 2006), similar to eIF4GII silencing.

Cell size and proliferation are in large part controlled by the PI3K/AKT/mTOR pathway (Sarbassov et al., 2005). Treatment of cells with the mTOR inhibitor rapamycin, or specific silencing of the mTOR partner protein raptor, blocks proliferation and diminishes cell size (Kim et al., 2002; Sarbassov et al., 2005), similar to the phenotype of eIF4GII-silenced cells. Accordingly, we found that raptor depletion also restricts cells at G1, similar to eIF4GII depletion (Fig. 2 D). Combined silencing of eIF4GII and raptor slightly increased arrest of cells in G1 and
mTOR complex 1 (mTORC1) is composed of the kinase mTOR, and the proteins raptor and mLST8, which collectively mediate many mTOR kinase functions (Guertin et al., 2006). We asked whether serum- and insulin-mediated phosphorylation of eIF4GI at position S1108 is mediated by mTORC1 because eIF4GI phosphorylation is coordinately decreased by serum withdrawal or hypertonic stress, both of which also inhibit mTORC1 activity (Raught et al., 2000). eIF4GI phosphorylation in leucine-starved cells increased upon leucine restoration, and was reduced by raptor silencing (Fig. 3 A). Drug inhibition of mTOR was shown by abolition of ribosomal protein S6 phosphorylation at mTOR target sites S240/S244 (Fig. 3 A). Furthermore, eIF4GI is reportedly phosphorylated at position S1108 with activation of the mTOR pathway and nutrient sensing (Raught et al., 2000). To determine whether eIF4GI is directly connected to the mTOR pathway, we examined its phosphorylation by mTOR in response to nutrient signaling.

In *Saccharomyces cerevisiae*, eIF4GI is connected to the mTOR pathway in response to nutrient cues that control eIF4GI stability (Berset et al., 1998). Consistent with the results in yeast, we found that inhibition of mTOR function with LY294002 and rapamycin for 12 h reduced eIF4GI levels by half, without altering levels of eIF4E or eIF4A (Fig. 3 A). Drug inhibition of mTOR was shown by abolition of ribosomal protein S6 phosphorylation at mTOR target sites S240/S244 (Fig. 3 A). Furthermore, eIF4GI is reportedly phosphorylated at position S1108 with activation of the mTOR pathway and nutrient sensing (Raught et al., 2000). To determine whether eIF4GI is directly connected to the mTOR pathway, we examined its phosphorylation by mTOR in response to nutrient signaling.

Inhibition of proliferation (Fig. 2 D, Fig. S4, C and D; also shown later). eIF4GI is therefore linked to the mTOR network to influence cell size and proliferation.
and eIF4GI-silenced MCF10A cells that had been starved for leucine for 48 h, or starved and allowed to recover by the addition of leucine for 12 h. eIF4GI-depleted cells were unable to recover cell size after leucine restitution, similar to mTORC1 inhibition (Fig. 3, E and F). In fact, reduction of eIF4GI more severely blocked cell size recovery than did inhibition of mTORC1 by depletion of raptor (Fig. 3 F).

Although DAP5 silencing also prevented full cell size recovery, it was not as severe as eIF4GI or raptor depletion, whereas eIF4GII and Paip1 silencing had no effect (Fig. 3 F, and unpublished data). An inability to sense nutrients or growth factors, which depends in large part on the mTOR signaling pathway, can lead to mitochondrial dysfunction due to an inability to use extracellular nutrients (Kundu and Thompson, 2005). We tested whether eIF4GI-depleted cells, like growth factor–deprived or mTOR-inhibited cells, exhibit mitochondrial impairment (Lum et al., 2005; Schieke and Finkel, 2006).

To determine whether eIF4GI is directly phosphorylated by mTOR, immunoprecipitated mTORC1 complex from MCF10A cellular lysates was mixed with isolated S6K1 or eIF4GI (obtained from cells treated with rapamycin and LY294002 to reduce phosphorylation). Immunoprecipitated mTORC1 phosphorylated S6K1 at T389 (P-S6K) and eIF4GI at S1108 (P-eIF4GI) in a Wortmannin-sensitive manner (Fig. 3 D). eIF4GI therefore undergoes mTORC1-dependent phosphorylation in response to growth factors and amino acids. mTORC1 kinase activity has also been linked to eIF4GI abundance (Berset et al., 1998) and effective eIF4F complex formation (Vary et al., 2007).

Inhibition of mTORC1 signaling leads to an inability to control cell size in response to nutrients, such as leucine (Kim et al., 2002; Sarbassov et al., 2005). The effect of eIF4GI silencing on cell size was examined by forward scatter flow cytometry as a measure of cell diameter to compare control and eIF4GI-silenced MCF10A cells that had been starved for leucine for 48 h, or starved and allowed to recover by the addition of leucine for 12 h. eIF4GI-depleted cells were unable to recover cell size after leucine restitution, similar to mTORC1 inhibition (Fig. 3, E and F). In fact, reduction of eIF4GI more severely blocked cell size recovery than did inhibition of mTORC1 by depletion of raptor (Fig. 3 F). Although DAP5 silencing also prevented full cell size recovery, it was not as severe as eIF4GI or raptor depletion, whereas eIF4GII and Paip1 silencing had no effect (Fig. 3 F; and unpublished data). An inability to sense nutrients or growth factors, which depends in large part on the mTOR signaling pathway, can lead to mitochondrial dysfunction due to an inability to use extracellular nutrients (Kundu and Thompson, 2005). We tested whether eIF4GI-depleted cells, like growth factor–deprived or mTOR-inhibited cells, exhibit mitochondrial impairment (Lum et al., 2005; Schieke and Finkel, 2006).
results were obtained in HeLa cells as well (not depicted).

Depletion of eIF4GI, but not other translation factors (depletion shown in Fig. S4 A), resulted in a significant decrease in mitochondrial membrane potential (decreased loading of the membrane permeable fluorescent dye, TMRE) in MCF10A cells compared with control cells. (Fig. 3, G and H). Similar results were obtained in HeLa cells as well (not depicted).

Moreover, we can exclude technical explanations for these results such as abnormal mTOR activation by high amino acid or serum levels in the medium because MCF10A cells are cultivated in low amino acid medium with minimal serum.
whereas HeLa cells are cultivated in more enriched medium. If diminished mitochondrial function in eIF4GI-depleted cells phenocopies that of mTOR inhibition, it should be a result of an inability to use extracellular nutrients. Consequently, cellular energy stores should also be depleted, leading to an increased AMP/ATP ratio and subsequent AMPK activation (Kundu and Thompson, 2005). Indeed, ATP content is reduced by 45% in eIF4GI-depleted cells compared with control cells, which correlates with a threefold increase in activated (T172-phosphorylated) AMPK (Fig. 3 H), consistent with a key role for eIF4GI in maintenance of bioenergetics. In agreement with diminished mitochondrial function, DAP5 silencing did not activate AMPK (shown later in Fig. 5 A). An energetic defect exists in eIF4GI-silenced cells that mimics the defect observed with mTOR inhibition or nutrient deprivation.

**eIF4GI depletion induces autophagy**

A key phenotype of mTORC1 inhibition is induction of autophagy. During autophagy cells engulf large portions of cytoplasm into autophagosomes, which fuse with lysosomes to recycle cellular components as energy sources (Kundu and Thompson, 2005). We tested whether eIF4GI depletion results in autophagy. A widely used marker for autophagy is the intracellular distribution of LC3 protein. The LC3-I form migrates as a 16-kD band in SDS-PAGE, but is lipidated during autophagy to produce a 14-kD LC3-II form, and incorporated into growing autophagosomes (Kabeya et al., 2000). A GFP-LC3 fusion protein was expressed in 293 cells (to demonstrate cell type independence), followed by transfection of control or eIF4GI-siRNAs. GFP-LC3 presented as a diffuse cytoplasmic stain in nontransfected and control siRNA transfected cells, but was found clustered and in vacuoles in nutrient-starved or eIF4GI-silenced cells (Fig. 4 A). Moreover, treatment of MCF10A cells with the membrane-permeable dye monodansylcadaverine (MDC), which binds specifically to autophagosomes (Yoshimori, 2007), was used to visualize autophagy induction by eIF4GI depletion. It should be noted that the LC3 antiserum detects the faster-migrating LC3-II form more efficiently than the LC3-I form (Mizushima and Yoshimori, 2007).

To compare these results to depletion of other translation factors, we expressed GFP-LC3 in MCF10A cells and silenced eIF4GI, DAP5, eIF4GII, or eIF4E, or inhibited protein synthesis with puromycin. These cells also showed autophagy induction as visualized by GFP-LC3 clustering with DAP5 depletion, but not with silencing of eIF4GII or eIF4E. Furthermore, overall inhibition of protein synthesis with puromycin did not induce autophagy (Fig. 4 D). DAP5 depletion shares some of the characteristics observed for eIF4GI silencing, including similar effects on translation rates, reduced cellular proliferation, autophagy induction, and altered cell size regulation in response to nutrients. However, DAP5-silenced cells do not demonstrate the morphological changes, alterations in mitochondrial activity, or AMPK activation that were observed in eIF4GI-depleted cells. These data indicate a potential role for eIF4GI and DAP5 in overlapping but distinct pathways. In contrast, eIF4E silencing, although decreasing translation rates to a similar extent as eIF4GI or DAP5 silencing (Fig. S4 B), did not cause any of these same alterations including induction of autophagy (Fig. 4 D, and unpublished data) (Svitkin et al., 2005). These results therefore disclose a specific function for eIF4GI in cellular bioenergetics and suggest that eIF4GI is connected to the mTORC1 nutrient-sensing pathway. Collectively, these results also exclude trivial explanations for induction of autophagy such as the cell culture medium, leucine and other amino acid levels, or special growth supplements in medium such as hydrocortisone, insulin, and epidermal growth factor.

Because both eIF4GI and DAP5 silencing reduce protein synthesis rates, and combined depletion strongly arrests translation, we determined whether one of these two factors could physiologically substitute for the other. We characterized cell proliferation rates in control cells and MCF10A cells engineered to overexpress DAP5 (Fig. 4, E–G). DAP5 overexpression had little effect on overall translation or proliferation rates (Fig. 4 F, and unpublished data), but prevented cell growth arrest by DAP5 depletion (Fig. 4, F and G). Conversely, DAP5 overexpression did not block growth rate inhibition by eIF4GI-silencing, demonstrating that DAP5 and eIF4GI are not physiologically interchangeable (Fig. 4, F and G).

**eIF4GI silencing and decreased cellular proliferation are associated with diminished translation of E3 ubiquitin ligase Skp2 mRNA**

Because eIF4GI silencing results in increased G1 checkpoint accumulation, we investigated the effect on Skp2 mRNA translation. Skp2 promotes ubiquitin-dependent proteasome degradation of cell cycle inhibitor p27 (Pagano, 2004). eIF4GI silencing strongly increased levels of p27 protein, consistent with loss of Skp2 expression (Fig. 5 A). Inhibition of the proteasome with MG132 only minimally increased Skp2 levels in eIF4GI-silenced cells, suggesting that eIF4GI silencing primarily inhibits Skp2 mRNA translation (Fig. 5 B), also supported by decreased protein synthesis of Skp2 without alteration of mRNA levels (Fig. 5, C and E). In contrast, DAP5 depletion increased p27 levels without altering Skp2 levels (Fig. 5 E). Thus, despite similar effects on growth, eIF4GI and DAP5 perform distinct functions (Lee and McCormick, 2006). This result is consistent with qRT-PCR analysis of total and polysome-bound Skp2 mRNA (Fig. 5, C and D), a significant fraction of which shifts to lighter polysomes with eIF4GI depletion. In contrast, Hsp27 mRNA shifts to heavier polysomes with eIF4GI depletion, consistent with the array analysis (described later) and increased protein levels (Fig. 5 D). These results show that eIF4GI silencing impairs cell proliferation, in part, by reducing the translation of Skp2 mRNA and thereby increasing the abundance of p27 protein.
Polysomal association of a subset of mRNAs is altered by eIF4GI depletion, mediated in part by the presence of upstream ORFs and low mRNA abundance.

We explored whether translation of a small subset of mRNAs might be highly sensitive to reduction of eIF4GI levels. We identified the constellation of polysome-associated mRNAs in control and eIF4GI-silenced cells using gene expression arrays, comparing polysome mRNA levels to total mRNA abundance. Polysome profiles for control and eIF4GI-silenced cells derived from three independent studies were similar, displaying a slight decrease in polysome content in eIF4GI-depleted cells. Reduction in eIF4GI levels was evident in all polysome fractions, eliminating the possibility of translation by a small subset of mRNAs.

**Figure 4.** Autophagy induction and reduced cellular proliferation in eIF4GI-silenced cells. (A) 293T cells were transfected with a plasmid encoding GFP-LC3 and treated as shown. Nuclei were stained with propidium iodide (red). Bar, 5 μM. (B) MCF10A cells were treated as indicated and autophagosome formation established by MDC loading. Error bars represent standard deviation, calculated from at least three independent experiments. Statistical analysis used the t test; *, P < 0.05. (C) Endogenous LC3 cleavage in control and eIF4GI-silenced MCF10A cells determined by immunoblot. (D) MCF10A cells expressing AcGFP-LC3 transfected with (A) control (0–5% with dots), (B) eIF4GI (>90% with dots), (C) DAP5 (>90% with dots), (D) eIF4GII (0–5% with dots), (E) eIF4E (<5% with dots) siRNAs, or (f) treated with 200 μM puromycin (0–5% with dots) for 2 h. AcGFP-LC3 clustering analyzed by confocal microscopy. The number of cells with LC3-GFP dots were quantified in five fields of similar numbers of cells, t test (P < 0.001). Bar, 5 μM. (E) Immunoblot analysis showing increased levels of DAP5 in pBMN-DAP5-I-GFP transduced cells compared with vector control. (f) Growth rates on siRNA transfected cells (†, control; ▲, DAP5; and ■, eIF4GI). (G) Growth ratios determined from data shown in F.
pool of polysome-associated eIF4GI (Fig. 6 A). eIF4GI reduction did not alter total abundance or polysome association of the majority of mRNAs (~70%). The remaining mRNAs displayed significant changes in polysome association, overall abundance, or both (P < 0.05; SAM, false discovery rate of 5%). Hierarchical clustering analysis revealed four sets of mRNAs in eIF4GI-silenced cells (Fig. 6 B). (1) No overall mRNA change but decreased polysome association (134 genes); (2) decreased polysome association despite an increase in overall mRNA level (31 genes); (3) increased polysome association with no alteration in mRNA level (143 genes); and (4) increased polysome association despite an overall decrease in mRNA level (31 genes; see DNA microarray data section of Materials and methods). Gene array data were validated by performing immunoblot analysis on some target genes (Fig. 6 C) and real-time quantitative RT-PCR. For instance, eIF4GI-silenced cells showed increased Hsp27 and KLF4 protein levels and decreased levels of caspase 3, ARALAR, and MYCBP (and Skp2, as shown earlier), confirming array predictions at the mRNA and protein levels (Fig. 6 C).

A number of mRNAs revealed in these clusters are consistent with the eIF4GI-silencing phenotype. In particular, reduction in polysome association of mRNAs encoding mitochondrial regulatory proteins, such as ARALAR, a mitochondrial solute carrier that regulates ATP production in response to Ca\(^{2+}\) signals, and AKAP10, an anchoring protein that targets protein kinase A to mitochondria, are consistent with altered signaling to mitochondria, which could be responsible for some of the bioenergetic defects observed in eIF4GI-silenced cells (Wang et al., 2001). Translational alterations in eIF4GI-silenced cells likely also trigger indirect gene expression changes. For example, eIF4GI-silenced cells demonstrate diminished levels of the myc coactivator MYCBP, and increased levels of proliferation inhibitor KLF4 (Fig. 6 C) (Rowland and Peeper, 2006). In contrast, mRNAs encoding enzymes involved in branched-chain amino acid and fatty acid catabolism are increased in polysomes of eIF4GI-depleted cells, indicative of induction of a general catabolic state. Of interest, the PI3K/AKT/mTOR pathway regulates \(\beta\)-oxidation (Deberardinis et al., 2006), and multiple mRNAs involved in this process are increased in polysomes of eIF4GI-depleted cells. This increase in catabolic pathway components is likely a response to diminished nutrient sensing and mitochondrial activity. mRNAs encoding factors that participate in mitosis, particularly cytokinesis, were down-regulated in polysomes from eIF4GI-depleted cells. Altered cytokinesis with eIF4GI silencing was also reported previously (Coldwell and Morley, 2006). Interestingly, several pro-apoptotic mRNAs, including caspase 3 and Bid, were largely excluded from eIF4GI-depleted polysomes.
decreased in polysomal association with eIF4GI silencing have a higher mean folding energy than those that increase in poly- some association, suggesting that they are more highly struc- tured (Fig. S5 C). Nevertheless, insertion of a stable hairpin in the 5’UTR of a reporter mRNA did not further impair transla- tion rates with eIF4GI silencing, demonstrating that increased secondary structure is not suffi  cient to impart an increased re- quirement for eIF4GI (Fig. S5 D).

Interestingly, the 3’UTR of mRNAs down-regulated in polysomes from eIF4GI-silenced cells were longer than those that are up-regulated, suggesting the involvement of sequences or structural elements (Table I). Of note, the 3’UTR of certain plant RNA viruses binds specifi cally to eIF4GI and the mRNA 5’UTR, providing a cap-independent bridge to the ribosome. The mRNAs reduced in polysomes by depletion of eIF4GI are twice as likely When apoptosis is blocked, growth factor deprivation typically induces autophagy by preventing utilization of extracellular nutrients (Kundu and Thompson, 2005). The bioenergetic de- fects with eIF4GI silencing do not trigger apoptosis, but may promote cell death through autophagy. Conversely, mRNAs encoding several proteins involved in stress responses, including Hsp27, were increased in their polysomal association in eIF4GI-silenced cells.

The four clusters of mRNAs were examined for com- mon 5’ and 3’UTR elements that might play a role in their translation response to eIF4GI-depletion (Karolchik et al., 2004; Mignone et al., 2005; Huang et al., 2006). The 5’ UTR of mRNAs that decreased in polysomal association with eIF4GI silencing tended to be longer than those that are up-regulated (Table I). Although this trend is not statistically significant, mRNAs that decreased in polysomal association with eIF4GI silencing have a higher mean folding energy than those that increase in poly- some association, suggesting that they are more highly struc- tured (Fig. S5 C). Nevertheless, insertion of a stable hairpin in the 5’UTR of a reporter mRNA did not further impair translation rates with eIF4GI silencing, demonstrating that increased secondary structure is not sufficient to impart an increased re- quirement for eIF4GI (Fig. S5 D).

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to contain upstream ORFs (uORFs) in their 5′ UTRs (Skp2 mRNA contains two uORFs) (Table I). Because eIF4GI is necessary for translation reinitiation following an upstream ORF in vitro (Poyry et al., 2003), we examined the effect of uORFs on mRNA translation in eIF4GI-depleted cells. A bicistronic reporter mRNA was expressed in which the cap-dependent ORF was under the control of a wild-type 5′ UTR fragment of MDA-7 that contains three uORFs, or a mutant MDA-7 5′ UTR that harbors AUG to UUG mutations to eliminate the uORFs (Fig. 6 D). The wild-type 5′ UTR decreased translation by almost half in control cells and 80% in eIF4GI-depleted cells, in contrast to DAP5 depletion, which did not further decrease translation of the uORF-mRNA (Fig. 6 D). Similar results were obtained with a monocistronic firefly luciferase mRNA (Fig. S5, A and D). An upstream ORF in the wild-type PPP3CB 5′ UTR did not reduce translation in control cells compared with the non-ORF 5′ UTR, but did so by ∼60% in eIF4GI-silenced cells (Fig. S5, normalized for equal mRNA). Similar results were obtained with the 5′ UTR of the UBE4A mRNA (unpublished data).

The uORF-mediated suppression of mRNA translation in eIF4GI-silenced cells could be prevented by overexpression of the uORF-containing mRNA (Fig. S5 B). We therefore re-ranked the mRNAs in our microarray analysis based on total mRNA abundance, then segregated these mRNAs into abundance quartiles ranging over several hundred fold. We evaluated the percentage of eIF4GI-dependent mRNAs (clusters 1 and 2, 165 genes) that classify to each quartile (Table II). Remarkably, 54% of mRNAs decreased in polysomes with eIF4GI silencing belong to the lowest abundance quartile, whereas only 9.7% belong to the highest abundance quartile (total adds to slightly more than 100% due to multiple targets for the same mRNA in the microarray). Moreover, 92% of the mRNAs containing uORFs belong to the two lower abundance quartiles (Q3 and Q4). These data indicate that an important previously unrecognized function of eIF4GI is to promote selective translation of low abundance mRNAs.

### Table I. Sequence features of mRNAs altered in polysomal association of eIF4GI-depleted cells

| Feature          | Decreased in eIF4GI-depleted polysomes | Increased in eIF4GI-depleted polysomes |
|------------------|----------------------------------------|----------------------------------------|
| 5′ UTR           |                                        |                                        |
| Average length   | 1,422                                  | 1,659                                  |
| Range            | 79.30%                                 | 38.70%                                 |
| Median           | 42                                      | 26                                     |
| Longer than average (740) | 79%                                     | 38%                                    |
| 3′ UTR           |                                        |                                        |
| Average length   | 15                                     | 10                                     |
| Range            | 98–5,443                               | 15–7,486                               |
| Median           | 1,422                                  | 567                                    |
| Longer than average (740) | 79%                                     | 38%                                    |
| % containing uORF| 42                                     | 20                                     |
| % ≥ 2 uORF       | 26                                     | 7.7                                    |
| % containing GC-rich repeats | 21                                     | 6.2                                    |

### Table II. Comparison of total mRNA abundance and polysomal decrease in eIF4GI-depleted cells (clusters 1 and 2)

| Intensity quartile | Percentage of mRNAs from clusters 1 and 2 | Number of targets |
|--------------------|--------------------------------------------|-------------------|
| Q1 (398 targets)   | 9.7                                        | 16/165            |
| Q2 (398 targets)   | 15                                         | 24/165            |
| Q3 (398 targets)   | 28                                         | 46/165            |
| Q4 (400 targets)   | 54                                         | 90/165            |

#### Discussion

In budding yeast, eIF4G is present at sub-stoichiometric concentrations compared with ribosomes (von der Haar and McCarthy, 2002), and nutritional deprivation triggers translational downregulation and decreased levels of eIF4G (Berset et al., 1998). Our results indicate that although an artificially low level of eIF4G is not greatly limiting for translation of most mRNAs, high levels are essential for the strong translation of a subset of mRNAs whose products are involved in regulation of mitochondrial activity, cellular bioenergetics, and cellular proliferation. It was also noted by others that eIF4GI silencing only partially diminishes protein synthesis rates (Coldwell and Morley, 2006). How does protein synthesis take place with such low levels of eIF4G in silenced cells? Importantly, we find that even a moderate decrease in the levels of members of the eIF3 complex results in a significant reduction in overall protein synthesis rates. It has been reported that eIF3 can promote the association of mRNA with ribosomes in the absence of eIF4G in yeast (Jivotovskaya et al., 2006), suggesting that eIF4G may act after 40S ribosome subunit association with mRNA, and is not necessary for ribosome scanning on unstructured mRNAs in vitro (Pestova and Kolupaeva, 2002). Thus, translation initiation might proceed on many mRNAs through an alternate mechanism that involves little requirement for eIF4G. Additionally, our results with eIF4G and DAP5 co-silencing add to the growing evidence that different eIF4G family members might promote translation of overlapping and distinct groups of mRNAs (Lee and McCormick, 2006; Baker and Fuller, 2007; Franklin-Dumont et al., 2007).

It is striking that polysome association of mRNAs encoding regulators of nutrient utilization and mitochondrial regulation were decreased in eIF4G-silenced cells, including AKT3 (glucose homeostasis), AKAP10 and ARALAR (signaling to mitochondria) (Wang et al., 2001; Brozinick et al., 2003), as well as mRNAs encoding mitochondrial ribosomal proteins, which is also consistent with the regulation of eIF4G levels and activity in response to cellular nutrition and growth factor status. Because activated AMPK inhibits the mTOR pathway by directly phosphorylating TSC2, an inhibitor of the mTOR-activating factor Rheb (Inoki et al., 2003; Connolly et al., 2006), the bioenergetic alterations resulting from mitochondrial dysfunction in eIF4G-silenced cells likely act in a feedback loop to down-regulate mTOR activity, thereby promoting a nutrient deprivation phenotype.

As has been previously shown, deprivation of growth factor signaling, when coupled with an inability to undergo...
apoptosis, leads to starvation, withdrawal from the cell cycle, and autophagy, despite the abundance of extracellular nutrients (Lum et al., 2005). eIF4GI-silenced cells display all of these characteristics, particularly an inability to sense nutrients and maintain bioenergetics (i.e., mitochondrial dysfunction and AMPK activation), but without undergoing apoptosis, which is likely due to decreased translation of pro-apoptotic factor mRNAs. The reduced proliferation rate of eIF4GI-silenced cells can likely be accounted for by the decrease in polysome association of key transcription factors and kinases involved in cell cycle regulation (see DNA microarray data section of Materials and methods), including Skp2 (Fig. 5). In addition, reduction of mitogenic signals and decreased mTOR signaling lead to withdrawal from the cell cycle (Lum et al., 2005). Supporting our findings, eIF4GI was recently identified in a high-throughput screen as a factor that regulates cell size and cell cycle progression (Bjorklund et al., 2006). In fact, eIF4GI was categorized as a member of a “signaling” network that includes raptor, a crucial component of the mTORC1 signaling pathway, which we showed here to interact with and to phosphorylate eIF4GI in vitro. eIF4GI silencing in that screen led to increased GI content and decreased cell size, observed in our study as well. The finding that eIF4GI abundance is responsive to mTORC1 signaling and similarities between mTOR pathway inhibition and eIF4GI silencing suggests that the PI3K/AKT/mTOR axis communicates with eIF4GI during nutrient sensing to control bioenergetics. Of note, eIF4GI silencing increases p27 stability and abundance, as has been noted in mTORC1 inhibited cells (Nourse et al., 1994). In the lower metazoan C. elegans, nutritional deprivation, TOR, and eIF4G silencing all lead to remarkably similar phenotypes (Long et al., 2002).

Our findings also suggest the intriguing possibility that the eIF4G family of factors might favor translation of partially distinct classes of mRNAs. For instance, eIF4GII or Paip1 silencing does not result in metabolic disturbances. Indeed, it has been proposed that eIF4GII is regulated during differentiation by Ca\textsuperscript{2+}/calmodulin-dependent protein kinase I (CaMKI) and may selectively translate developmental mRNAs (Qin et al., 2003; Caron et al., 2004). Although the depletion of DAP5 similarly decreases translation rates (~30%) as observed with eIF4GI depletion, only some of the metabolic alterations associated with eIF4GI depletion are apparent in DAP5-depleted cells (Lee and McCormick, 2006). Further, our results show that DAP5 silencing does not specifically decrease translation of uORF-containing mRNAs, unlike eIF4GI depletion, nor does it substitute for eIF4GI to promote cell proliferation. Supporting our findings, eIF4GI was recently identified in a high-throughput screen as a factor that regulates cell size and cell cycle progression (Bjorklund et al., 2006). In fact, eIF4GI was categorized as a member of a “signaling” network that includes raptor, a crucial component of the mTORC1 signaling pathway, which we showed here to interact with and to phosphorylate eIF4GI in vitro. eIF4GI silencing in that screen led to increased GI content and decreased cell size, observed in our study as well. The finding that eIF4GI abundance is responsive to mTORC1 signaling and similarities between mTOR pathway inhibition and eIF4GI silencing suggests that the PI3K/AKT/mTOR axis communicates with eIF4GI during nutrient sensing to control bioenergetics. Of note, eIF4GI silencing increases p27 stability and abundance, as has been noted in mTORC1 inhibited cells (Nourse et al., 1994). In the lower metazoan C. elegans, nutritional deprivation, TOR, and eIF4G silencing all lead to remarkably similar phenotypes (Long et al., 2002).

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Fig. 7 outlines the control of translation by the mTOR signaling network and places our results in the context of known and potential regulatory components of the eIF4G family. In summary, the phenotypic similarities of eIF4GI silencing and mTORC1 inhibition described here indicate that eIF4GI is an essential factor of the nutrient-sensing pathway. These data also suggest that the function of overexpressed eIF4GI in tumors may involve suppression of autophagy-mediated cell death.
Samples were pulse-labeled for 30 min with 10 μCi of [35S]-methionine and metabolic labeling of cells and preparation of extracts image processing with Photoshop 7 software. Inc.) and converted into JPEG files, pseudo-colored, and merged for microscopy studies.

Membrane potential assay, autophagy induction, and MDC labeling Mitochondrial membrane potential was measured by incubating control or elf4G1-silenced cells for 30 min with 50 mM of tetramethyl rhodamine ethyl ester (TMRE; Invitrogen) followed by FACS analysis. Autophagy was induced by amino acid starvation. Indicated cells were washed three times with PBS and incubated in HBSS for 30 min at 37°C. Autophagic vacuoles in starved or siRNA-transfected MCF10A cells were labeled by incubation with 0.05 mM MDC (Invitrogen) in PBS at 37°C for 10 min (Biederbick et al., 1995). Cells were washed three times with PBS and lysed in buffer containing 0.1% Triton X-100 and 10 mM Tris-HCl, pH 8. Incorporated MDC was measured by fluorescence photometry (excitation wavelength 380 nm, emission filter 525 nm) in a FluoroCount microplate reader, and normalized to DNA levels.

Confocal microscopy studies After treatment, cells were seeded into 6-well plates with coverslips and grown for 24 h to ~70% confluency. After three PBS washes, cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in PBS for 10 min at 25°C. Fixed cells were washed once with PBS and permeabilized in 0.25% Triton in PBS for 10 min at 25°C, washed with PBS and blocked in 10% BSA in PBS for 1 h at 25°C. GFP-LC3 transfected or transfected cells were washed and mounted onto slides. For antibody staining for fluorescence microscopy, cells were incubated with 100 μl of primary antibody in 3% BSA in PBS (1:100 affinity-purified elf4G1 anti-serum, and 1:100 mouse monoclonal 12-28 (BD Biosciences), or 1:100 rabbit anti-β-actin (AbCam) antibody) overnight in a humidified chamber at 4°C. After three PBS washes, cells were incubated with secondary antibody in 3% BSA in PBS (1:100 FITC-conjugated donkey anti-rabbit and 1:100 TRITC-conjugated donkey anti-mouse; Jackson Immunoresearch Laboratories) at 25°C for 1 h. Coverslips were mounted on slides using Vectashield mounting medium with propidium iodide (Vector Laboratories). Confocal immunofluorescence microscopy and image acquisition were performed at room temperature using a confocal scanning laser microscope (LSM 510 META; Carl Zeiss, Inc.) using an oil immersion Plan Apochromat 1.4 NA 63x objective. The laser scan head is equipped with a META detector (multi-channel broad spectrum photon-multiplying tube arrays) and two additional single photon multipliers that assemble imaging pixels by pixel using Lasersharp 2000 software (Carl Zeiss, Inc.) and converted into JPEG files, pseudo-colored, and merged for image processing with Photoshop 7 software.

Metabolic labeling of cells and preparation of extracts Cells were pulse-labeled for 30 min with 10 μCi of [35S]-methionine and cysteine mix per ml (Easytag Express Protein Labeling Mix; PerkinElmer) in DMEM without methionine and cysteine containing 2% dialyzed FBS. After treatment, cells were washed twice in ice-cold 1x PBS and lysed in ice-cold buffer A (0.5% i godeal [vol/vol], 150 mM NaCl, 50 mM Hepes, pH 7.0, 2 mM Na2VO4, 25 mM β-glycerophosphate, and complete protease inhibitor mix; Roche). Debris was removed by centrifugation at 4°C for 10 min and the supernatants were stored at -80°C. Protein concentrations were determined by the Bradford method (Bio-Rad Laboratories). Specific activity was determined by trichloroacetic acid precipitation and liquid scintillation.

For immunoprecipitation of [35S]-labeled proteins, cells were labeled in Easytag mix as described above for 2 h, lysed in buffer A, and sonicated with four pulses of 5 s each. For determination of LC3, KLF4, and MYCBLP levels, cells were lysed in RIPA buffer (1% i godeal, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris, pH 8.0, 2 mM Na2VO4, 25 mM β-glycerophosphate, and complete protease inhibitor mix; Roche) and protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad Laboratories).

Polysome gradients, RNA extraction, and microarray analysis 48 h after siRNA transfection, MCF10A cells were seeded in 15-cm plates and harvested at 50% confluency (three plates per condition). Cells were treated with 100 μg/ml cycloheximide (Calbiochem) in complete medium for 5 min at 37°C, collected in ice cold PBS supplemented with complete protease inhibitor without EDTA (Roche) and 100 μg/ml cycloheximide. All subsequent steps were performed at 4°C. Cells were washed twice in PBS with 100 μg/ml cycloheximide, pelleted, and resuspended in 700 μl polysome isolation buffer (200 mM Tris, pH 7.5, 100 mM NaCl, and 30 mM MgCl2). After 5 min incubation, 250 μl detergent buffer (1% Triton, 0.2 M sucrose in polysome isolation buffer) was added and cells were lysed with a dounce homogenizer with 10 strokes. lysates were clarified by centrifugation at 13,000 g for 10 min at 4°C and supernatants were transferred to new tubes containing 100 μl heparin buffer (10 mg/ml heparin·[Sigma-Aldrich], 1.5 M NaCl in polysome isolation buffer). Samples were layered onto 11-ml 10–50% sucrose (Sigma-Aldrich) gradients in polystyrene extraction tubes (3.3 ml each), spun at 19,500 rpm for 1 h in SW41 rotor (Beckman Coulter) at 4°C. Gradients were collected from the bottom up with three 100 μl fractions each. RNA was isolated by extraction with phenol/chloroform followed by chloroform extraction, and precipitated overnight with 1 volume isopropanol and 0.1 volume NaOAc (pH 5.2) at -20°C. Fractions were centrifuged at 13,000 g for 10 min to collect the RNA, washed twice with 70% EtOH, and resuspended in 100 μl nuclelease-free water. Fractions 6–12, representing polysomes, were combined and extracted twice with phenol and twice with chloroform, followed by isopropanol precipitation. 10 μg of pooled RNA were used for microarray analysis using the one-cycle cDNA synthesis GeneChip kit and hybridized to GeneChip Human Genome U133A 2.0 chips, both from Affymetrix, according to the manufacturer’s instructions. For normalization, total RNA was extracted from cells using Trizol and further purified through RNeasy mini-eilute columns (Qiagen). RNA quantity and quality were determined using a bioanalyzer (Agilent Technologies), and samples were stored in nuclelease-free water at -80°C. mRNA targets with splice variants that alter 5’ and 3’ UTRs were omitted from the UTR analyses.

MITT proliferation assay Cell growth was measured by a standard MTT Cell Proliferation Assay. In brief, cells infected with shRNA-expressing lentiviruses and selected with puromycin and/or G418 were plated in quadriplicate (per time point) in 96-well format and subsequently quantified four times over a course of 72 h. Wells were treated with 20% sterile-filtered 5 mg/ml MTT (Sigma-Aldrich) in DMEM over 6 h at 37°C. The reduced formazan was then solubilized in a 1:1 DMF/20% SDS solution overnight. Colorimetric quantification was performed with a TECAN Sunrise microplate reader at an OD of 570 nm, with a negative reference at 450 nm. Relative growth rates were determined from the first-order logarithmic regression analysis of the colorimetric change over time per cell line (P < 0.01). Results were normalized to the nonsilencing shRNA control cell line.

DNA constructs, transfections, transductions, and luciferase assays Plasmid pE GFP-LC3 was from T. Yoshimori (National Institute for Basic Biology, Okazaki, Japan). 293T cells were transfected by the CaPO4 method. pBabe-AcGFP-LC3 was generated by subcloning the LC3 fragment from EGFP-LC3 into the pBabe-AcPlacI (BD Biosciences), and for further subcloning into the pHSN and pSall sites of AcPlacI, those same sites in pBABE-puro. The pRl-HCV-IRES-FL plasmid (Tischendorf et al., 2004) was a gift from M. Krüger (Medizinische Hochschule Hannover, Hannover, Germany). Duplexed oligos spanning bases 17 to 105 of the 5’ UTR of mda-7/IL24 with our without uORFs (wild-type or AUG to UUG mutants) were purchased from Integrated DNA Technologies and ligated into the EcoRV site of the pRl-HCV-IRES-FL plasmid. pCMVLuc was generated by insertion of HindIII-XhoI fragment carrying for luciferase from pSP-luc+ (Promega) into the same sites of the plasmid.
pcDNA3.1 (Invitrogen). Duplexed oligos spanning the entire 5′ UTR of PPP3CB with our without uORFs (wild-type or AUG to UUG mutants) were ligated into blunt-ended HindIII-cut pCMVlac. Constructs were verified by DNA sequencing. pcRif and pstemRif were provided by G. Goodall (Institute of Medical and Veterinary Science, Adelaide, Australia). The indicated concentrations of plasmids were transfected into MCF10A cells in 6-cm dishes at ~50% confluency 48 h after siRNA transfection using Firefly Luciferase or Dual Luciferase Reporter Assay Systems (Promega). HA-GST-p85 SK61 pRKS5 was from D.M. Sabatini (Whitehead Institute, Cambridge, MA; Addgene plasmid 8466). pSSPAGI was a gift of R. Lloyd (Baylor College of Medicine, Houston, TX). The elf4GI primers were used to amplify elf4GIc (the nomenclature of Byrd et al. [2002]). The amplified products were cloned into pcDNA3.1 (Invitrogen) and sequenced. The plasmids were a gift of Dr. Weinberg, Whitehead Institute. Targeted knockdown of mTOR, elf4GIc, and 4E-BP1 was performed essentially as described, with modifications (Kim et al., 2002). 293T cells were transfected with HA GST SK61 or myc-His elf4GI plasmids and 24 h later incubated with 10 μM YM201636 and 100 nM rapamycin for 12 h. For mTOR immunoprecipitation, MCF10A cells were lysed in buffer B (40 mM Hepes, pH 7.4, 120 mM NaCl, 1 mM EDTA, and 0.3% CHAPS) supplemented with complete protease inhibitor tablet (Roche) and 1× Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Lysates were clarified by centrifugation at 0.8 g for 10 min. After pre-clearing, 500 μg of MCF10A lysate were immunoprecipitated with mTOR antibody and protein A/G-agarose slurry. Treated 293T cells expressing either SK61 or elf4GIc were lysed in Buffer A, and immunoprecipitated with either HA or myc antibodies and protein A/G-agarose slurry. Captured immunoprecipitates were washed in their respective buffers three times, then washed two more times in buffer B without EDTA. mTOR immunoprecipitates were combined with either SK61 or elf4GI immunocomplexes and incubated with 40 μl of kinase buffer [25 mM Hepes-KOH, pH 7.4, 50 mM KCl, 20% Glycerol, 10 mM MgCl2, 4 mM MnCl2, 1 mM DTT, and 500 μM ATP] for 15 min at 37°C. The reaction was stopped by addition of 10 μl 6X SDS-loading buffer and proteins were separated by SDS-PAGE followed by immunoblotting. Quantification of bands was performed by densitometry using Quantity-One software (Bio-Rad Laboratories).

Quantitative RTPCR RNA was extracted using Trizol (Invitrogen), and in the case of luciferase reactions, DNase treated using Turbo DNA-free kit (Ambion). Single step qRT-PCR reactions were performed using SYBR Green Quantitative RTPCR kit (Sigma-Aldrich) in a Lightcycler (Roche) according to manufacturer’s instructions. Primer pair sequences used are available upon request. mRNA changes were quantified using the ΔΔCt method using β-actin mRNA as control (Winer et al., 1999). For qRT-PCR analysis of light and heavy poly- somes, fractions were pooled into unbound mRNA and subunits (1–4), monosomes and light (5–8), and heavy (9–12) polysomal pools. Virtually no RNA was amplifiable in the first pool, so comparisons were made between light and heavy polysomes only, normalized to β-actin and setting the sum of mRNA in the two pools to 100%.

Ribosome half-transit time measurements Measurement of ribosome half-transit time was performed as described, with modifications (Rivuschkin et al., 2005; Sivan et al., 2007). Equal numbers of control or elf4GI overexpressing cells were cultured in medium supplemented with 5% dialyzed FBS and 10 μCi of [35S]-methionine and cysteine mix per ml. At the indicated times, 0.25 ml aliquots were mixed with 1.75 ml ice-cold PBS with 100 μg/ml cycloheximide. Cells were lysed in ribosome buffer (20 mM Hepes [KOH], pH 7.2, 10 mM NaCl, 3 mM MgCl2, 0.5% Igepal, 0.1 mM DTT, 100 μg/ml cycloheximide, and protease inhibitor cocktail) for 20 min, nuclei and mitochondria were pelleted by centrifugation at 4°C, maximum speed for 10 min. 50% of this post-mitochondrial supernatant (PMS) was depleted of ribosomes through ultracentrifugation at 100,000 g for 20 min at 4°C to generate post-ribosomal supernatant (PRS). Incorporated radioactivity was measured in equal aliquots of PMS and PRS samples, and the ribosome half-transit time was determined as the displacement in time of the PMS (total protein) and PRS (released protein) linear regression trends.

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DNA microarray data
The DNA array analysis was performed at the NYU Cancer Institute Genomics Facility. Samples were processed in triplicate for each experimental condition. The preparation of cRNA probes and hybridization to GeneChip HGU133A 2.0 arrays followed the recommendations of the manufacturer (Affymetrix). Raw data were normalized by model-based expression index algorithm using dChip (Li and Wong, 2001) filtered on presence/absence calls using GeneSpring software version 7.2 (Silicon Genetics/Agilent). Differentially abundant mRNAs were identified in a cross-validation approach using TM4 analysis software (Saeed et al., 2003) by combining t test with P value cutoff of 0.05 and/or SAM (Tusher et al., 2001) with false discovery rate (FDR) set to 5%. The complete set of microarray data was deposited for public access to the NCBI Gene Expression Omnibus database under the accession number GSE10111.

Online supplemental material
Fig. S1 shows that elf4GI silencing was sustained and in most cells. Fig. S2 demonstrates that elf4GI silencing does not strongly decrease protein synthesis in primary and highly transformed cells, despite disassembly of elf4F complexes, and can be rapidly recovered after hypoxia or hyper-tonic stresses that inhibit initiation and elongation. Fig. S3 demonstrates that ribosome transit time on mRNAs is not significantly altered by elf4GI silencing. Fig. S4 shows effects of silencing different elf4F family members on protein synthesis and cellular proliferation rates. Fig. S5 provides evidence that elf4GI silencing particularly impairs translation of mRNAs containing uORFs. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200710215/DC1.

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