Regulation of eukaryotic translation initiation factor 6 dynamics through multisite phosphorylation by GSK3

Courtney F. Jungers1, Jonah M. Elliff, Daniela S. Masson-Meyers1, Christopher J. Phiel1*, and Sofia Origanti1,3,*,†

From the 1Department of Biological Sciences, Marquette University, Milwaukee, Wisconsin, USA, the 2Department of Integrative Biology, University of Colorado, Denver, Colorado, USA, and the 3Department of Biology, Saint Louis University, St. Louis, Missouri, USA

Eukaryotic translation initiation factor 6 (eIF6) is essential for the synthesis of 60S ribosomal subunits and for regulating the association of 60S and 40S subunits. A mechanistic understanding of how eIF6 modulates translation in response to stress, specifically starvation-induced stress, is lacking. Here we show a novel mode of eIF6 regulation by glycogen synthase kinase 3 (GSK3) that is predominantly active in response to serum starvation. Both GSK3α and GSK3β phosphorylate human eIF6. Multiple residues in the C terminus of eIF6 are phosphorylated by GSK3 in a sequential manner. In response to serum starvation, eIF6 accumulates in the cytoplasm, and this altered localization depends on phosphorylation by GSK3. Disruption of eIF6 phosphorylation exacerbates the translation inhibitory response to serum starvation and stalls cell growth. These results suggest that eIF6 regulation by GSK3 contributes to the attenuation of global protein synthesis that is critical for adaptation to starvation-induced stress.

Eukaryotic translation initiation factor 6 (eIF6) is a key modulator of translation initiation that regulates the biogenesis and availability of the 60S ribosomal subunits (1–3). eIF6 directly associates with pre-60S complexes in the nucleolus and is exported into the cytoplasm in complex with the 60S where it aids in 60S maturation (4–8). The well-characterized role of eIF6 is its anti-association activity that prevents interactions between the 60S and 40S ribosomal subunits (2, 9–12). Structural and biochemical studies indicate that eIF6 binds to 60S at the 40S-binding interface and sterically hinders association of the 40S subunits (10–16). Release of eIF6 from the mature 60S allows association of the 40S–mRNA complex, which leads to active 80S formation and translation initiation (10–15). An inhibition of eIF6 release (or premature release) from the 60S subunits can greatly influence intersubunit association and translation initiation. A block in eIF6 release leads to an increase in the fraction of eIF6-bound 60S subunits that are unable to join 40S, which hinders the assembly of active 80S monosomes (10–13, 15, 16). Alternatively, insufficient levels of eIF6 seem to lead to spurious association of 60S with the 40S subunits that are devoid of mRNA. This causes an increase in the assembly of inactive 80S monosomes, which impairs translational response to growth stimuli as seen in eIF6−/− mice (17–19). Thus, an impairment of eIF6 function can substantially affect ribosome levels, limiting protein synthesis specifically in response to growth stimuli, and this deregulation has been shown to contribute to the underlying pathologies of diseases such as Shwachman–Bodian–Diamond syndrome, cancer, and certain metabolic disorders (13, 17–20).

In response to stimuli such as insulin or phorbol esters, primary hepatocytes and mouse embryonic fibroblasts (MEFs) derived from the eIF6−/− mice do not up-regulate protein synthesis, unlike the WT cells (17–19). Similar defects in translation are also observed in vivo, where livers of eIF6−/− mice are smaller and exhibit an accumulation of inactive/empty (mRNA-free) 80S ribosomes compared with WT (17–19). The lack of insulin-dependent stimulation of protein synthesis in the eIF6−/− mice has been associated with a reprogramming of fatty acid synthesis and glycolytic pathways with implications for muscle, liver, and fat metabolism (19, 21).

In terms of mechanism, the increased formation of inactive 80S complexes in the eIF6−/− cells is attributed to an impairment of its anti-association function in the cytoplasm (17–19). Interestingly, such an accumulation of inactive 80S monosomes is commonly observed in cells subjected to stress, especially stress induced by nutrient deprivation or limitation (22–24). Starvation or nutrient limitation in yeast and mammalian cells invokes an adaptive metabolic response that conserves energy by restricting global protein synthesis and leads to an accumulation of inactive (empty) 80S ribosomes along with an increase in the pool of free 60S subunits (22–33). The role of eIF6 as a ribosome anti-association factor and 60S biogenesis factor in modulating this starvation response has not been thoroughly explored. Given the key role of eIF6 in regulating translational response to insulin and growth factors in an mTOR-independent manner (19, 34), it is important to understand the mechanisms that control eIF6 in response to growth inhibitory stress responses. To address this, here we report a novel regulation of eIF6 by glycogen synthase kinase 3 (GSK3) that is active under conditions of serum starvation-induced stress.

Global proteomic and biochemical studies indicate that human and murine eIF6 is phosphorylated at multiple sites, and the majority of these sites are conserved and cluster around the C-terminal tail (35–42). However, most of these phosphorylation sites have not been validated in vivo, and the identities of the associated kinases have not been clarified. It is also unclear whether these uncharacterized phosphorylation sites carry any functional relevance. Here we report a novel...
regulation of eIF6 by GSK3 that is active under conditions of serum starvation-induced stress. Based on sequence-motif prediction analysis, we identified the presence of a GSK3-specific motif within the C-terminal tail of eIF6. Previous studies have shown that GSK3 is a unique kinase that is activated in response to growth inhibitory conditions such as starvation or under resting states and is inhibited in response to insulin and other growth stimulatory conditions by AKT and mTORC1-p70S6K1-dependent phosphorylation (43–49). GSK3 plays a prominent role in translational control by inhibiting the nucleotide exchange function of the translation initiation factor: eIF2β, which is reversed in response to insulin (43, 50–52). GSK3 phosphorylates the Ser-540 site in the e subunit of eIF2B to inhibit its activity (43, 50–52). Here, we show that GSK3 also influences translation by regulating human eIF6 through multisite phosphorylation at Ser-243, Ser-239, Ser-235, and Thr-231 sites. GSK3 interacts with endogenous eIF6, and phosphorylation by GSK3 alters the subcellular localization of eIF6 in response to serum starvation. Interestingly, the phosphodead mutant and the C-terminal deletion mutant displayed increased levels of free 60S subunits in response to serum starvation. Altering the steady-state levels of ribosomal subunits even by a small percentage can have detrimental effects as seen in several ribosomopathies (53, 54). Expression of the phosphodead mutant decreased translation rates and stalled cell growth in response to serum starvation. Based on these findings that eIF6 is a novel substrate of GSK3, we propose a model wherein GSK3-dependent phosphorylation of eIF6 contributes to translation inhibition in response to serum starvation by regulating 60S availability.

**Results**

**GSK3 phosphorylates the C-terminal tail of eIF6**

Sequence analysis of the C terminus of human eIF6 revealed the presence of potential candidates for phosphorylation by GSK3. We identified two such multisite motifs (motif 1 and 2) in the C terminus that are highly conserved in higher eukaryotes from *Xenopus* to mammals (Fig. 1A) but show variability in lower eukaryotes such as yeast. Canonically, GSK3 phosphorylates multiple residues on its substrates in a sequential manner starting with the C terminus. GSK3 often works synergistically with a priming kinase that phosphorylates a priming site located 4 residues away from the GSK3 recognition site (Fig. 1A); however, GSK3 can also work independently of a priming kinase (43, 45, 46, 55–57). The potential priming sites on eIF6 are indicated in Fig. 1A. To determine whether eIF6 is a substrate of GSK3, we purified full-length human eIF6 from *Escherichia coli* and incubated recombinant eIF6 with GSK3. GSK3α and GSK3β are two isoforms of GSK3 that are largely redundant in their activities. Human eIF6 was phosphorylated by both the isoforms of GSK3 (Fig. 1, B and C). We also observed autophosphorylation of GSK3 as previously reported (45). The kinases were used at low concentrations that would not be detected by Coomassie staining, but the autophosphorylation showing similar kinase activities could be detected. Phosphorylation of eIF6 was detected by the incorporation of radiolabeled phosphate in eIF6 in the presence of GSK3 only. Control reactions lacking eIF6 or GSK3 did not exhibit a band at these molecular weights highlighting specificity.

To validate these results and to determine whether eIF6 obtained under a physiological context was also a substrate for GSK3, eIF6 was immunoprecipitated from HCT116 cells that were briefly serum-starved, to capture potentially preprimed eIF6. We observed that the immunoprecipitated eIF6 is also phosphorylated by GSK3β (Fig. 1D), thus confirming eIF6 as a bona fide target of GSK3. Also, GSK3β displayed a time-dependent activation in response to serum starvation, which was verified by probing for the loss of inhibitory phosphorylation of the serine 9 residue that is phosphorylated by kinases such as AKT (Fig. S1, A and B) (44, 46–49, 55, 56).

We next tested for specificity of phosphorylation by eliminating the two multisite motifs in the C terminus by creating a deletion mutant that lacks the last 36 amino acid residues (eIF6-ΔC36). GSK3β did not phosphorylate eIF6-ΔC36, whereas it phosphorylated the full-length (FL) eIF6, suggesting that the last 36 residues are critical for phosphorylation (Fig. 1D). We did observe a faint background signal for eIF6-ΔC36 incubated with kinase (Fig. 1D, lane 3). Prolonged exposures of the blots have captured a similar nonspecific band for the Myc-empty vector incubated with the kinase (Fig. S1C), which could be associated with immunoprecipitation.

We also observed a doublet of eIF6 that was more obvious for the immunoprecipitated substrate, and such a doublet has been observed before (14). This gel effect likely arises from the high cysteine content of human eIF6 (9 Cys residues for a 26-kDa protein); when denatured, the exposed Cys residues likely form nonspecific disulfide bonds that are resistant to reduction. Also, the addition of multiple negatively charged phosphates could further alter its gel migration patterns. The intensity of doublet varied based on the concentration of recombinant eIF6 (Fig. S1D) and also based on resolving gel percentage and concentration of DTT in sample buffer (data not shown). Both the bands of the doublet were confirmed to be full-length recombinant human eIF6 by MS (data not shown). Such a denaturing gel effect has been observed for other cysteine-rich proteins (58).

**GSK3 phosphorylates multiple sites within the last 20 amino acid residues of eIF6**

To identify the specific motif that is phosphorylated by GSK3, we analyzed a C-terminal tail deletion mutant lacking the last 20 residues (motif 2) (eIF6-ΔC20). Indeed, deletion of just motif 2 greatly reduced eIF6 phosphorylation (Fig. 2A) and confirmed that motif 2 is critical for recognition by GSK3β. In this case, motif 2 is in the disordered region of the C-terminal tail of eIF6 that is predicted to protrude outside the core structure, which would be favorable for regulatory interactions (10, 16). Also, all the predicted sites indicated in motif 2 were previously identified to be modified by phosphorylation in global proteomic studies, including those performed under cellular conditions of stress (35–41).
Computational analyses indicated that the C-terminal tail carrying motif 2 is highly conserved in vertebrates (especially birds and mammals), and analysis of 50 species of vertebrates indicated 100% identity in the GSK3-specific motif 2 in the C-tail (Fig. 2B). In addition, the serine/threonine residues at 239 and 243 are highly conserved in lower eukaryotes including budding and fission yeast.

To identify the specific residues in motif 2 that are phosphorylated by GSK3β, we carried out nanoscale liquid chromatography with tandem MS analysis (nano-LC–MS/MS) on the immunoprecipitated eIF6 sample incubated with and without GSK3β. MS analysis revealed that the Thr231 residue was phosphorylated by GSK3β (Fig. 2C and Fig. S2), and its detection by MS indicated a better preservation of the Thr-231 phosphorylation site. The relative abundances of the peptides in samples incubated with and without the kinase were found to be identical (Fig. S2, A–D). The technical limitations of MS to capture and accurately identify two or more sites of phosphorylation within a single peptide, especially when sample levels are limiting, made it challenging to capture other C-terminal phospho sites (59, 60). However, these results validated that motif 2 in the C-terminal tail is phosphorylated by GSK3β.

**GSK3 phosphorylates multiple sites on eIF6 in a sequence**

To directly test whether other GSK3-specific sites in motif 2 are phosphorylated, we used the site-directed mutagenesis approach in which the indicated serine or threonine sites of phosphorylation within motif 2 were substituted with alanines (Fig. 2D). In the presence of multiple and adjacent recognition sites, GSK3 phosphorylates residues in a sequence starting from the C terminus. Examples of sequential phosphorylation...
Regulation of eIF6 by GSK3

A

GSK3β + - +
eIF6-FL + - -
eIF6-ΔC20 - + +

Autorad.

p-GSK3β
p-eIF6

myc (blot)

B

KLNEAQPS'TIATSMDRSLDSLT

Consensus
Sequence
Identity
Coverage

C

114.69134 57.54931 L
258.74927 114.79707 N
377.17699 179.80207 E
486.21397 214.61863 A
595.27595 278.43981 Q
655.32332 257.16830 P
740.35734 370.68231 S
821.37135 461.18352 T
914.41542 517.73752 I
1019.45253 553.24950 A
1123.54521 603.77374 T
1227.54261 647.38636 S
13124.61272 712.81800 N
14175.11995 88.06311 L

D

GSK3

KLNEAQPS'TIATSMDRSLDSLT

231 235 239 243

phosphorylated
T231

E

GSK3β:

55 kDa-
35 kDa-
25 kDa-

p-GSK3β
p-eIF6

myc (blot)

F

% change in phosphorylation relative to eIF6-FL

FL  S243A

****
Regulation of eIF6 by GSK3

include substrates such as β-catenin and glycogen synthase that carry three and four GSK3-specific sites, respectively (61–65). Substitution of the potential priming site serine 243 with alanine resulted in a significant 40% reduction in phosphorylation compared with eIF6-FL (full-length and WT) (Fig. 2, E and F). This suggested that in the absence of the Ser-243 site, phosphorylation of the Ser-239, Ser-235, and Thr-231 sites are reduced. However, it indicated that the initiating phosphorylation at Ser-243 is required for robust phosphorylation of eIF6. It also suggested that the Ser-239 site could serve as a potential initiation site in the absence of the Ser-243 site. The significance of the other three sites was confirmed by substitution of the Thr-231, Ser-235, and Ser-239 sites with alanines (Fig. 2E). Mutation of these three residues greatly reduced phosphorylation of eIF6, suggesting that they are also the key sites of phosphorylation (Fig. 2F).

These results were further validated by analysis of the individual site mutants (Fig. S3A). The T231A mutation reduced phosphorylation by 40%, indicating that it is one of the sites of phosphorylation (Fig. S3, A and B), which was consistent with MS analysis. It also indicated that in the absence of the Thr-231 site, the other sites in the sequence are phosphorylated. Both the S235A and S239A mutations markedly reduced phosphorylation of eIF6. Altogether, the studies with the single-site, multisite, and deletion mutants and MS analysis indicate that the Ser-243, Ser-239, Ser-235, and Thr-231 sites are key sites of phosphorylation.

Further to determine whether phosphorylation of eIF6 is sequential, we used a classical approach of incubating the substrate peptide (C-terminal peptide of eIF6) with the kinase, followed by MS analysis (Fig. 3A). MS analysis revealed that the Ser-243 site is phosphorylated by GSK3. Doubly phosphorylated peptides were also detected with phosphorylation at both the Ser-243 and Ser-239 sites (Fig. 3A). These results strongly indicated that GSK3 sequentially phosphorylates eIF6 starting with the Ser-243 site followed by the Ser-239 site. Because of the technical limitations associated with MS, detection of peptides with triple or quadruple phosphorylation were found to be at or below baseline (Fig. 3A). There are several other serine and threonine residues in the C-tail; however, peptides with phosphorylation at other sites could not be detected. MS analysis only detected phosphorylation in the GSK3-specific motif, which highlighted the specificity of phosphorylation.

GSK3 often works in concert with priming kinases that phosphorylate the initiating/priming site in the sequence. It is currently unclear whether the initiating Ser-243 site is also recognized by another priming kinase in vivo. It is also unclear as to the likely effect of enhanced priming phosphorylation of the Ser-243 site on the subsequent sites in the sequence. We therefore synthesized a phosphopeptide with phosphorylation at the Ser-243 site (Fig. 3B) and subjected it to kinase assay. MS analysis revealed that GSK3β phosphorylated the phosphopeptide at subsequent Ser-239 and Ser-235 sites (Fig. 3B and Fig. S3C) sequentially. Detection of quadruple phosphorylation was at or below baseline (Fig. 3B). However, the Thr-231 site was identified earlier in our MS analysis of immunoprecipitated eIF6 (Fig. 2C). Interestingly, for both the primed and nonprimed peptide analysis, we were unable to detect any doubly phosphorylated Ser-235/Ser-243 or Thr-231/Ser-243 peptides or singly phosphorylated peptides with just the Ser-239, Ser-235, or Thr-231 phospho sites or peptides with phosphorylation at other serines and threonines in the C terminus. This clearly indicated that phosphorylation is specific and only occurs in a sequence. These MS results in combination with the analyses of site-specific mutants strongly indicate that the phosphorylation of eIF6 by GSK3β occurs in a sequence starting with the Ser-243 site, followed by the Ser-239, Ser-235, and Thr-231 sites.

Cellular phosphorylation of eIF6

Because GSK3 is activated under serum-starved conditions, we also determined whether eIF6 is phosphorylated in serum-starved 293T cells by MS. Nano-LC–MS/MS analysis revealed that the C-terminal tail of eIF6 is phosphorylated and that the sites of phosphorylation mapped to the Ser-243 site (Fig. S4A) or the Ser-239 site (Fig. S4B). As mentioned before, these phospho sites have been detected on endogenous eIF6 by several other phosphoproteomic studies.

We next tested whether GSK3β interacts with eIF6 under serum-starved conditions. GST–GSK3β interacted with endogenous eIF6 in serum-starved cells (Fig. 4A). The interaction was found to be specific to WT–GST–GSK3β because no such interaction was observed with either the empty vector or with a priming-site recognition mutant of GSK3 (GST–GSK3β-R96A) (Fig. 4, A and B). These results clearly indicate that there is a cellular interaction between GSK3β and eIF6.

Altered subcellular localization of eIF6 in response to starvation is regulated by GSK3

We then determined the effect of GSK3-dependent phosphorylation on eIF6 levels and/or localization. We observed
that the total levels of eIF6 were not altered by overexpression of GST-GSK3\(\beta\), suggesting that GSK3 may not regulate the total eIF6 protein levels (Fig. 4, A and B). This was also confirmed by probing for eIF6 protein levels in response to long-term serum starvation when GSK3 is fully active. We did not detect significant changes in total eIF6 protein levels even after 18 h of starvation, suggesting that GSK3 does not regulate the stability or synthesis of eIF6 protein (Fig. 4, C and D).
Regulation of eIF6 by GSK3

Figure 4. Cellular interactions between GSK3 and eIF6. A and B, to capture interactions between GST-GSK3β and endogenous eIF6, HCT116 cells were transfected with GST-GSK3β-WT or empty vector (A), and 293T cells were transfected with GST-GSK3β-WT or GST-GSK3β-R96A mutant (B), 24 h after transfection, the cells were serum-starved for 4 h. The blots were probed with the indicated antibodies. Each experiment was repeated three and two independent times, respectively. Whole-cell lysates (Input) of samples used for the pulldowns were analyzed by Western blotting and probed with the indicated antibodies (lanes 3 and 4). β-Actin and α-tubulin were used as the loading control. C, lysates of HCT116 cells were collected just prior to serum starvation at 0 h and again at 4, 8, and 18 h of starvation. The samples were analyzed by Western blotting, and the blots were probed with anti-eIF6 and anti-α-tubulin antibodies (loading control) (n = 3). D, total eIF6 protein levels were quantitated using the blots represented in C. The values represent the S.E. of three independent experiments. All time points were compared with the 0-h time point, and no significant differences were found, as determined by one-way analysis of variance (Dunnett’s multiple comparison test).

Because the total levels of eIF6 were unaltered, we next tested whether GSK3 regulates eIF6 function by altering its subcellular localization in response to serum starvation. Interestingly, immunofluorescence staining of endogenous eIF6 showed that eIF6 was predominantly localized to the cytoplasm in response to serum starvation in HeLa and HCT116 cells (Fig. 5, A–C, and Fig. S5, A and B). Serum starvation caused cytoplasmic (punctate) accumulation of eIF6, which was reversed by the readdition of serum, indicating that the response is prompt (Fig. 5A). These results were also confirmed by using a different mAb specific for eIF6, which ruled out nonspecific staining associated with the antibody (Fig. 5B). To further determine whether this altered subcellular localization depended on GSK3 activity, HeLa cells were treated with the GSK3 inhibitor LiCl. Treatment with the GSK3 inhibitor rescued eIF6 localization to the nucleus (Fig. 5C). Because LiCl could have other cellular targets, we also used CHIR99021, a highly selective GSK3-specific inhibitor, which also rescued eIF6 localization to the nucleus (Fig. S5B) (66, 67). The potency of the GSK3-specific inhibitor was confirmed by probing for a loss of phosphorylation of β-catenin at GSK3-specific sites (Ser-33/Ser-37/Thr-41) (61–63, 68), which was also validated with SB415286 treatment—another GSK3-specific inhibitor (Fig. S5, C and D). Inhibitor treatment resulted in greater than 50% loss in β-catenin phosphorylation, suggesting that GSK3 is inhibited under these conditions (Fig. S5, C and D). Similar effects on localization were observed for a normal rat intestinal epithelial cell line (RIE-1) treated with the GSK3-specific inhibitor. In RIE-1 cells, eIF6 shows predominant cytoplasmic accumulation in response to serum starvation as shown by fewer nuclei (pink) co-stained with eIF6 antibody, which is reversed by inhibition of GSK3 (Fig. S6A).

We further quantitated eIF6 levels in the nuclear and cytoplasmic fractions by subcellular fractionation. The purity of different fractions was confirmed by probing for topoisomerase IIβ, a nuclear marker, and α-tubulin, a cytoplasmic marker. eIF6 was distributed almost equally between the nuclear and cytoplasmic fractions in serum-fed controls (53% nucleus and 47% cytoplasm), and no significant difference was found between the two fractions (Fig. 6, A and B). Similar to the imaging results, we found that in the serum-starved cells, there was a significant decrease in eIF6 levels in the nucleus with a corresponding increase in the cytoplasm (~32% in nucleus and ~68% in cytoplasm) (Fig. 6, A, C, D, and E). Inhibition of GSK3 completely restored the nuclear–cytoplasmic distribution as seen in the CHIR99021-treated cells (54% nucleus, 46% cytoplasm) (Fig. 6, D and F).

To determine the significance of phosphorylation in regulating the subcellular localization of eIF6 in response to starvation, we generated Myc-tagged phosphodead mutant of eIF6 (eIF6-ΔC) in which all four sites of phosphorylation (Ser-243, Ser-239, Ser-239, and Thr-231) were substituted with alanines. We also generated the Myc-tagged eIF6-ΔC mutant that lacked the last 20 amino acid residues in the C terminus, including the
GSK3-specific sites of phosphorylation. To analyze the effect of Myc-tagged WT and mutants in the absence of endogenous eIF6, they were either expressed transiently (Fig. 6, G and H) or stably (Fig. S6, B–D) in a cell line that was stably knocked down for eIF6 (eIF6-KD) (Fig. S6, B and C). Endogenous eIF6 levels were reduced to greater than 85% by using shRNA targeted against eIF6 (Fig. S6, B and C). Analysis of the subcellular localization of the phosphodead mutants revealed that eIF6-4A and eIF6-ΔC do not accumulate in the cytoplasm in response to starvation unlike the WT cells (Fig. 6, G–I, and Fig. S7, A–C). The total input levels of eIF6 under all conditions tested are shown in Fig. S7 (D–F). These results strongly suggest that eIF6 exhibits predominant cytoplasmic localization in response to serum starvation, and this altered localization is regulated by GSK3-dependent phosphorylation of the C-terminal tail.
Expression of the phosphodead mutant decreases translation rates and stalls cell growth

We performed the polysome profile assay to determine the physiological effect of disrupting the GSK3-specific sites of phosphorylation on translation rates. Polysome profiles of starved (WT control) cells compared with the serum-fed cells showed that serum starvation causes a profound inhibition of translation, as shown by an increase in the levels of free 60S, 40S, and 80S subunits with a concomitant decrease in the heavier polysome levels (Fig. S8), and this was consistent with previous reports (22–33). Interestingly, in response to starvation, cells stably expressing the phosphodead eIF6-4A mutant and eIF6-D4C mutant in the eIF6-KD cell line, displayed a significant 20–25% increase in the steady-state levels of free 60S subunits compared with eIF6-WT (Fig. 7, A–D). A consistent increase in the levels of 80S monosomes and a further reduction in heavier polysome levels that were already quite reduced because of serum starvation was also observed for both mutants (Fig. 7, A and C). These results suggest that translation is further inhibited in the mutant cells under starved conditions. This was also...
confirmed by measuring the incorporation rate of L-azido-L-methionine (AHA), an analog of methionine. Compared with eIF6-WT, a significant reduction in AHA incorporation (∼25%) was observed for the eIF6-4A mutant relative to eIF6-WT under starvation conditions, suggesting that the translation rate is much more impaired in these cells (Fig. 7).

We then determined whether the altered translation profile in the eIF6-4A mutant affected cell growth in response to...
Regulation of eIF6 by GSK3

starvation. As shown in Fig. 7 (F and G), there was a significant 20–25% decrease in the number of viable eIF6-4A mutant cells compared with eIF6-WT. Analysis of the MTS assay further indicated that even in the absence of serum, cell proliferation was not fully inhibited for eIF6-WT but was completely stalled for the eIF6-4A mutant (Fig. 7G). These results indicate that the eIF6-4A mutant significantly impacts cell growth in response to starvation.

Discussion

The metabolic response to starvation is to conserve energy and limit processes with high-energy requirements such as translation and ribosome biogenesis (22–31). Here, we show that the regulation of translation initiation factor eIF6 by GSK3 affects translation response to starvation. Despite its extensive role in metabolism, eIF6 function in starvation response is poorly understood. We show that eIF6 is regulated in response to starvation-induced stress and that the regulation hinges on the GSK3 signaling pathway that is prolifically active under such nutrient-deprived conditions. This study also identifies GSK3 as one of the kinases that phosphorylates the multiple sites in the C-terminal tail of eIF6. Although several global proteomic and biochemical studies have shown that the C-terminal tail of eIF6 is heavily phosphorylated, the identity of the kinases involved, other than PKCβII, were largely unknown. Our results show that GSK3 sequentially phosphorylates the C-terminal tail of eIF6 at Ser-235, Ser-239, Ser-235, and Thr-231 sites, with Ser-235 being the initiation site in the sequence. It is currently unclear whether GSK3 works in concert with a priming kinase in cells to enhance phosphorylation at the initiation site.

Interestingly, one of the sites of phosphorylation, the Ser-235 site, is also phosphorylated by the RACK1-PKCβII complex (12). Substitution of the serine 235 site with alanine blocks eIF6 release from 60S and inhibits active 80S formation (12). Apart from in vitro studies, the Ser-235 site has also been shown to be important for normal growth and translation in vivo (17, 18). Studies in eIF6+/− mouse embryonic fibroblasts reconstituted with the S235A mutant show that the Ser-235 site is critical for eIF6 function in stimulating translation in response to insulin and phorbol esters (17–19). It is not uncommon for a single site to be regulated by multiple kinases in a context-dependent manner. Because PKCβII is active under conditions of growth and proliferation, whereas GSK3 is active under growth-deprived conditions, the two kinases could phosphorylate the Ser-235 site based on the polarizing cellular cues of growth and starvation or resting. However, in the case of eIF6 phosphorylation by GSK3, additional sites are also phosphorylated, which could further perturb the local protein structure and alter eIF6 localization.

We observed predominant cytoplasmic accumulation of eIF6 in response to serum starvation and inhibiting the GSK3-dependent phosphorylation of eIF6 rescues the subcellular distribution. Because GSK3 is predominantly cytoplasmic with a small nuclear fraction, it is likely that phosphorylation by GSK3 leads to increased cytoplasmic retention of eIF6. In our study, the predominant cytoplasmic accumulation of eIF6 in response to starvation was consistently observed among different cell lines including the normal RIE-1, HCT116, and HeLa cells. A previous study showed that the nuclear import of eIF6 was mediated by calcium-activated calcineurin phosphatase, whereas the nuclear export was mediated by phosphorylation of eIF6 at Ser-174/175 residues by nuclear casein kinase 1 in COS-7 cells (69). Thus, our results and previous data suggest that altering the subcellular localization of eIF6 may be a predominant mode of regulating eIF6 function, and the underlying signaling mechanisms may vary based on the growth stimulus and stressed states. However, it is yet to be understood whether this change in localization is due to altered association with 60S subunit or due to altered association with a nuclear-export factor.

We and others (22–33) have shown that in response to nutrient deprivation, protein synthesis is inhibited with a decrease in polysome levels along with a corresponding increase in free 60S subunits and inactive 80S monosomes. Even mild changes to the 60S or 40S levels can drastically impact cell growth as seen in ribosomopathies. Our results indicate that phosphorylation of the C-tail of eIF6 and its cytoplasmic accumulation is important to maintain a basal level of protein synthesis under starved conditions. It is unclear as to how eIF6 function in 60S maturation is altered in response to starvation and whether the effects on 60S availability are direct or indirect. eIF6 is known to work with upstream regulators of 60S maturation, such as Shwachman–Diamond Syndrome factor and the GTPase EFL1 (elongation factor-like 1) complex, and RPL10 that facilitate eIF6 release, and it remains to be determined how such release is affected in response to stress (4, 11, 13, 20, 70). However, these results indicate that any disruption of GSK3-dependent phosphorylation of eIF6 can shift the balance from adaptation to enhanced sensitivity to starvation. Analysis of eIF6 phosphorylation in the free eIF6 pool that is unbound to 60S relative to those that are bound to 60S will help to understand the potential effects of phosphorylation on eIF6 release.

Based on these results, we propose the following model, in which in response to nutrient or growth-limiting conditions, AKT signaling is inactivated, which in turn activates GSK3, and GSK3 phosphorylates eIF6 at multiple sequential sites. Phosphorylation by GSK3 enhances cytoplasmic accumulation of eIF6. The timely release of eIF6 from the 60S in the late stages of maturation is critical for the 60S and 40S subunits to associate and initiate translation. Phosphorylation at the Ser-235 site has been shown to facilitate eIF6 release under fed conditions. Because this site and other sequential sites are phosphorylated under starvation conditions by GSK3, it is likely that eIF6 release from the 60S permits 60S association with the limited pool of mRNA-bound 40S to engage in translation even under starvation conditions. This regulation ensures that a basal rate of translation continues during starvation to maintain slow growth, which is essential to adapt to starvation and to permit recovery upon nutrient addition. This model is based on the results that in the absence of eIF6 phosphorylation, the rates of translation are further decreased under serum starvation conditions, and there is an increase in free 60S pool that is not engaged in translation. Future studies will assess the role of phosphorylation in altering the dynamics of eIF6 and 60S
interaction and determine the mechanism of altered subcellular localization.

**Experimental procedures**

**Cell culture and transfections**

HCT116 cells (human colorectal carcinoma line) (ATCC) were maintained in McCoy’s 5A medium supplemented with 10% fetal bovine serum (FBS), and 100 μg/ml penicillin and 100 μg/ml streptomycin (Gibco). HEK293T/17 and HeLa cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco). For transfections, ~2 × 10⁶ HCT116 and 2.5 × 10⁶ 293T cells were collected per 60-mm dish. Each dish was transfected with 4 μg of plasmid DNA and 20 μl of Lipofectamine 2000 (Invitrogen). 24 h later, the cells were washed twice in PBS and serum-starved for 4 h using respective media with 0.1% FBS only. 4 h later, the cells were washed twice in PBS and collected in mammalian cell lysis buffer (MCLB) (50 mM Tris-Cl, pH 8.0, 5 mM EDTA, 0.5% Igepal, 150 mM sodium chloride) that was supplemented with the following inhibitors just before lysis: 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium fluoride, 10 mM β-glycerophosphate, 1 mM sodium vanadate, 2 mM DTT, 1 × protease inhibitor mixture (Sigma–Aldrich), 1 × phosphatase inhibitor mixture (Santa Cruz Biotechnology). The lysates were rocked for 15 min at 4 °C, followed by centrifugation at 14,000 rpm for 10 min at 4 °C.

**Plasmid constructions**

To clone human elf6, mRNA was extracted from MCF7 (ATCC) cells using RNAqueous total RNA isolation kit (Invitrogen), and cDNA was synthesized using the Superscript first-strand cDNA synthesis kit (Invitrogen). Using PCR, elf6 ORF was cloned into the pCMV-Myc plasmid (Clontech) using the respective forward and reverse primers carrying EcoRI and XhoI restriction sites: 5‘-TATAAGAATTCTAATGGCG-GTCCGAGCTTCGTTCGAGAAC-3’ and 5‘-TATAACTCGAGTCAAGGTAGGCTGTCAATGAGGAAC-3. GST-tagged GSK3β constructs were cloned into the pDEST™27 vector (Thermo Fisher Scientific) as described previously (71). All of the pCMV and pcDNA3.1–Myc–elf6 phospho-site mutants were generated using the Q5® site-directed mutagenesis kit (New England Biolabs) using the forward and reverse primers Table S1. All plasmid constructs used in this study were verified by Sanger sequencing (Genewiz).

**Generation of stable cell lines expressing elf6-WT and mutants**

HCT116 cells were transfected with pLKO.1 vector expressing either an elf6 targeted shRNA (CCGGGTGCACTCCTACGACTTCAATTTCGAGAATTTGCTTTGCAGATGCACTTTTGG, Sigma, TRC327700) or a control nontargeting shRNA (Sigma) as described above. Stably transfected clones were selected in 2 μg/ml puromycin and maintained in 0.5 μg/ml puromycin. ~30 clones were screened for elf6 knockdown (elf6–KD) by Western blotting. To express elf6 WT and mutants in the elf6–KD cell line, the cells were stably transfected with pcDNA3.1 plasmid expressing either the Myc-tagged elf6-WT, elf6-4A, elf6–ΔC, or the empty vector. To evade shRNA targeting, mismatches were generated in the Myc-elf6-WT, Myc-elf6–4A, or Myc-elf6–ΔC plasmids using site-directed mutagenesis. All stably transfected clones were selected in 500 μg/ml Geneticin and 0.5 μg/ml puromycin. ~25 clones were screened for expression of Myc-elf6-WT or mutant by Western blotting. All stable lines were maintained in 100 μg/ml Geneticin and 0.5 μg/ml puromycin.

**Cell viability assays**

7,000 HCT116 cells expressing elf6-WT or elf6-4A were cultured per well of a 96-well plate in McCoy’s culture medium without phenol red. After overnight incubation, the cells were serum-starved for 24 h in McCoy’s medium supplemented with 0.1% FBS. 20 μl of MTS solution (CellTiter 96® Aqueous One solution reagent, Promega) was added to each well, and the plates were incubated for 1 h. Absorbances were read at 490 nm (Spectra Max i3x, Molecular Devices). To obtain background-corrected absorbances, average absorbance values of the control wells containing media and MTS only were subtracted from all other absorbances. For trypan blue assay, HCT116 cells expressing elf6-WT or elf6-4A were plated as indicated for polysome profile assays. The cells suspended in culture media were mixed with trypan blue dye at 1:1 ratio, and the numbers of blue-stained (dead) cells and unstained (viable) cells were counted using a hemocytometer.

**Immunoprecipitation**

For immunoprecipitation of Myc-elf6, or Myc-empty vector, lysates with 1.2 mg of total protein were suspended in a final volume of 500 μl of MCLB buffer supplemented with inhibitors. The lysates were precleared with 20 μl of protein A/G plus–conjugated agarose beads (Santa Cruz Biotechnology) and 1 μl of normal mouse IgG (Santa Cruz Biotechnology). Protein A/G plus–agarose beads were washed in MCLB buffer before use. For preclearing, the lysates were rotated for 20 min at 4 °C. Precleared lysates were collected by centrifugation at 3500 rpm for 3 min at 4 °C, and the beads were discarded. Precleared lysates were incubated with 20 μl of protein A/G plus–agarose beads and 11 μl of Myc-tag antibody (9E10) (Santa Cruz Biotechnology) and rotated overnight at 4 °C. The samples were washed three times in MCLB buffer, and the beads were collected by centrifugation at 3000 rpm for 3 min and washed for a fourth time in incomplete kinase buffer (50 mM Tris, pH 7.4, 10 mM MgCl₂, 2 mM DTT), and after the final wash, the beads were suspended in 45 μl of incomplete kinase buffer. Immunoprecipitation of the Myc-tagged phospho-site mutants of elf6, elf6–ΔC20, and Myc-elf6–WT was carried out as described above except that 1.5 mg of total protein was used for the assay. For the Myc-elf6–ΔC36 mutant, because of a small reduction in its expression, 2 mg of total protein was used for the assay. This ensured that the total elf6 protein input for the kinase assays were comparable between elf6–WT and the elf6–ΔC36 mutant.
Regulation of eIF6 by GSK3

In vitro kinase assay

For the in vitro kinase assays, Myc-tagged versions of eIF6 were immunoprecipitated as indicated above, and the immunoprecipitated beads were incubated with or without 350 units of recombinant GSK3β (rabbit skeletal muscle) (New England Biolabs) or 0.7 unit/µg of recombinant human GSK3α (Abcam) and 1× hot kinase buffer (50 mM Tris, pH 7.4, 50 mM MgCl₂, 10 mM DTT, 50 µM cold ATP, [γ-³²P]ATP) and incubated at 30 °C for 30 min. For kinase reactions with pure eIF6 protein, codon-optimized recombinant human His₆-eIF6 was cloned into RSF-Duet vector and purified from E. coli as described previously, except purification using a nickel–nitrilotriacetic acid column was followed by negative selection using Heparin column (72). 2.5 µg recombinant-eIF6 protein was incubated with the kinase as described above. Kinase reactions with the phospho-site mutants of eIF6 and eIF6-WT control were set up as detailed above, except that the reactions were incubated at 30 °C for shorter duration of 25 min to capture time-dependent kinetic differences. Kinase reactions were analyzed by SDS-PAGE and exposed to X-ray film for autoradiography. For Western blotting of the kinase assay, duplicate kinase reactions were simultaneously set up as described above except that the reactions were set up using the cold kinase buffer (50 mM Tris, pH 7.4, 50 mM MgCl₂, 10 mM DTT, 250 µM cold ATP). The blots were probed with anti-eIF6 mAb (1:1000) or anti-Myc-tag mAb (1:750) and anti-GSK3 antibody (1:750 in 5% milk with TBS-T) and incubated overnight. Secondary antibodies (Jackson ImmunoResearch) were diluted 1:30000 in TBS-T. The blots were developed using ECL Western blotting substrate (Pierce). For Western blotting of nuclear and cytoplasmic fractions, 20 µg of total protein was loaded per lane and probed with the following primary antibodies dissolved in TBS-T: α-tubulin (DM1A) (1:3000, 1 h), and anti-eIF6 antibody (D1696) (1:1000, overnight) (Cell Signaling Technology).

Pulldown assay

To capture interactions with the endogenous eIF6, HCT116 cells were transfected with GST-GSK3β or empty vector as described above. Cell lysis was carried out in MCLB buffer, and a small fraction was saved for analysis of input levels. For the pulldown assays, 1.1 mg of total protein suspended in 500 µl of MCLB buffer and supplemented with inhibitors was incubated with 50 µl of GSH–agarose resin beads (Gold Biotechnology) and rotated overnight at 4 °C. The next day, the samples were washed three times in MCLB buffer supplemented with inhibitors and resuspended in (30 µl) of MCLB buffer and 2× Laemmli buffer. The samples were then analyzed by Western blotting. For the pulldown of GST-GSK3β WT and GST-GSK3β-R96A, 293T cells were transfected with the respective vectors, and 1.9 mg of total protein was used for the pulldown analysis.

Western blotting

For Western blotting, proteins were resolved by SDS-PAGE. For all blots, ~20–40 µg of total protein was loaded per lane, except for the kinase reactions where samples were prepared as described above. The proteins were transferred onto nitrocellulose membranes (0.45 µm; Bio-Rad Laboratories). The blots were blocked either for 1 h or overnight in 5% nonfat dry milk diluted in TBS-T. The following primary antibodies dissolved in TBS-T were used: anti-GST (1:1000, overnight), anti-α-tubulin (1:1000, overnight), anti–β-actin (1:3000, 1 h), anti-eIF6, anti-GSK3β, anti-phospho GSK3β (Ser-9), anti-vinculin, and anti-Myc-tag antibodies (1:1000, overnight) (Cell Signaling Technology). To assay for GSK3β interaction with endogenous eIF6, the blots were probed with anti-eIF6 mAb (1:500) and incubated overnight (Cell Signaling Technology). To assay for β-catenin phosphorylation, 100 µg of total protein was loaded per lane, and the blots were probed with anti–phospho-β-catenin (Ser-33/Ser-37/Thr-41) antibody (1:750 in 5% milk with TBS-T) and incubated overnight. Secondary antibodies (Jackson ImmunoResearch) were diluted 1:30000 in TBS-T. The blots were developed using ECL Western blotting substrate (Pierce). For Western blotting of nuclear and cytoplasmic fractions, 20 µg of total protein was loaded per lane and probed with the following primary antibodies dissolved in TBS-T: α-tubulin (DM1A) (1:3000, 1 h), and anti-eIF6 antibody (D1696) (1:1000, overnight) (Cell Signaling Technology).

Mass spectrometry

For MS, 7 × 10⁶ 293T cells were plated per 10-cm plate and transfected as indicated before. 24 h later, the cells were serum-starved in Dulbecco’s modified Eagle’s medium containing 0.1% FBS for 3 h. The cells were lysed in MCLB buffer supplemented with inhibitors. Immunoprecipitations were carried out as previously described using 3 mg of total protein. For the kinase reactions, 15 µl of Myc-eIF6-bound beads were incubated with/without recombinant GSK3β (NEB) and suspended in cold kinase buffer and incubated at 30 °C for 30 min. The reactions were washed twice in incomplete kinase buffer and resuspended in incomplete kinase buffer and 1.5× Laemmlı buffer. The samples were boiled and analyzed by SDS-PAGE followed by Coomassie staining. Stained gels were sent to the University of Wisconsin–Madison mass spectrometry facility.

Enzymatic "in gel" digestion

In-gel digestion and mass spectrometric analysis was done at the mass spectrometry facility of the Biotechnology Center at the University of Wisconsin–Madison. In short, Coomassie-stained gel pieces were destained twice for 5 min in MeOH: H₂O:NH₄HCO₃ (50%:50%:100 mM), dehydrated for 5 min in ACN:H₂O:NH₄HCO₃ (50%:50%:25 mM) and then once more for 30 s in 100% ACN, dried in a SpeedVac for 2 min, rehydrated completely and reduced in 25 mM DTT (25 mM NH₄HCO₃) for 30 min at 56 °C, alkylated by solution exchange with 55 mM iodoacetamide (in 25 mM NH₄HCO₃) in the dark at room temperature for 30 min, washed once in 25 mM NH₄HCO₃, dehydrated twice for 5 min in ACN:H₂O: NH₄HCO₃ (50%:50%:25 mM) and then once more for 30 s in 100% ACN, dried in a SpeedVac again, and finally rehydrated with 20 µl of trypsin digestion solution (10 ng/µl trypsin (Promega) in 25 mM NH₄HCO₃ and 0.01% ProteaseMAX w/v (Promega)). Additional 30 µl of rehydration solution (25 mM NH₄HCO₃ and 0.01% ProteaseMAX w/v (Promega)) was added to facilitate complete rehydration with excess overlay needed for peptide extraction. The digestion was conducted for 3 h at 42 °C. Peptides generated from digestion were transferred to a new tube and acidified with 2.5% TFA to 0.3% final concentration. Gel pieces were extracted further with ACN:H₂O:TFA.
Nano-LC–MS/MS

Peptides were analyzed by nano-LC–MS/MS using the Agilent 1100 nanoflow system (Agilent) connected to a hybrid linear ion trap–Orbitrap™ mass spectrometer (LTQ-Orbitrap Elite™, Thermo Fisher Scientific) equipped with an EASY-Spray™ electrospray source. Chromatography of peptides prior to mass spectral analysis was accomplished using a capillary emitter column (PepMap® C18, 3 μM, 100 Å, 150 × 0.075 mm, Thermo Fisher Scientific) onto which 3 μl of extracted peptides was automatically loaded. The nano-HPLC system delivered solvents A: 0.1% (v/v) formic acid, and B: 99.9% (v/v) acetonitrile, 0.1% (v/v) formic acid at 0.5 μl/min to load the peptides (over a 30-min period) and 0.3 μl/min to elute peptides directly into the nano-electrospray with gradual gradient from 3% (v/v) B to 20% (v/v) B over 17 min and concluded with 5 min fast gradient from 20% (v/v) B to 50% (v/v) B, at which time a 5 min flush-out from 50–95% (v/v) B took place. As peptides eluted from the HPLC-column/electrospray source survey, MS scans were acquired in the Orbitrap with a resolution of 120,000 followed by MS2 fragmentation of 20 most intense peptides detected in the MS1 scan from 350 to 1800 m/z; redundancy was limited by dynamic exclusion.

Data analysis

Raw files were searched against Uniprot human amino acid sequence database (downloaded on October 31, 2016) containing a list of common contaminants (67,042 total entries) using Sequest HT search engine within Proteome Discoverer suite (version 2.2.0.388). Full trypsin specificity, two missed cleavages, 15 ppm for precursor, and 0.6 Da for fragment mass tolerance plus fixed carbamidomethylation (Cys) and dynamic oxidation (Met), deamidation (Asn/Gln), and phosphorylation (Ser/Thr) were selected in the search parameters. A strict confidence threshold of 0.01 false discovery rate in decoy database searching on protein and peptide levels were applied. In addition, at least two peptides per protein plus 0.01 q value high confidence threshold was selected to affirm proper identification.

MS of synthetic elf6 phosphopeptide and nonphosphorylated peptide

100 μM of the synthesized phosphopeptides (Aapptec) or nonphosphorylated peptides were incubated with 350 units of recombinant GSK3β (New England Biolabs) and cold kinase buffer. The reactions were incubated for 20 min at 30°C. The reactions were then quenched by 40 mM EDTA and by freezing.

Nanos-LC–MS/MS

100 μM of synthesized version of human elf6 phosphopeptide was acidified with 2.5% TFA to 0.4% final and solid phase extracted (ZipTip C18 pipette tips, Millipore) according to the manufacturer’s protocol. Peptide was eluted off ZipTip C18 tip with 2 μl of ACN:H2O:TFA (70%:29.9%:0.1%) and diluted to 20 μl of final volume with 0.1% formic acid. Mass spectrometric analysis followed using the Agilent 1100 nanoflow system (Agilent) connected to a hybrid linear ion trap–Orbitrap™ mass spectrometer (LTQ-Orbitrap Elite™, Thermo Fisher Scientific) equipped with an EASY-Spray™ electrospray source. Chromatography of peptides prior to mass spectral analysis was accomplished using capillary emitter column (PepMap C18, 3 μM, 100 Å, 150 × 0.075 mm, Thermo Fisher Scientific) onto which 2 μl of extracted peptides was automatically loaded. Nano-HPLC system delivered solvents A: 0.1% (v/v) formic acid, and B: 99.9% (v/v) acetonitrile, 0.1% (v/v) formic acid at 0.50 μl/min to load the peptides (over a 30-min period) and 0.3 μl/min to elute peptides directly into the nano-electrospray with gradual gradient from 3% (v/v) B to 20% (v/v) B over 17 min and concluded with 5 min of fast gradient from 20% (v/v) B to 50% (v/v) B, at which time a 4-min flush-out from 50–95% (v/v) B took place. As peptides eluted from the HPLC-column/electrospray source survey, MS scans were acquired in the Orbitrap with a resolution of 120,000 followed by MS2 fragmentation of 20 most intense peptides detected in the MS1 scan from 350 to 1800 m/z; redundancy was limited by dynamic exclusion. Raw files were searched against targeted amino acid sequence database containing a list of common laboratory contaminants plus a sequence of synthetic version of human elf6 (202 total entries) using Sequest HT search engine within Proteome Discoverer suite (version 2.2.0.388). No-enzyme specificity, 0 missed cleavages, 15 ppm for precursor, and 0.6 Da for fragment mass tolerance plus dynamic oxidation (Met), deamidation (Asn/Gln), and phosphorylation (Ser/Thr) were selected in the search parameters. Confidence threshold of 0.02 false discovery rate in decoy database searching on protein and peptide levels were applied. Dynamic phosphorylation cascade distributions were manually interrogated for precursor ion abundance and quality of MS/MS fragmentation.

MS of serum-starved samples

For cellular MS analysis, Myc-elf6 was immunoprecipitated from 293T cells that were serum-starved for 24 h. Immunoprecipitations were carried out as previously described except that 6 mg of total protein was used for analysis, and both a faster and slower migrating species of elf6 was excised from Coomassie-stained gel and subjected to MS analysis as described above. Phosphopeptides were only detected in the slower migrating/super-shifted band. Table S2 summarizes the list of phosphopeptides indicated in this study.

Immunofluorescence staining and microscopy

50,000 HCT116 cells were plated in each well of a 12-well plate carrying poly-o-lysine–coated glass coverslips (Neuvitro). After overnight incubation, the cells were either serum-fed with McCoy’s 5A medium containing 10% FBS or serum-
Regulation of eIF6 by GSK3

starved in medium containing 0.1% FBS, and 24 h later, the cells were treated with either 0.1% DMSO (vehicle) or 10 μM CHIR99021 (Tocris). The cells were fixed overnight in 2% paraformaldehyde/PBS. For immunostaining, fixed cells were permeabilized with 2% Triton X-100/PBS for 20 min and incubated in blocking buffer (2% BSA, 0.1% Igepal, PBS) for 30 min. The following primary antibodies diluted in the blocking buffer were used for staining: anti-eIF6 mAb (D16E9) (1:300) (Cell Signaling Technology) or anti-eIF6 mAb (1:350) (Santa Cruz Biotechnology), and incubated in a humidified chamber at room temperature for 1 h. Coverslips were washed with 0.1% Igepal/PBS and incubated with Cy3-conjugated AffiniPure donkey anti-rabbit IgG secondary antibody (Jackson ImmunoResearch) diluted in blocking buffer (1:300) at room temperature for 1 h in the dark. Coverslips were washed in 0.1% Igepal/PBS and mounted using ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). Immunofluorescence studies in HeLa cells were carried out as described above. Staining of normal rat intestinal epithelial cells (RIE-1) was carried out as described above except that 30,000 cells were plated per well, and the cells were probed with anti-eIF6 mAb (D16E9) (1:200) (Cell Signaling Technology). RIE-1 cells were cultured and maintained as described previously (73, 74). For imaging, the slides were analyzed using the Leica DM6 B upright fluorescent microscope. The images were acquired using Leica DFC 3000G (Bin 1 × 1, Gamma1) camera and processed by Leica LAS X imaging software.

Subcellular fractionation

700,000 HCT116 cells were plated per 100-mm dish, and 24 h later, the cells were serum-starved in medium containing 0.1% FBS or fed with medium containing 10% FBS for an additional 24 h. To assay the effect of inhibitors, serum-starved cells were treated with either 0.1% DMSO (vehicle) or 10 μM CHIR99021 (Tocris). For subcellular fractionation of WT and mutants, 1.2 million cells were plated per 60-mm dish. To assay for total protein input, the cells were lysed in MCLB buffer supplemented with inhibitors as previously described. To extract cytoplasmic fractions, the cells were collected in cell lysis buffer (10 mM HEPES, pH 7.5, 10 mM KCl, 0.1 mM EDTA, 0.5% Igepal) supplemented with the following inhibitors: 10 mM β-glycerophosphate, 1 mM sodium vanadate, 2 mM DTT, 1× protease inhibitor mixture (Sigma–Aldrich), 1× phosphatase inhibitor mixture (Santa Cruz Biotechnology), and 0.5 mM PMSF. Nuclear pellets were solubilized on ice for 30 min followed by centrifugation at 12,000 × g for 15 min at 4°C. Both nuclear and cytoplasmic fractions were analyzed by Western blotting.

AHA translation assay

To measure the rate of AHA incorporation, the Click-iT AHA kit was utilized, and the assay was performed as per the manufacturer’s instructions (Life Technologies) as reported before (76). 15,000 cells were plated per well of a 96-well plate, and after overnight incubation they were washed twice in 1× PBS. The cells were serum-starved in medium containing 0.1% FBS. 24 h later, the cells were incubated in methionine-free medium containing 0.1% FBS and Click-iT AHA reagent (50 μM) for 30 min at 37°C in a CO2 incubator. The cells were fixed, and the azide–alkyne cycloaddition reaction was carried out using azide conjugated to Alexa Fluor 488, and nuclei were stained with Hoechst 33342. Fluorescence intensity was measured using a microplate reader.

Polysome profile analysis

Polysome profile analysis was carried out as described previously, except 1.5 million cells were plated per 10-cm plate for eIF6-WT and eIF6-4A mutant serum-fed controls, and 2 million cells were plated per 10-cm plate for serum-starved samples (50–60% cell density) (73, 74). The cells were treated with 100 μg/ml cycloheximide (Tocris) for 5 min at 37°C and washed with ice-cold 1× PBS buffer containing 100 μg/ml cycloheximide on ice. The cells were lysed in the polysome lysis buffer (50 mM HEPES, pH 7.4, 250 mM KCl, 5 mM MgCl2, 250 mM sucrose, 1% Triton X-100, 1% sodium deoxycholate, 100 μg/ml cycloheximide, 100 units/ml Superserin (Invitrogen); 0.25× protease inhibitor mixture EDTA free (Sigma), and 1× phosphatase inhibitor mixture (Santa Cruz Biotechnology). ~10.5 OD260 were loaded per linear sucrose gradient (20–47%) for all assays and for the comparison of fed versus starved, and ~9.5 OD260 were loaded for comparison of serum-starved mutants versus WT. Within each experiment, the same OD units of lysate in same volume (600 μl) was layered per sucrose gradient (20 mM HEPES, pH 7.4, 200 mM KCl, 10 mM MgCl2, 100 μg/ml cycloheximide, RNase-free sucrose), and gradients were centrifuged at 35,000 rpm for 3 h 20 min using a SW41 Ti rotor (Beckman). Absorbance was followed at 254 nm. The area under the curve (AUC) was measured using the Peak Chart software (Brandel).

Data availability

The MS data have been uploaded to MassIVE under accession number MSV000085596. All other data have been included in the article.

Acknowledgments—We thank Dr. Grzegorz Sabat and Dr. Greg Barrett-Wilt at the University of Wisconsin–Madison Mass Spectrometry Core for helping us with the generation and analysis of mass spectrometry data. We also thank Dr. Edwin Antony for
 Regulation of eIF6 by GSK3

The nuc(e)lar Tif6p and Eif1lp are required for a late cytoplasmic step of ribosome synthesis. *Mol. Cell* 8, 1363–1373 CrossRef Medline

Russell, D. W., and Spremulli, L. L. (1979) Purification and characterization of a ribosome dissociation factor (eukaryotic initiation factor 6) from wheat germ. *J. Biol. Chem.* 254, 8796–8800 Medline

Gartmann, M., Blau, M., Armache, J. P., Mielke, T., Topf, M., and Beckmann, R. (2010) Mechanism of eIF6-mediated inhibition of ribosomal subunit joining. *J. Biol. Chem.* 285, 14848–14851 CrossRef Medline

Weis, F., Giudice, E., Churcher, M., Jin, L., Hilcenko, C., Wong, C. C., Traynor, D., Kay, R. R., and Warren, A. J. (2015) Mechanism of eIF6 release from the nascent 60S ribosomal subunit. *Nat. Struct. Mol. Biol.* 22, 914–919 CrossRef Medline

Ceci, M., Gaviraghi, C., Gorni, C., Sala, L. A., Offenhäuser, N., Marchisio, P. C., and Biffo, S. (2003) Release of eIF6 (p27BBP) from the 60S subunit allows 80S ribosome assembly. *Nature* 426, 579–584 CrossRef Medline

Finch, A. J., Hilcenko, C., Basse, N., Drynan, L. F., Goyenechea, B., Menne, T. F., González Fernández, A., Simpson, P., D’Santos, C. S., Arends, M. J., Donadieu, J., Bellanne-Chantelot, C., Costanzo, M., Boone, C., McKenzie, A. N., et al. (2011) Uncoupling of GTP hydrolysis from eIF6 release on the ribosome causes Shwachman–Diamond syndrome. *Genes Dev.* 25, 917–929 CrossRef Medline

Pesce, E., Minici, C., Bassler, J., Hurt, E., Degano, M., Calamita, P., and Biffo, S. (2015) Direct and high throughput (HT) interactions on the ribosomal surface by iRJA. *Sci. Rep.* 5, 15401 CrossRef Medline

Klinge, S., Voigs-Hoffmann, F., Leibundgut, M., Arpagaus, S., and Ban, N. (2011) Crystal structure of the eukaryotic 60S ribosomal subunit in complex with initiation factor 6. *Science* 334, 941–948 CrossRef Medline

Groft, C. M., Beckmann, R., Sali, A., and Burley, S. K. (2000) Crystal structures of ribosome-anti-assocation factor IF6. *Nat. Struct. Biol.* 7, 1156–1164 CrossRef Medline

Gandin, V., Miluzio, A., Barbieri, A. M., Beugnet, A., Kiyokawa, H., Marchisio, P. C., and Biffo, S. (2008) Eukaryotic initiation factor 6 is rate-limiting in translation, growth and transformation. *Nature* 455, 684–688 CrossRef Medline

Miluzio, A., Beugnet, A., Gross, S., Brina, D., Mancino, M., Campaner, S., Amati, B., de Marco, A., and Biffo, S. (2011) Impairment of cytoplasmic eIF6 activity restricts lymphomagenesis and tumor progression without affecting normal growth. *Cancer Cell* 19, 765–775 CrossRef Medline

Brina, D., Miluzio, A., Ricciardi, S., Clarke, K., Davidsen, P. K., Viero, G., Tebaldi, T., Offenhäuser, N., Rozman, J., Rathkolb, B., Neschen, S., Klingenspor, M., Wolf, E., Gailus-Durner, V., Fuchs, H., et al. (2015) eIF6 coordinates insulin sensitivity and lipid metabolism by coupling translation to transcription. *Nat. Commun.* 6, 8261 CrossRef Medline

Wong, C. C., Traynor, D., Basse, N., Kay, R. R., and Warren, A. J. (2011) Defective ribosome assembly in Shwachman–Diamond syndrome. *Blood* 118, 4305–4312 CrossRef Medline

Clarke, K., Ricciardi, S., Pearson, T., Bharudin, I., Davidsen, P. K., Bonomo, M., Brina, D., Scagliali, A., Simpson, D. M., Beynon, R. J., Khanim, F., Ankers, J., Sarzynski, M. A., Ghosh, S., Pisconti, A., et al. (2017) The role of Eif6 in skeletal muscle homeostasis revealed by endurance training co-expression networks. *Cell Rep.* 21, 1507–1520 CrossRef Medline

Ashe, M. P., De Long, S. K., and Sachs, A. B. (2000) Glucose depletion rapidly inhibits translation initiation in yeast. *Mol. Biol. Cell* 11, 833–848 CrossRef Medline

van den Elzen, A. M., Schuller, A., Green, R., and Séraphin, B. (2014) Dom34-Hbs1 mediated dissociation of inactive 80S ribosomes promotes restart of translation after stress. *EMBO J.* 33, 265–276 CrossRef Medline

Liu, B., and Qian, S. B. (2016) Characterizing inactive ribosomes in translational profiling. *Translation (Austin)* 4, e1138018 CrossRef Medline

Jastrzebski, K., Hannan, K. M., Tchoubrieva, E. B., Hannan, R. D., and Pearson, R. B. (2007) Coordinate regulation of ribosomal biogenesis and function by the ribosomal protein S6 kinase, a key mediator of mTOR function. *Growth Factors* 25, 209–226 CrossRef Medline

Iadevia, V., Liu, R., and Proud, C. G. (2014) mTORC1 signaling controls multiple steps in ribosome biogenesis. *Semin. Cell Dev. Biol.* 36, 113–120 CrossRef Medline

Krokowski, D., Gaccioli, F., Majumder, M., Mullins, M. R., Yuan, C. L., Papadopoulo, B., Merrick, W. C., Komar, A. A., Taylor, D., and

References

1. Brina, D., Gross, S., Miluzio, A., and Biffo, S. (2011) Translational control by 80S formation and 60S availability: the central role of eIF6, a rate-limiting factor in cell cycle progression and tumorigenesis. *Cell Cycle* 10, 3441–3446 CrossRef Medline

2. Brina, D., Miluzio, A., Ricciardi, S., and Biffo, S. (2015) eIF6 anti-association activity is required for ribosome biogenesis, translational control and tumor progression. *Biochim. Biophys. Acta* 1849, 830–835 CrossRef Medline

3. Miluzio, A., Beugnet, A., Volta, V., and Biffo, S. (2009) Eukaryotic initiation factor 6 mediates a continuum between 60S ribosome biogenesis and translation. *EMBO Rep.* 10, 459–465 CrossRef Medline

4. Malyutin, A. G., Musalaoglar, S., Patchett, S., Frank, J., and Johnson, A. W. (2017) Nmd3 is a structural mimic of eIF5A, and activates the cgPITase Lsg1 during 60S ribosome biogenesis. *EMBO J.* 36, 854–868 CrossRef Medline

5. Ma, C., Wu, S., Li, N., Chen, Y., Yan, K., Li, Z., Zheng, L., Lei, J., Woolford, J. L. Jr., and Gao, N. (2017) Structural snapshot of cytoplasmic pre-60S ribosomal particles bound by Nmd3, Lsg1, Tif6 and Reh1. *Nat. Struct. Mol. Biol.* 24, 214–220 CrossRef Medline

6. Kater, L., Thoms, M., Barrio-García, C., Cheng, J., Ismail, S., Ahmed, Y. L., Bange, G., Kressler, D., Bernhagenou, O., Sinning, I., Hurt, E., and Beckmann, R. (2017) Visualizing the assembly pathway of nucleolar pre-60S ribosomes. *Cell* 171, 1599–1610.e4 CrossRef Medline

7. Basu, U., Si, K., Warner, J. R., and Maitra, U. (2001) The *Saccharomyces cerevisiae* TIF6 gene encodes translation initiation factor 6 is required for 60S ribosomal subunit biogenesis. *Mol. Cell Biol.* 21, 1453–1462 CrossRef Medline

8. Senger, B., Lafontaine, D. L., Grindorge, J. S., Gadol, O., Camasses, A., Sanni, A., Garnier, J. M., Breitenbach, M., Hurt, E., and Fasiolo, F. (2001)
Regulation of eIF6 by GSK3

Hatzoglou, M. (2011) Characterization of hibernating ribosomes in mammalian cells. *Cell Cycle* 10, 2691–2702 CrossRef Medline

28. Manning, B. D. (2013) Adaptation to starvation: translating a matter of life or death. *Cancer Cell* 23, 713–715 CrossRef Medline

29. Nielsen, P. J., Duncan, R., and McConkey, E. H. (1981) Phosphorylation of ribosomal protein S6. Relationship to protein synthesis in HeLa cells. *Eur. J. Biochem.* 120, 523–527 CrossRef Medline

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

31. Tzamarias, D., Roussou, I., and Thireos, G. (1989) Coupling of GCN4 initiation of translation independently of elf2α phosphorylation. *J. Cell Sci.* 128, 4420–4427 CrossRef Medline

32. Yoshikawa, H., Larance, M., Harney, D. J., Sundaramoorthy, R., Ly, T., Frame, S., and Cohen, P. (2001) GSK3 takes centre stage more than 20 years after its discovery. *Biochem. J.* 359, 1–16 CrossRef Medline

33. Olsen, J. V., Vermeulen, M., Santamaria, A., Kumar, C., Miller, M. L., Jen-...
modulate glycogen metabolism and gene transcription. Chem. Biol. 7, 793–803 CrossRef Medline
67. Kramer, T., Schmidt, B., and Lo Monte, F. (2012) Small-molecule inhibitors of GSK-3: structural insights and their application to Alzheimer’s disease models. Int. J. Alzheimers Dis. 2012, 381029 CrossRef Medline
68. Hagen, T., Di Daniel, E., Culbert, A. A., and Reith, A. D. (2002) Expression and characterization of GSK-3 mutants and their effect on β-catenin phosphorylation in intact cells. J. Biol. Chem. 277, 23330–23335 CrossRef Medline
69. Biswas, A., Mukherjee, S., Das, S., Shields, D., Chow, C. W., and Maitra, U. (2011) Opposing action of casein kinase 1 and calcineurin in nucleo-cytoplasmic shuttling of mammalian translation initiation factor eIF6. J. Biol. Chem. 286, 3129–3138 CrossRef Medline
70. Patchett, S., Musalgaonkar, S., Malyutin, A. G., and Johnson, A. W. (2017) The T-cell leukemia related rpl10-R98S mutant traps the 60S export adapter Nmd3 in the ribosomal P site in yeast. PLoS Genet. 13, e1006894 CrossRef Medline
71. Zeidner, L. C., Buescher, J. L., and Phiel, C. J. (2011) A novel interaction between glycogen synthase kinase 3α (GSK-3α) and the scaffold protein receptor for activated C-kinase 1 (RACK1) regulates the circadian clock. Int. J. Biochem. Mol. Biol. 2, 318–327 Medline
72. Si, K., Chaudhuri, J., Chevesich, J., and Maitra, U. (1997) Molecular cloning and functional expression of a human cDNA encoding translation initiation factor 6. Proc. Natl. Acad. Sci. U.S.A. 94, 14285–14290 CrossRef Medline
73. Origanti, S., and Shantz, L. M. (2007) Ras transformation of RIE-1 cells activates cap-independent translation of ornithine decarboxylase: regulation by the Raf/MEK/ERK and phosphatidylinositol 3-kinase pathways. Cancer Res. 67, 4834–4842 CrossRef Medline
74. Origanti, S., Nowotarski, S. L., Carr, T. D., Sass-Kuhn, S., Xiao, L., Wang, J. Y., and Shantz, L. M. (2012) Ornithine decarboxylase mRNA is stabilized in an mTORC1-dependent manner in Ras-transformed cells. Biochem. J. 442, 199–207 CrossRef Medline
75. Schreiber, E., Matthias, P., Müller, M. M., and Schaffner, W. (1989) Rapid detection of octamer binding proteins with “mini-extracts,” prepared from a small number of cells. Nucleic Acids Res. 17, 6419 CrossRef Medline
76. Signer, R. A., Magee, J. A., Salic, A., and Morrison, S. J. (2014) Haematopoietic stem cells require a highly regulated protein synthesis rate. Nature 509, 49–54 CrossRef Medline

Regulation of eIF6 by GSK3

J. Biol. Chem. (2020) 295(36) 12796–12813 12813