THE EFFECTS OF SINGLE AMINO ACID DEPRIVATION ON TRANSLATION AND THE INTEGRATED STRESS RESPONSE

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This thesis was guided by three principle research objectives. The first objective was to determine if GCN2 or eIF2α was required for the induction of the integrated stress response (ISR) by single essential amino acid deprivation. The second objective was to characterize the previously identified GCN2-eIF2α-independent activation of the ISR. The third objective was to characterize the mechanism by which single essential amino acid deprivation inhibits translation.

To answer the question as to whether single essential amino acids can induce the ISR independent of GCN2 and stimulate phosphorylation of eIF2α, we conducted experiments where GCN2 knockout (GCN2(-/-)) and non-phosphorylatable-eIF2α (eIF2α(S51A)) MEF cells were exposed to media lacking leucine, arginine, histidine or methionine. In GCN2(-/-) and eIF2α(S51A) MEFs, expression of the transcription factor ATF4 was reduced compared to the wild-type when exposed to essential amino acid deprivation. ATF4 expression, however, remained significantly increased under methionine deprivation. These results demonstrate the presence of a GCN2-eIF2α-independent signaling pathway that activates the ISR.

In order to characterize the GCN2-eIF2α-independent signaling pathway, we assessed whether other genes regulated by ATF4 also were upregulated independent of GCN2 or eIF2α. In mutant MEFs, methionine deprivation resulted in the continued upregulation of 4EBP1 and ASNS, two genes containing the ATF4 promoter region termed the CARE. In addition, when

ATF4 was knocked down, methionine deprivation no longer increased the expression of 4EBP1 and ASNS in mutant cells. These results indicate that the GCN2-eIF2α-independent pathway can upregulate CARE containing genes, and that the GCN2-eIF2α-dependent and independent pathways converge at ATF4.

In order to determine whether single essential amino acids have different effects on translation, polysome profiling was performed on HEK 293T cells exposed to media deficient in histidine, arginine, leucine or methionine. Methionine and leucine deprivation were shown to drastically reduce the amount of polysomes, in a manner independent of 4EBP1 or eIF2α phosphorylation. In addition, transfection of a 4EBP1 mutant that constitutively binds eIF4E did not inhibit cell growth or translation. These results indicate that essential amino acids, when deprived, have effects on translation independent of eIF2 and 4EBP1, and 4EBP1 phosphorylation status alone does not inhibit global translation.

BIOGRAPHICAL SKETCH

Kevin Michael Mazor was born on April 9, 1988 in Houston, Texas to Pam and Michael Mazor. As a child, he could be found walking circles around the playground, engaging in fighter battles with his toy X-Wing. After retiring from the rebellion, he became a student of Sherlock Holmes, who kindled in him a desire not just to see, but observe. After learning that a forensic scientist has to deal with blood and dead bodies, he decided something biochemical was more to his liking (as molecules don't bleed). He attended Michigan State University, where he earned BS degrees in both Nutritional Sciences and Biochemistry and Molecular Biology (2006–2010). It was at Michigan State where his love of the scientific method and experimental design blossomed and he learned that experimental failure was always an option. In order to fully develop his deductive reasoning skills and learn more about the fundamental ways in which the body works, he decided to pursue his Ph.D. He began the doctoral program in Molecular Nutrition at Cornell University in 2010. During this process he has grown and changed. Although his current future is different than his future appeared six years ago, he will use the knowledge and skills he learned and continue to always think like a scientist.

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It is not shocking to say that I would not have gotten here without the help of countless people -- whether known to me or not. I would, therefore, like to take this time to thank those people who have profoundly influenced my life to this point.

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LIST OF ABBREVIATIONS

4EBP1: eIF4E Binding Protein 1

ASNS: Asparagine Synthetase

ATF4: Activating Transcription Factor 4

C/EBP: CCAAT-Enhancer-Binding Proteins

CARE: C/EBP-ATF Binding Element

CHOP: CCAAT/enhancer-binding protein homologous protein

eIF2: Eukaryotic Initiation Factor 2

eIF2B: Eukaryotic Initiation Factor 2B

eIF2α: Alpha Subunit of eIF2

eIF4E: Eukaryotic Initiation Factor 4E

eIF4G: Eukaryotic Initiation Factor 4G

GCN2: General Control Nonderepressible 2, also known as eIF2α Kinase 4

GDP: Guanosine-5' Diphosphate

GTP: Guanosine-5' Triphosphate

HEK: Human Embryonic Kidney

ISR: Integrated Stress Response

MEF: Mouse Embryonic Fibroblasts

mTORC1: Mechanistic Target of Rapamycin Complex 1

PIC: Preinitiation Complex

S6K1: P70-S6 Kinase 1

$tRNA_e^{Met}$: Elongator Methionine tRNA

$tRNA_i^{Met}$: Initiator tRNA

uORF: Upstream Open Reading Frame

CHAPTER 1

AN INTRODUCTION AND LITERATURE REVIEW

Introduction

It has long been known that mammals require an exogenous source of certain amino acids, termed essential or indispensable, in order to synthesize all of the proteins required for life. These particular amino acids are essential dietary components due to the body's inability to synthesize them de novo. When any of these essential amino acids is absent or deficient in the diet, global translation can be suppressed while, at the same time, translation of certain transcription factors can increase in order to allow the cell to cope with the stress. These responses are mediated by two well studied pathways: (a) mechanistic target of rapamycin complex 1 (mTORC1) (1) and the downstream targets of its serine/threonine kinase activity, and (b) the integrated stress response (ISR) pathway consisting of general control nonderepressible 2 (GCN2, i.e. eIF2αK4) and its target, the alpha subunit of eukaryotic initiation factor 2 (eIF2α), as well as the subsequent increase in translation of activating transcription factor 4 (ATF4) and its transcriptional effects (2). Although the responses of these pathways to total and single essential amino acid deprivation have been rather extensively studied, the relative roles of these two pathways and the exact mechanisms by which different essential amino acids regulate these pathways are not well understood. Recent studies have demonstrated possible mechanisms for how mTORC1 senses leucine, arginine and glutamine as well as mechanisms for GCN2-independent upregulation of ATF4 in the absence of methionine. The current review aims to summarize the current knowledge on how mTORC1 and the ISR pathway are regulated by amino acids, focusing primarily on how the lack of specific amino acids is sensed and how this leads to inhibition of mTORC1 signaling and activation of the ISR pathway.

Historical Perspective

The determination of which amino acids were essential to mammals was first done in growing rats (reviewed in Stewart 1943). Essential amino acids were determined feeding diets deficient in each amino acid to young rats and following their growth rate. If the lack of an amino acid in the diet resulted in growth inhibition, the amino acid was assumed to be essential. In order to determine if growing rats had different amino acid needs than adult rats, nitrogen balance experiments were conducted with adult rats (4, 5). These experiments, along with similar experiments in mice and dogs (6, 7), showed that the essential amino acids were phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine and histidine. Cysteine or tyrosine was essential only if methionine or phenylalanine, respectively, was insufficient. Arginine was determined to be essential only for growing rats. The nitrogen balance experiments were then repeated in humans to determine the specific amounts of each essential amino acid that are required for nitrogen balance (8–11). The Food and Agriculture Organization /World Health Organization (2007) continues to update the requirements. The current recommendations, as well as those from 1985, are shown in Table 1.

Table 1.1. Current and past essential amino acids requirements. Adapted from "Protein and Amino Acid Requirements in Human Nutrition" WHO 2007

Amino Acid	**mg amino acid/ kg body weight per day**	
	2007 Recommendations	1985 Recommendations
Histidine	10	8-12
Isoleucine	20	10
Leucine	39	14
Lysine	30	12
Methionine + cysteine	15	13
Phenylalanine + tyrosine	25	14
Threonine	15	7
Tryptophan	4	3.5
Valine	26	10

Since the discovery of the essential amino acids, pathways for the synthesis of other amino acids have been elucidated (12) and it is clear that the nine truly essential amino acids are not able to be synthesized by mammalian cell lines due to a lack of enzymes that catalyze their synthesis (13). However, it should be noted that the synthesis of some nonessential amino acids depend on tissue-specific pathways. In these cases, individual tissues or cell types may require a supply of additional amino acids. For example, de novo synthesis of arginine depends on citrulline synthesis in the intestine and its conversion to arginine in the kidney (14). Therefore, in addition to the essential amino acids, mammalian cells grown in cell culture may require arginine, cysteine, glutamine and tyrosine for optimal growth (15).

Determination of the effects that each individual amino acid has on cell growth and translation began with polysome profiling experiments in the 1960's, which demonstrated that an absence of one essential amino acid could affect the stability of hepatic polysomes (16). This was expanded upon when it was demonstrated that tryptophan deprivation shifted translating ribosomes from the polysome fractions to the lighter monosome and disome fractions (17, 18). In liver of fasted mice that were given amino acids by tube-feeding, the lack of tryptophan resulted in a greater fraction of ribosomes in the monosome and disome fractions, whereas the absence of threonine, isoleucine or methionine in the intubated amino acid mixture had no effect on the polysome profile (19). However, Munro and coworkers demonstrated, in an in vitro cell-free system, that medium lacking one essential amino was able to shift ribosomes from the monosome to the polysome fraction (20), suggesting tryptophan was not unique in its ability to inhibit polysome formation. Munro and coworkers hypothesized that tryptophan was the least abundant amino acid in liver and, hence, more readily depleted in liver. Therefore, in the work done by Sidransky et al. (19) the length of time rats were fed amino acid-deficient diets may not

have been sufficient to deplete liver levels of essential amino acid other than tryptophan. As such, a shift in ribosomes away from the polysome fraction was only observed in the livers of rats fed the tryptophan deficient diet. In order to emulate long term starvation Munro and coworkers fed rats a diet limiting in either threonine or isoleucine for several days in order to deplete the liver levels of these amino acids. When these rats were fasted overnight and then fed diets lacking threonine or isoleucine for several days, ribosomes were shown to shift away from the polysome fraction to the monosome fraction, demonstrating that essential amino acids other than tryptophan can inhibit translation in the liver of whole animals (21). Yokogoshi and Yoshida (1980) reported similar findings in rats that were adapted to a protein-free diet and then given access to diets that contained all amino acids except the one under investigation, with rats having access to the diet for 5 hours (22). A lack of tryptophan, methionine/cystine, threonine, phenylalanine/tyrosine, leucine, isoleucine, valine, or lysine significantly increased the proportion of ribosomes in the monosome-disome fractions.

Experiments investigating the effects of deprivation of a single essential amino acid on translation have also been done in human cells (23) and isolated rat hepatocytes (24). These studies also support the conclusion that a lack of any essential amino acid will result in the depletion of polysomes and a lower rate of protein synthesis. In Hela cells, methionine, histidine, or valine were deprived for 2 hours and protein synthesis rates and polysome profiles were then determined. The deprivation of any of the tested essential amino acids reduced the percentage of ribosomes in polysomes and decreased protein synthesis. In control cells 68% of ribosomes were in the polysome fraction, and this was decreased to 26, 28 and 30% under methionine, histidine and valine deprivation, respectively. In rat hepatocytes, cells suspended in medium lacking lysine, methionine or tryptophan decreased protein synthesis by 26, 50 and 36%, respectively, as

measured between 1 and 2 h after suspension in treatment medium and the addition of radiolabeled leucine.

Overall, these early studies demonstrated that certain amino acids were essential for growth or nitrogen balance and that a lack of an essential amino acid in the diet or cell medium impaired the capacity for protein synthesis as assessed by polysome profiles or incorporation of label into proteins. However, beyond the obvious role of amino acids as substrates for protein synthesis, little was known about how amino acids act to regulate the processes involved in protein synthesis. The role of amino acids in regulating translation and the mechanisms by which this occurs are active areas of current research.

An Overview of Translation Initiation

In eukaryotes, translation initiation consists of multiple steps: formation of the 43S preinitiation complex (PIC), cap recognition and formation of the 48S PIC, scanning down the mRNA, until the start codon is recognized, and finally formation of the 80S ribosome at the start codon. These events are facilitated by multiple initiation factors to ensure that the ribosome, containing the charged initiator tRNA, begins translation at the correct start site (2).

Initiator tRNA

In eukaryotes translation is initiated using a unique tRNA aminoacylated with methionine. The structure of the initiator tRNA is highly conserved across all forms of life (25). In eukaryotes, both the initiator (tRNAiMet) and elongater tRNAMet(tRNAeMet) are aminoacylated with methionine (Met-tRNAiMet, Met-tRNAeMet) by methionyl-tRNA synthetase (MRS) with no unique specificity (26). Structural differences between the initiator and elongator tRNAMet prevent the initiator tRNAMet from binding the eukaryotic elongator

factor 1A (eEF1A), the protein that brings charged tRNAs to the ribosome during translation elongation, while promoting its binding to eukaryotic elongator factor 2 (eIF2) (27, 28).

Changes in the cellular concentration of tRNAiMet have consequences for translation initiation. Yeast growth rate decreases when concentrations of Met-tRNAiMet are low (29), and an increase in tRNAiMet has been implicated in cancer development (30).

43S PIC formation

The so-called ternary complex is formed when aminoacylated Met-tRNAiMet binds to eIF2-GTP. eIF2 is a GTPase with 3 subunits (α, β, and γ). eIF2γ binds GTP, and eIF2α and eIF2β function to bind Met-tRNAiMet (31–33). The affinity of eIF2 for Met-tRNAiMet is roughly 15-fold higher when GTP is bound to eIF2 than when GDP is bound, decreasing Kd values from 150 nM with eIF2-GDP to 9 nM with eIF2-GTP. In addition, Met-tRNAiMet binding to eIF2-GTP is facilitated by the presence of methionine on the tRNAiMet as non-acylated tRNAiMet has a Kd value of roughly 130 nM for both eIF2-GTP and eIF2-GDP (34). Thus, methionine charging of tRNAiMet is necessary for ternary complex formation.

The 43S preinitiation complex is formed when an active ternary complex binds to the 40S ribosomal subunit in conjunction with eukaryotic initiation factor 3 (eIF3) and eukaryotic initiation factor 1A (eIF1A). eIF3 is a large multi-subunit protein that both facilitates mRNA cap complex recognition and inhibits premature 60S subunit binding (35), while eIF1A functions to transfer Met-tRNAiMet to the 40S subunit (36).

Cap recognition

In eukaryotes, the 5′ methyl-cap is involved in the binding of the 43S PIC to the mRNA. The 5′ cap is recognized and bound by eukaryotic initiation factor 4E (eIF4E). eIF4E also binds

eukaryotic initiation factor 4G (eIF4G) and 4A (eIF4A) to form the eIF4F complex. eIF4A is an RNA helicase that functions to remove any secondary structure present in the mRNA, while eIF4G acts as a scaffolding protein that is recognized by eIF3 in order to bind the 43S PIC to the mRNA, forming the 48S PIC. The 48S PIC then scans down the mRNA until the Met-tRNAiMet binds to the start codon. After recognition of the start codon, the GTP associated with eIF2 is hydrolyzed by eukaryotic initiation factor 5 (eIF5) and the resulting eIF2-GDP dissociates from the 48S PIC. After eIF2 dissociation, eIF5B-GTP binds to the PIC and promotes the release of the other initiation factors as well as the association of the 60S ribosomal subunit. After the GTP of eIF5B-GTP is hydrolyzed, eIF5B-GDP dissociates, leaving the 80S with the Met-tRNAiMet in the P site (2, 37, 38).

Regulation of Translation Initiation by mTORC1

It has been established that mTORC1 regulates cell growth and autophagy based on the presence of amino acids. Recently, great strides have been made on uncovering the mechanisms involved in the signaling of amino acid availability to mTORC1.

mTORC1 regulates translation initiation through eIF4E binding protein 1

Formation of the eIF4F complex and cap recognition is dependent on eIF4G binding to eIF4E. eIF4E binding proteins (4EBPs) are a class of small largely unstructured proteins that share the same binding site as eIF4G on eIF4E. Therefore, 4EBPs act as competitive inhibitors for the formation of the eIF4F complex. Of the 4EBPs, 4EBP1 is the most ubiquitous and is thought to be a primary regulator of cap-dependent translation initiation. (39–42) 4EBP1 binding to eIF4E is regulated by the phosphorylation state of 4EBP1. Hypophosphorylated 4EBP1 is active and binds to eIF4E, thereby inhibiting translation. Conversely, hyperphosphorylated 4EBP1 is inactive allowing eIF4G to bind eIF4E and activate translation initiation (43–45). 4EBP1 is

phosphorylated by mTORC1 in response to signals such as insulin and amino acids. In the presence of such signals mTORC1 is active and phosphorylates 4EBP1, inhibiting 4EBP1 binding to eIF4E and allowing the formation of the eIF4F complex. When mTORC1 is inactive, 4EBP1 remains hypophosphorylated and prevents eIF4G from binding to eIF4E, inhibiting translation initiation (45–49).

mTORC1 activation by amino acids

How mTORC1 senses the presence of amino acids is not fully understood although the growing understanding of the role of amino acids on mTORC1 is that inputs from amino acid signaling stem from multiple receptor proteins that have actions converging on a complex of mTORC1 with other proteins on the lysosomal membrane.

The tumor suppressor complex (TSC1/2) is a known regulator of mTORC1 activity in response to growth factor/insulin signaling and to the cellular energy state, but it does not appear to be the mediator of amino acid signals (50–57). The main mediator of TSC1/2 signaling to mTORC1 is Ras Homolog Enriched In Brain (Rheb) which is normally localized to the lysosomal membrane (51, 52, 54). Rheb is a small GTPase, and the GTP-loaded form of Rheb is known to be critical for activation of mTORC1's serine/threonine kinase activity. Under nutrient insufficient conditions TSC1/2 binds to Rheb and stimulates Rheb's intrinsic GTPase activity, which converts Rheb-GTP to Rheb-GDP. Because Rheb-GDP binds strongly to the TSC1/2 complex, this prevents Rheb from being able to associate with mTORC1. In nutrient-sufficient conditions, insulin signaling activates the phosphoinositide 3-kinase/Akt signaling pathway, which results in inhibitory phosphorylation of TSC2 which in turn stimulates the dissociation of the TSC1/2 complex from Rheb, leaving Rheb available for mTORC1 association. This induced release of the TSC1/2 complex from lysosomal Rheb also permits Rheb-GTP loading, which is

required for mTORC1 activity. Thus, the presence of insulin and sufficient energy promotes mTORC1 activity by allowing the loading of Rheb with GTP and allowing the association of mTORC1 with Rheb at the lysosomal membrane. Both association with Rheb and the GTP loading of Rheb are required for mTORC1 activation.

Rheb-GTP association and activation of mTORC1 have been shown to be dependent on amino acids, but by mechanisms that do not involve TSC1/2 (52, 58). Either total amino acid deprivation or leucine deprivation inhibited the association of mTORC1 with Rheb-GTP and the activation of mTORC1's kinase activity without altering the guanine nucleotide status of Rheb. This suggests that amino acids primarily regulate the association of mTORC1 with Rheb rather than the guanine nucleotide state of Rheb. Indeed, in the presence of insulin, the addition of amino acids has been shown to promote the localization of mTORC1 to the lysosomal membrane where it associates with Rheb-GTP leading to the activation of mTORC1 (50, 55).

The association of mTORC1 with the lysosomal membrane is dependent on a lysosome-associated complex that consists of a vacuolar adenosine triphosphatase (v-ATPase), Ragulator, a heterodimer of Ras-related GTPases termed the Rag GTPases, the lysosomal transmembrane protein SLC38A9, and vacuolar protein sorting-34 (VPS34). These, and perhaps other, proteins that associate with mTORC1 at the lysosomal membrane are involved in the pathways by which amino acids exert their activation effects on mTORC1.

Ragulator is a pentameric protein complex that associates with the lysosomal membrane and functions to localize the Rag GTPases to the lysosomal membrane (59, 60). The Rag GTPases are heterodimers between Rag A or B (Rag A/B) and Rag C or D (Rag C/D); thus the heterodimer could be RagA/RagC, Rag A/RagD, RagB/RagC or RagB/RagD (61, 62). In addition, Ragulator acts as a guanine nucleotide exchange factor (GEF) for Rag A/B to load

GTP. For mTORC1 to be localized to the lysosome, Rag A/B in the Rag dimer must be loaded with GTP and Rag C/D in the Rag dimer must be loaded with GDP (59, 60, 63–65). Thus, the action of Ragulator on Rag A/B is a critical step in the localization and activation of mTORC1 on the lysosomal membrane.

Several studies have suggested that amino acids from within the lysosome activate mTORC1 by signaling through the v-ATPase. This mechanism was shown to involve the direct interaction of the v-ATPase with Ragulator and Rag GTPases, as disruption of the proton gradient across the lysosomal membrane did not inhibit amino acid signaling to mTORC1. The Fo domain of the v-ATPase was shown to constitutively bind Ragulator complex, and the F1 domain of v-ATPase was shown to interact with the Rag GTPases associated with Ragulator when amino acids were sufficient (66). The association of the v-ATPase with Ragulator and the Rag GTPases activates the GEF domain of Ragulator protein complex, causing Rag A/B to load GTP (60). A different but incompletely elucidated mechanism has been suggested for the glutamine-specific regulation of mTORC1. This glutamine-specific regulation was not dependent upon Ragulator or Rag A/B but did require the v-ATPase and the lysosomal localization of mTORC1 (85).

In addition to the effect of Ragulator on Rag A/B GTP loading, Rags are regulated by two GTPase activating proteins (GAPs): the GATOR and the Folliculin (FLCN)-folliculin interacting protein (FNIP) complexes, which regulate RagA/B and RagC/D, respectively (64, 65). Regulation of the guanine nucleotide state of the Rags appears to be a central mechanism by which protein associations with the Rag/Ragulator complex and mTORC1 activity are regulated.

GATOR acts as a GAP for Rag A and Rag B. GATOR is made up of two sub-complexes: GATOR1 and GATOR2. GATOR1 is a GAP for Rag A/B. Mutation of GATOR1 to inactivate

its GAP activity resulted in mTORC1 becoming insensitive to amino acid deprivation, demonstrating that GATOR1 acts to convert RagA/B to its inactive GDP-bound form. GATOR2 however was shown to be a negative regulator of GATOR1. When GATOR2 was inhibited, mTORC1 activity was suppressed (64). The process is further regulated by proteins that can inhibit GATOR2. Studies have shown that Sestrins and CASTOR1 associate with GATOR2 in amino acid-dependent manners (67–70). In the absence of amino acids, Sestrins and CASTOR1 associate with GATOR2 and prevent GATOR2 from inhibiting GATOR1, with consequent inactivation of mTORC1. In the presence of amino acids, however, Sestrins or CASTOR1 release from GATOR2, allowing for GATOR2 to inhibit GATOR1, leading to the activation of mTORC1 (67, 68).

The FLCN-FNIP complex acts as a GAP for Rag C and Rag D. The FLCN-FNIP complex associates with the Rag heterodimer and is present on the lysosomal membrane. The FLCN-FNIP complex activates the hydrolysis of the GTP bound to Rag C/D, converting Rag C/D to the GDP-bound form that is needed for mTORC1 association with Rheb-GTP and mTORC1 activation (60, 64, 65). Thus, GATOR and FLCN-FNIP regulate the conversion of Rag A/B-GTP and Rag C/D-GTP to Rag A/B-GDP and Rag C/D-GDP, respectively. However, amino acids inhibit GATOR1's GAP activity so Rag A/B remains GTP-loaded while FLCN-FNIP's GAP activity activates Rag C/D GTPase so that the Rag C/D is in the GDP form. Hydrolysis of the GTP bound to Rag C/D to GDP in conjunction with GTP loading on Rag A/B localizes mTORC1 to the lysosomal membrane.

SLC38A9 was only recently found to be associated with the Ragulator and Rag dimers at the lysosomal membrane as a result of studies in HEK293T cells (71–73). SLC38A9 is an 11-pass transmembrane protein of the lysosomal membrane that acts as a low-affinity, relatively

nonspecific amino acid transporter (71). Despite its ability to transport most amino acids, SLC38A9 shows a preference for cationic amino acids (arginine, lysine and histidine). SLC38A9 localizes with the Ragulator/Rag complex in an amino acid-independent manner, and the cytosolic N-terminal tail of SLC38A9 is sufficient to mediate this association and to contribute to the activation of mTORC1 by Rheb (71, 72). Full length SLC38A9 with its transmembrane domain, however, is able to signal amino acid availability and enhance mTORC1 activity. Knockout of SLC38A9 in HEK293T cells strongly repressed the activation of mTORC1 by arginine (71). Exactly how SLC38A9 signals amino acid availability remains unclear, but it appears to be mediated through amino acid-sensitive associations of full-length SLC38A9 with the Rag GTPases and to depend on the lysosomal concentration of amino acids (71, 72). The observation that depletion of SLC38A9 resulted in the retention of mTORC1 at the lysosome under both amino acid-sufficient and amino acid-starved conditions suggests that SLC38A9 is required for the release of Rag/Ragulator-bound mTORC1 from the lysosomal membrane (72). Thus, SLC38A9 could play an important role in the downregulation of mTORC1 activity in response to amino acid deprivation.

The autophagy regulator vacuolar protein sorting-34 (VPS34) has also been shown to be necessary in signaling of amino acid availability to mTORC1. In HEK293 cells, VPS34 knockdown inhibited the phosphorylation of p70-S6 kinase 1 (S6K1) by mTORC1 when amino acids were added back to amino acid-deficient medium. In addition, VPS34 was shown to be pulled down with mTORC1 in mTORC1 immunoprecipitation assays, and overexpression of VPS34 was shown to increase S6K1 phosphorylation in a dose-dependent manner, demonstrating that VPS34 increases mTORC1 kinase activity on its S6K1 target (58). VPS34-null murine embryonic fibroblasts (MEFs) were also shown to have impaired amino acid

signaling to mTORC1 as cells adapted to amino acid deficient medium failed to phosphorylate 4EBP1 and S6K1 when amino acids were added to the growth media (74, 75). The signaling of amino acid availability to mTORC1 through VPS34 was shown to be dependent on intracellular calcium levels and calmodulin (76). When Hela cells were supplemented with amino acids after being exposed to amino acid-deficient medium for 2 h, intracellular calcium levels increased, and if cells were incubated with either a chelator for calcium or an inhibitor of calmodulin, mTORC1 stimulated phosphorylation of S6K1 did not occur. It was demonstrated that the increase in intracellular calcium upon amino acid supplementation resulted in the association of calmodulin with the VPS34-mTORC1 complex, leading to the activation of mTORC1. How the calcium-dependent VPS34 mTORC1 activation integrates with Rag-GTPase and Rheb-mediated stimulation of mTORC1, however, is still unknown.

Unique role of leucine in mTORC1 activation

It is well-established that mTORC1 is particularly sensitive to leucine status (77–79). Based on deprivation of individual essential amino acids, the lack of leucine was most effective at reducing 4EBP1 phosphorylation In fact, when cells were supplied all amino acids except leucine, 4EBP1 remained dephosphorylated, demonstrating the unique ability of leucine to activate mTORC1's serine/threonine kinase activity (80). Though the influence of leucine on mTORC1 has long been known, the mechanism by which mTORC1 senses leucine status has remained controversial. Several recent lines of research suggest that leucine acts in a manner differently than other amino acids in regulating mTORC1 activity (69, 81, 82).

Wolfson et al., working with HEK293T cells, demonstrated that Sestrin2 acts as a leucine sensor in vivo (69). Sestrin2 was shown to be specific for leucine at physiological concentrations of amino acids, with binding of Sestrin2 to isoleucine, arginine or methionine occurring only at well

above physiological concentrations. Leucine-bound Sestrin2 dissociates from GATOR2, allowing GATOR2 to inhibit GATOR1 and prevent the hydrolysis of RagA/B-GTP to inactive RagA/RagB-GDP and, thus, to promote mTORC1 localization to the lysosomal membrane. Although Sestrin2 is the most studied, Sestrin1 is also sensitive to leucine, whereas Sestrin3 is not (69).

Han et al. (81), also working with HEK293T cells, demonstrated a different mechanism by which leucine may have a unique effect on mTORC1. They showed that leucine availability can signal mTORC1 through the leucyl-tRNA synthetase (LRS). The LRS is a component of the multi-tRNA synthetase complex (MSC), which consists of nine tRNA synthetases and three nonenzymatic components (82). LRS, but not isoleucyl-tRNA synthetase, was shown to colocalize with mTORC1 when leucine was supplemented to leucine-deprived HeLa cells demonstrating that LRS binds mTORC1 separate from the MSC. Leucine binding was also shown to be required for LRS-mediated activation of mTORC1. When the leucine-binding domain of LRS was mutated, the response of S6K1 phosphorylation to leucine supplementation was inhibited. Leucine-bound LRS was shown to associate with mTORC1 and Rag D and act as a GAP for Rag D to hydrolyze the GTP to GDP. LRS aminoacylation mutants were shown to stimulate the leucine-dependent activation of mTORC1, demonstrating that the aminoacylation activity is not required for the ability of LRS to activate mTORC1.Thus, leucine-bound Sestrin2 acts to suppress GATOR1's GAP activity on Rag A/B promoting GTP loading of Rag A.B, whereas leucine-bound LRS acts as a GAP for Rag C/D to keep it in the GDP form. Working together, these two proteins could be responsible for maintaining the Rag dimer in the correct guanine nucleotide state for mTORC1 localization and activation.

In contrast to an effect of leucine that is mediated by regulating the ability of the

Rag/Ragulator complex to associate with mTORC1 and then localize mTORC1 to the lysosome, Averous et al. (83), working with serum-fed MEFs, found that leucine activated mTORC1 without any detectable change in the lysosomal localization of mTORC1. As expected, MEFs exposed to amino acid-deficient medium exhibited a decrease in mTORC1 activity and lysosome association. However, when all amino acids except leucine were added to the deprived cells, mTORC1 was shown to localize to the lysosome but remain inactive. Only when leucine was added back did mTORC1-stimulated phosphorylation of 4EBP1 and S6K1 return to the levels observed in control cells. In addition, when Rag C/D was knocked down in cells grown in complete medium, mTORC1 localization to the lysosome was inhibited but mTORC1 activation was not. Rag C/D knockdown prevented the complete inhibition of mTORC1 by deprivation of either total amino acids or leucine alone but did not prevent an increase in mTORC1's kinase activity with addition of either total amino acids or leucine to the deficient medium. In total these results suggest that the Rag GTPases are required for mTORC1 localization to the lysosome and for the complete inactivation of mTORC1 due to amino acid deprivation. However, mTORC1 localization to the lysosome and activation of mTORC1 were shown to be largely independent, with leucine being the primary regulator of mTORC1 activation and with total amino acids being the primary regulator of mTORC1 association with the lysosome.

Unique role of arginine on the regulation of mTORC1 through CASTOR1

Like leucine, arginine has also been shown to specifically activate mTORC1 by mechanisms involving Rag GTPases. Studies in intestinal epithelial cells showed that the addition of either arginine or leucine to amino acid-deprived cells resulted in an increase in mTORC1 activity. The activity of mTORC1 was more sensitive to leucine availability, but only arginine stimulated mTORC1 activity by a mechanism that was dependent upon the expression

of cationic amino acid transporters, suggesting leucine and arginine have different mechanisms by which they stimulate mTORC1 (84).

Arginine appears to signal to mTORC1 through two mechanisms. The first is through the lysosomal transmembrane amino acid transporter SLC38A9 (71). As previously discussed, SLC38A9 signals the presence of lysosomal amino acids to mTORC1. The ability of arginine supplementation to increase mTORC1 activity in HEK293T cells was reduced by knockout of SLC38A9, whereas the ability of leucine supplementation to activate mTORC1 was not substantially reduced by knockout of SLC38A9. Therefore, based on the observation that mammalian lysosomes contain high concentrations of arginine (85), Wang et al. (71) suggested that lysosomal arginine concentration signals to mTORC1 through SLC38A9. However, because SLC38A9 knockout did not totally eliminate the ability for arginine to stimulate mTORC1, a second arginine sensing mechanism must exist.

This additional mechanism was demonstrated to involve the CASTOR proteins by Chantranupong et al. (70) who found that, in HEK 293T cells, CASTOR1 and CASTOR2 form homo- and hetero-dimers with each other, but only CASTOR1 was able to bind arginine. Leucine or lysine did not bind to CASTOR1, demonstrating the specificity of CASTOR1 for arginine. Arginine binding to the CASTOR1 homodimer or the CASTOR1-CASTOR2 heterodimer was shown to disrupt the association between CASTOR1 and GATOR2. This disruption of the CASTOR1-GATOR2 complex releases GATOR2 and allows it to inhibit GATOR1's GAP activity on Rag A/B, with RagA/B-GTP promoting mTORC1 binding and activation. In addition, in SLC38A9 null cells, knockdown of CASTOR1 was shown to make mTORC1 totally insensitive to arginine deprivation.

Unique role of glutamine in stimulating mTORC1 via Arf1

Recently, glutamine has also been shown to specifically regulate mTORC1 in a manner independent of the Rag GTPases but dependent on lysosomal localization of mTORC1 (86, 87). The mechanism of this regulation was recently shown by Jewell et al. (88). Addition of glutamine, but not leucine, stimulated mTORC1 activity in MEFs and HEK293A cells in which Rag A and Rag B had been knocked out. This glutamine-specific, Rag A/B-independent regulation of mTORC1 was shown to be dependent on the lysosomal localization of mTORC1 and the v-ATPase. However, Ragulator was not required for this glutamine-specific, Rag A/B-independent activation of mTORC1. In Rag A/B knockout HEK293A cells, knockdown of the ADP ribosylation factor 1 (Arf1), which is a GTPase , eliminated the ability of glutamine to stimulate mTORC1 activity, indicating that Arf1 is required for the glutamine-specific, Rag A/B-independent regulation of mTORC1.

Regulation of Translation Initiation and the Integrated Stress Response by GCN2

In addition to mTORC1, translation initiation is also regulated by the amount of ternary complex available for translation. GCN2 regulates ternary complex availability by phosphorylating eIF2 in the presence of uncharged tRNAs.

The GCN2/eIF2α/ATF4 pathway

After translation initiation, eIF2-GDP must be converted back to eIF2-GTP in order for initiation to begin again. This is accomplished by the guanine nucleotide exchange factor eukaryotic initiation factor 2B (eIF2B) (89, 90). eIF2B is present in a much lower concentration than eIF2 and therefore eIF2-GTP regeneration by eIF2B is used as a point of regulation in translation initiation (91). When one of the essential amino acids is deficient, the amount of aminoacylated tRNA for that amino acid decreases (1). The consequent increase in non-aminoacylated tRNAs is sensed by GCN2 (i.e., eIF2α kinase 4) (93). Uncharged tRNAs bind to

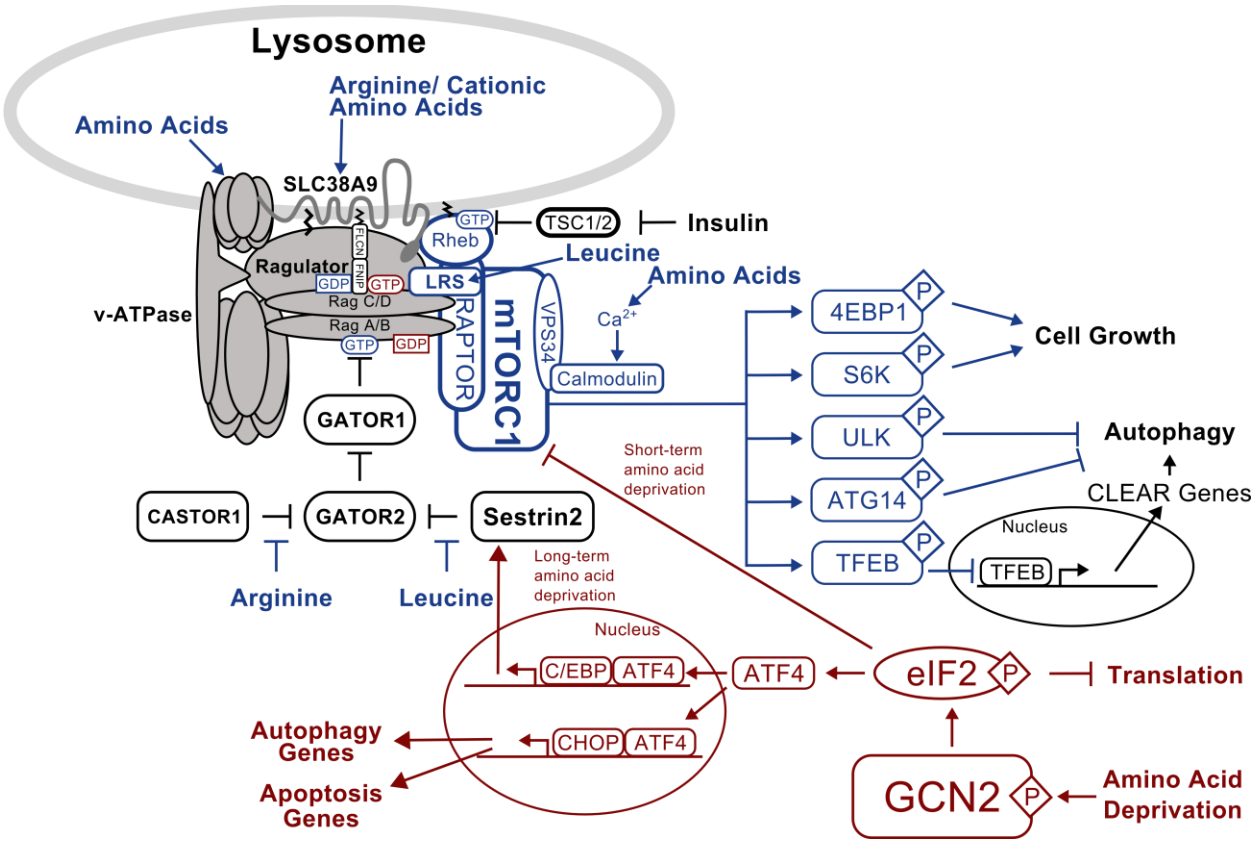

Figure 1.1 Regulation of mTORC1 activity by amino acids. Under amino acid sufficient conditions (Blue) the presence of lysosomal amino acids single to mTORC1 through the v-ATPase. The v-ATPase binds to Ragulator and the Rag GTPases and, in the presence of amino acids, promotes the guanine nucleotide exchange factor of Ragulator to load GTP onto RagA/B. The Folliculin (FLCN) – FNIP complex associates with the lysosome and RagC/D. In the presence of amino acids, the FLCN-FNIP complex acts as a GTPase activating protein (GAP) and activates the hydrolysis of GTP bound to Rag C/D to GDP. GTP-bound RagA/B and GDP-bound RagC/D are active and localize the mTORC1 complex to the lysosomal membrane. Once localized to the lysosome mTORC1 is activated by GTP bound Rheb in the presence of amino acids, insulin and other growth factors. An increase in intracellular calcium levels under amino acid sufficient conditions also activates mTORC1 by increasing calmodulin binding to VPS34 and mTORC1. Once active, mTORC1 promotes growth by phosphorylating p70-S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4EBP1) and inhibits autophagy through the phosphorylation of unc-51 like kinase complex (ULK), ATG14 and transcription factor EB (TFEB). Single essential amino acids also specifically activate mTORC1. The lysosomal concentration of arginine activates mTORC1 by signaling through the transmembrane protein SLC38A9, which associates with Ragulator and Rheb. Cytosolic amino acid concentrations of leucine and arginine signal through the GATOR proteins. GATOR1 acts as a GAP to RagA/B, preventing the formation of active, GTP-bound RagA/B. GATOR 2 is an inhibitor to GATOR1, activating mTORC1 by preventing GATOR1's hydrolysis of GTP bound to RagA/B. Sestrin2 and CASTOR1 are proteins that bind and inhibit GATOR 2, and have been shown to be sensors for arginine and leucine respectively. Both CASTIOR1 and Sestrin2 act through a similar mechanism. When the amino acid of interest binds (arginine to CASTOR1 and leucine to Sestrin2) the association between CASTOR1 or Sestrin2 and GATOR2 is disrupted. The release of GATOR2 promotes mTORC1 activation through the inhibition of GATOR1. In addition, leucine signals through the leucyl-tRNA synthetase (LRS) where leucine-bound LRS acts as a GAP for RagD and activates the hydrolysis of GTP bound to Rag D to GDP.

In the absence of amino acids those processes highlighted in blue are inhibited, preventing

Figure 1.1 (Continued) mTORC1 lysosomal localization and activation. This inhibits cell growth and promotes autophagy by preventing phosphorylation of S6K1, 4EBP1, ULK, ATG14 and TEFB. TEFB acts as a transcription factor that upregulates genes specific for the promotion of autophagy in the Coordinated Lysosomal Expression and Regulation (CLEAR) network.

In addition, the absence of amino acids activates the integrated stress response (Red) by promoting the phosphorylation of general control nonderepressible 2 (GCN2, ie eIF2αK4). GCN2 then phosphorylates the alpha subunit of the eukaryotic initiation factor 2 (eIF2). eIF2-P inhibits mTORC1, translation initiation as well as increases the translation of the transcription factor activating transcription factor 4 (ATF4). ATF4 then upregulates genes that contain a promotor sequence termed the CARE (C/EBP-ATF4 binding element). CARE-containing genes include Sestrin2, which when upregulated under amino acid deprivation helps inhibit mTORC1 activation. In addition, ATF4 forms a heterodimer with another CARE containing gene CHOP (CCAAT/enhancer-binding protein homologous protein) to increase transcription of genes that promote both autophagy and apoptosis.

the histidyl-tRNA synthetase–related regulatory domain on GCN2, causing it to dimerize and auto-phosphorylate, thus becoming activated (94–96). Active GCN2 then phosphorylates Ser51 of the alpha subunit of eukaryotic initiation factor 2 (eIF2α) (97–100). When eIF2α is phosphorylated, the affinity of eIF2-GDP for eIF2B is increased, resulting in phosphorylated eIF2-GDP acting as a competitive inhibitor of eIF2B. This prevents the reformation of eIF2-GTP, and translation initiation is inhibited by the reduced abundance of the ternary complex, decreasing overall protein synthesis (101).

Although eIF2α phosphorylation inhibits global protein synthesis, the translation of certain mRNAs are increased under these conditions. The most studied protein that is translationally regulated in such a way is activating transcription factor 4 (ATF4) (102, 103). The mechanism of the increased translation of ATF4 is outlined in Figure 1.2. The 5′ UTR of the ATF4 mRNA contains an inhibitory upstream open reading frame. After translation of this peptide, the 60S subunit releases, while the 40S subunit continues to scan down the mRNA. The rates at which the ternary complex is added back to the 40S subunit and at which subsequent formation of the 80S ribosome occurs depend on the abundance of eIF2α-GTP. Under nutrient-sufficient conditions with abundant eIF2-GTP, reformation of the preinitiation complex with the Met-tRNAiMet occurs readily and translation is reinitiated at an additional upstream start codon

that results in translation of an open reading frame that overlaps but is out-of-frame with the ATF4 open reading frame. However, if the eIF2-GTP level is low due to eIF2α phosphorylation, the reformation of the preinitiation complex takes longer, causing the 40S subunit to pass the start site for the dummy protein and in turn recognize the downstream ATF4 start site, resulting in increased translation of ATF4 (104–106).

ATF4 is a transcription factor that forms heterodimers with CCAAT-enhancer-binding proteins (C/EBP). The heterodimers recognize the consensus sequence of "TGATGXAAX" in gene promoter regions. This sequence is termed the CARE (C/EBP-ATF binding element) and C/EBP-ATF4 binding results in the upregulation of genes in the ISR essential for the protection of cells in a variety of stress conditions. These genes include eIF4E binding protein 1 (4EBP1), CCAAT/enhancer-binding protein homologous protein (CHOP, or DDIT3) asparagine synthetase (ASNS), the amino acid transporter solute carrier family 7 member 11(SLC7A11), and tRNA synthetases (103, 107–109)

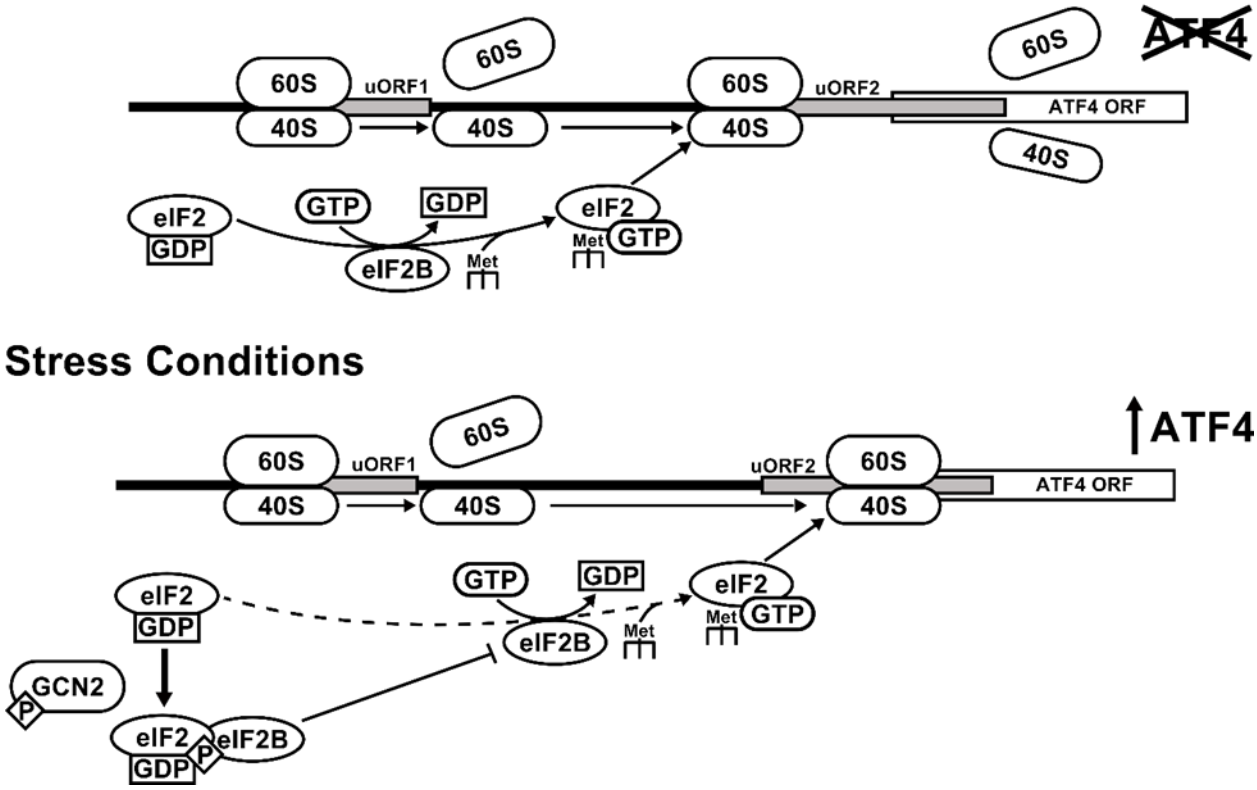

Figure 1.2. Regulation of ATF4 expression. ATF4 is regulated by the abundance of active eIF2-GTP. A small peptide is initially translated (uORF1), then the 60S subunit falls off and the 40S begins reading down the mRNA. In the presence of high eIF2-GTP, the 43S subunit is quickly regenerated, and the 60S subunit binds. The active ribosome then recognizes the start site of a dummy protein (uORF2) out of frame with the ATF4 ORF. In the presence of low eIF2-GTP, the complete ribosome takes longer to form, and the start site of the dummy protein is passed, leading to the recognition of the ATF4 translation start site. Adapted from Wek et al. (106).

Palii et al. (110) demonstrated that the GCN2 stress response pathway responds differently to deprivation of each individual essential amino acid. The increase in the proportion of eIF2α that was phosphorylated and the increase in abundance of ATF4 upon exposure of HepG2 cells to medium lacking a single essential amino acid varied widely for the different amino acids. The absence of leucine or threonine in the medium increased the phospho-eIF2α to total eIF2α ratio to 10-times the sufficient control ratio, whereas the absence of valine had no effect. ATF4 protein abundance increased many fold with a lack of methionine in the medium but was not increased at all with a lack of isoleucine. These observations suggest that each

essential amino acid, or the lack thereof, may regulate translation initiation and ISR to a very different extent, but the basis for such a broad range of responses is not clear. The degree of response could relate to the degree to which the cellular amino acid and amino acyl-tRNA pools are reduced by the lack of a single essential amino acid in the medium or diet, or the response to individual amino acid deficiencies could depend on additional signaling events or pathways besides the conventional GCN2/eIF2α-phosphorylation/ATF4 pathway.

Methionine-dependent, GCN2-independent regulation of the ISR

Recently studies have demonstrated that methionine deprivation increases the ISR through mechanisms independent of the GCN2 pathway, possibly involving responses to reduced S-adenosylmethionine or glutathione levels (111, 112). Tang et al. (111) demonstrated that, in both MCF7 breast cancer cells and PC3 prostate cancer cells, deprivation of any essential amino acid increased the ISR. However, methionine deprivation resulted in additional changes in the transcription of a variety of genes beyond those commonly induced in response to deprivation of any essential amino acid. These additional transcriptional responses to methionine deprivation were shown to correlate with a reduction of the cellular S-adenosylmethionine level, suggesting that a decrease in methylation reactions might be the link to unique transcriptional responses to methionine deprivation. The unique responses to methionine deprivation were reduced when the creatine biosynthetic pathway, a major consumer of methyl groups, was inhibited. Despite these associations, it is not clear whether the unique effects of methionine deprivation were due to S-adenosylmethionine levels, methylation reactions, or simply the change in methionine levels.

Wanders et al. (112) found that eIF2α phosphorylation remained elevated under methionine-restriction in liver of GCN2 knockout mice. The continued elevation of eIF2α phosphorylation and resulting ISR in the GCN2-null mice were shown to be due to the activation

of a different eIF2α kinase, PERK (eIF2α kinase 3). Methionine restriction was shown to result in significantly lower hepatic glutathione (GSH) levels in the GCN2-null mice. When the methionine restricted diet was supplemented with cysteine to increase GSH levels, the GCN2-independent increase in eIF2α phosphorylation was ablated. This demonstrates that a GCN2-independent increase of eIF2α phosphorylation under sulfur amino acid (methionine + cysteine) restriction can result from a decrease in GSH and subsequent oxidative stress and activation of PERK. Together, these studies suggest that methionine restriction has the ability to increase the ISR through mTORC1-and GCN2-independent mechanisms that might be dependent on reduction in cellular levels of the methionine metabolite S-adenosylmethionine, on the cysteine-containing tripeptide GSH, or on methionine itself.

GCN2-mTORC1 crosstalk

Given that both GCN2 activation and mTORC1 inhibition are involved in responses to essential amino acid deficiencies, it is reasonable to speculate that there might be crosstalk between the two signaling pathways. Indeed, there are many reports of evidence consistent with crosstalk between the GCN2 signaling cascade and mTORC1, but the molecular mechanisms have not been fully elucidated.

Anthony and coworkers (113) demonstrated that the decrease in the phosphorylation of mTORC1 targets, 4EBP1 and S6K1, in liver of wild-type mice fed a leucine-deficient diet was not observed when GCN2-null mice were fed the same diet. This suggests that GCN2 plays a role in the downregulation of mTORC1 kinase activity under conditions of leucine deprivation. In addition, MEFs exposed to the GCN2-activating compound L-asparaginase exhibited a reduction in the phosphorylation of S6K1 (114). This response did not occur in GCN2-null MEFs, further demonstrating its dependence on GCN2.

One mechanism by which GCN2 signaling may alter mTORC1 is by the ATF4-induced upregulation of expression of certain gains. Sestrin2 has been identified as a stress response protein that is induced by the eIF2α/ATF4 pathway and represents a link between GCN2 and mTORC1. Sestrin 2, regulates the GATOR complex, which in turn regulates the Rag A/B GTPase. Ye et al. (115) showed that Sestrin2 was required for the continued inactivation of mTORC1 in MEFs under long term amino acid deprivation. The effect of Sestrin2 on suppression of mTORC1 was also shown to be necessary for cell survival during glutamine deprivation. Thus, GCN2 activation under amino acid deprivation can inhibit mTORC1 activity through ATF4 induction of genes encoding proteins such as Sestrin2 that are required for mTORC1 inhibition.

Recently it was also shown that arginine and leucine deprivation inhibit mTORC1 through a GCN2-dependent, but ATF4 independent mechanism (116). The dephosphorylation of S6K1 or 4EBP1 in MEFs exposed to arginine or leucine deficient medium for 1 h was shown to be abolished when GCN2 was knocked out. In addition, mTORC1 was also shown to be insensitive to leucine deprivation when eIF2α was mutated at Ser51 and was unable to be phosphorylated. The GCN2/eIF2α phosphorylation-dependent inhibition of mTORC1, however, was shown to be independent of an increase in ATF4, and mTORC1 was still inhibited by leucine deprivation in ATF4-null MEFs. Interestingly, mTORC1 was also inhibited in GCN2-null MEFs exposed to amino acid-free medium, demonstrating GCN2 is not required for mTORC1 inhibition in response to total amino acid deprivation. Thus, GCN2/eIF2α-dependent but ATF4- independent mechanisms as well as GCN2-indepdantant mechanisms may also contribute to the inhibition of mTORC1 in response to amino acid deficiency. It is not yet clear whether these mechanisms require a deficiency of specific amino acids.

mTORC1 and GCN2 are regulators of autophagy

Under amino acid deprivation, mTORC1 and GCN2 not only act to inhibit translation initiation but also function in the regulation of macroautophagy. Macroautophagy, usually referred to as autophagy, involves the formation of a double-membrane cytoplasmic vesicle called an autophagosome. The autophagasome encloses cellular components that are subsequently degraded when the autophagosome combines with a lysosome creating an autophagolysosome (117, 118). The coordinated regulation of protein synthesis and autophagy functions to inhibit the synthesis of most proteins when amino acid pools are low while, at the same time, promoting the degradation of non-vital cellular components in order to increase the available pools of free-amino acids. Both actions contribute to a cell's ability to survive under amino acid-deficient conditions.

Autophagy is regulated by mTORC1. Among mTORC1's downstream targets are subunits of the ULK kinase complex (i.e., ULK1/2 and ATG13L subunits), VPS34-containing complexes (i.e., ATG14 subunit), and transcription factor EB (TFEB) (119). The ULK kinase complex is a serine/threonine protein kinase, and it stimulates autophagy by phosphorylating sites on subunits in the VPS34-containing complexes (i.e., Beclin1 and AMBRA1). VPS34 is a phosphatidyl inositol 3-kinase that converts membrane phosphatidyl inositol to phosphatidylinositol 3-phosphate (PtdIns3P). Increasing density of PI(3)P in the ER membrane promotes membrane expansion and development of the autophagosome (120). Under nutrient sufficient conditions, autophagy is maintained at basal levels by the inhibitory phosphorylation of ULK kinase complex and of ATG14-containing VPS34 complexes. Amino acid deprivation results in the inhibition of mTORC1 and, hence, the removal of its inhibitory effects on ULK kinase and VPS34 complexes, allowing both complexes to undergo marked activation. Activated

ULK kinase then phosphorylates AMBRA1, which translocates with VPS34, the VPS34 regulator Beclin-1 and the E3 ubiquitin protein ligase TRAF6 to the endoplasmic reticulum (ER) where TRAF6 stabilizes ULK kinase through lys-63-linked ubiquitination (121–123). ULK kinase then phosphorylates Beclin1 which promotes the activation of the kinase activity of VPS34 (124). VPS34 then phosphorylates phosphatidylinositol in the ER membrane, converting it to PtdIns3P. Generation of PtdIns3P at the site of autophagosome formation is necessary for expansion of the membrane to form a mature autophagosome.

In addition to the key roles of ULK kinase and VPS34, the suppression of TFEB phosphorylation by mTORC1 inhibition by amino acid deprivation results in the release of this transcription factor from cytoplasmic 14-3-3 binding protein that sequesters it in the cytoplasm. This release allows TFEB to move into the nucleus and upregulate the transcription of genes in the Coordinated Lysosomal Expression and Regulation (CLEAR) network (125–127). The CLEAR gene products act to increase lysosome formation and to enhance autophagy.

The activation of the GCN2/eIF2α phosphorylation/ATF4 pathway under amino acid deprivation also promotes autophagy. B'chir et al. (128, 129) studied the induction of autophagy genes and its dependence on GCN2, ATF4 and CHOP. They demonstrated that amino acid deprivation leads to a large induction of autophagy genes and that these genes are dependent on the GCN2/eIF2α phosphorylation/ATF4 signaling pathway (128). The CARE element of some of these genes was shown to require not only ATF4 but also CHOP, implying that ATF4 and CHOP form a heterodimer in order to increase expression of some autophagy genes. However, not all autophagy gene induction required CHOP, leading the authors to group the autophagy genes into three categories: (a) genes that contained a CARE and were ATF4-dependent but CHOP-independent, (b) genes that contained a CARE and were dependent on both ATF4 and CHOP

and (c) genes that did not contain a CARE and were dependent on both ATF4 and CHOP. The sequence at the site where ATF4 and CHOP bind in this third class is termed the CHOP Responsive Element (CHOP-RE). Expression of genes in all three categories were increased under leucine deprivation, but those genes where both ATF4 and CHOP bound the CHOP-RE variant of the CARE were more sensitive to leucine deprivation than the other two classes. B'chir et al, further elucidated the role CHOP has on the shift from pro-survival processes such as autophagy to pro-apoptotic processes under leucine deprivation (129). CHOP-knockout MEFs cultured in leucine-deficient medium for over 24 h were shown to have increased cell survival compared to wild-type cells and to have a delay in the production of the apoptotic marker, cleaved caspase-3. In addition, under long term leucine deprivation, CHOP- knockout MEFS demonstrated a larger increase in mRNA expression of a variety of autophagy genes compared to wild-type cells. These results demonstrate that under long term amino acid deprivation, CHOP functions to stimulate apoptosis by limiting the expression of genes required for autophagy. These studies therefore, demonstrate that the GCN2/eIF2α/ATF4 pathway can induce expression of autophagy genes, including CHOP, and that CHOP is vital in the balance between autophagy and apoptosis under long term leucine deprivation.

Regulation of autophagy by availability of single amino acids

Little is known about the effects of single essential amino acid deprivation on the regulation of autophagy, though single amino acids have been shown to have differential effects on the regulation of autophagy through mechanisms independent of mTORC1.

Single amino acids were shown to inhibit proteolysis, as measured by valine accumulation in cells exposed to cycloheximide, in isolated rat hepatocytes exposed to amino acid deprivation (130). The amino acids shown to inhibit proteolysis upon supplementation (leucine,

phenylalanine, tyrosine, glutamine, proline, histidine, tryptophan and methionine) were termed regulatory amino acids and supplementation of any one of these amino acids in amino acids deprived hepatocytes resulted in an inhibition of proteolysis, though supplementation with all of the regulatory amino acids was required for complete inhibition. It was suggested that amino acid availability inhibits autophagy by signaling through mTORC1 as Blommaart et al. (131), also working with rat hepatocytes and using valine accumulation as a marker of proteolysis, demonstrated that the relationship between S6K1 phosphorylation and the percentage that amino acid supplementation inhibits proteolysis was linear. This conclusion however was brought into doubt as Kanazawa et al. (132) demonstrated that the inhibition of proteolysis under amino acid supplementation was insensitive to rapamycin exposure, and that when leucine, tyrosine or glutamine were individually supplemented to amino acid deprived rat hepatocytes, inhibition of proteolysis and increase in S6K1 phosphorylation were not well correlated. Kanazawa et al. suggested that the differences between their results and those previously obtained by Blommaart et al. were due to the long exposure of the cells to cycloheximide that could result in an inhibition of autophagosome formation. These results, in addition to the previously discussed sections on mTORC1 regulation by specific amino acids, suggest that proteolysis can be regulated by amino acids both in an mTORC1-dependent and -independent manner and that this regulation could be amino acid specific.

Recent work by Angcajas et al. (134) also demonstrated that single amino acids can regulate autophagy as supplementation of amino acid deprived H4-II-E rat hepatoma cells with methionine, proline, arginine, cysteine, glutamate, or isoleucine inhibited autophagy as measured by the ratio of active to precursor of the microtubule-associated protein light chain 3 (LC3 ratio). Arginine supplementation was shown to be being particularly effective in inhibiting autophagy.

This inhibition was shown to be mTORC1-independent but dependent on intracellular nitric oxide (NO) as when NO synthesis was inhibited arginine supplementation was shown to have no effect on autophagy. This result was also confirmed in the human carcinoma cell line HepG2.

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CHAPTER 2

GCN2- AND EIF2α-PHOSPHORYLATION-INDEPENDENT, BUT ATF4-DEPENDENT, INDUCTION OF CARE-CONTAINING GENES IN METHIONINE-DEFICIENT CELLS

Introduction

The regulation of gene expression in response to changes in the nutritional environment, including the availability of amino acids, has been well documented in recent decades (reviewed in Baird and Wek 2012). The major signaling pathway for the regulation of mammalian gene expression in response to amino acid availability involves the alpha subunit of eukaryotic initiation factor 2 (eIF2α) kinase pathway. GCN2 (eIF2α kinase 4) is one of four mammalian eIF2α kinases (2), and GCN2 is specifically activated by "uncharged" tRNA accumulation during amino acid starvation (3–12). The lack of any essential amino acid can result in GCN2 activation and large changes in gene expression (13, 14). Other types of cellular stresses activate the other three eIF2α kinases, also leading to large changes in gene expression (2, 15, 16). Because different types of stress converge at the level of phosphorylated eIF2α, the eIF2α kinase-mediated response is often called the integrated stress response (1).

Eukaryotic initiation factor 2 (eIF2), which is a trimer of α, β, and γ subunits, is required for the initiation of mRNA translation (17, 18). It mediates the binding of the charged initiator tRNA (Met-$tRNA_i^{Met}$) to the ribosome in a GTP-dependent manner by formation of the ternary complex (eIF2-GTP-Met-$tRNA_i^{Met}$). Upon base pairing of the AUG start codon with Met-$tRNA_i^{Met}$, the GTP is hydrolyzed to GDP and the eIF2-GDP is released (17). For the eIF2 that is released to be used for a new round of translation initiation, the bound GDP must be exchanged for GTP by action of the guanine nucleotide exchange factor, eukaryotic initiation factor 2B

(eIF2B) (19–21).

The activation of any one of the four mammalian eIF2α kinases results in the phosphorylation of eIF2α (22). When eIF2α is phosphorylated, it binds tightly to eIF2B, which inhibits the guanine nucleotide exchange factor and prevents the formation of new eIF2-GTP (23, 24). Because eIF2-GTP is required for formation of new a ternary complex, the phosphorylation of eIF2α impacts global mRNA translation initiation (Anthony et al. 2001; Krishnamoorthy and Pavitt 2001; Wek et al. 2006; Baird and Wek 2012). Paradoxically, the lack of sufficient ternary complex, and hence 43S preinitiation complex, also drives an increase in the translation of activating transcription factor 4 (ATF4), a transcription factor that plays a key role in initiating the integrated stress response (1, 14). ATF4 mRNA contains upstream inhibitory open reading frames, and under normal conditions, translation is initiated at a short upstream open reading frame that encodes a small peptide. After translation of this short region of mRNA, the 60S subunit falls off and the 40S continues to slide down the mRNA searching for a new start site. Under nutrient-sufficient conditions with abundant eIF2-GTP, reformation of the ternary complex with Met-tRNA$_i^{Met}$ occurs readily, and this allows reinitiation at a subsequent start codon that is still upstream of the ATF4 start codon but has an open reading frame that overlaps (but is out-of-frame with) the open reading frame for ATF4. This results in translation of a functionally inert protein and bypass of the ATF4 start site. However, under stress conditions in which the reformation of eIF2-GTP is inhibited and the abundance of ternary complex is low, the 40S subunit is more likely to bypass the start site for the dummy protein before the 43S preinitiation complex is reformed, facilitating engagement with the downstream ATF4 start site and translation of ATF4 (1, 14, 22, 26).

When translationally induced, the transcription factor ATF4 enters the nucleus where it

forms heterodimers with CCAAT-enhancer-binding proteins (C/EBP) (14). The heterodimers recognize the consensus sequence of "TGATGXAAX" in gene promoter regions. This sequence is termed the CARE (C/EBP-ATF response element), and C/EBP-ATF4 binding results in the upregulation of genes essential for the protection of cells in a variety of stress conditions (14, 27, 28). These genes include the cationic amino acid transporter (CAT-1), system A neutral amino acid transporter 2 (SNAT2), asparagine synthetase (ASNS), eukaryotic initiator 4E binding protein 1 (4EBP1) and genes that function in the stimulation of autophagy, as well as ATF4 itself (14, 27, 29–32).

Despite the common role of eIF2α kinases and ATF4 in the integrated stress response, gene expression in response to various cellular stresses is not identical, suggesting the existence of additional signaling pathways that respond to the various types of stress (33). Even with similar stresses, such as the lack of one essential amino acid versus another essential amino acid, there can be a high degree of non-overlap of differentially regulated genes (34) or a high degree of variation in the extent of up- or down-regulation (35). Deval et al. (36) investigated the relative roles of GCN2 and mTORC1 in the differential expression of genes in response to leucine starvation and concluded that the GCN2 pathway accounted for the major, but not the total, response of gene transcription to leucine deprivation. Wanders et al. (37) recently demonstrated that GCN2-null mice were still able to respond to sulfur amino acid deprivation by eIF2α phosphorylation. Inclusion of cysteine in the diet prevented this response, and they attributed it to glutathione deficiency and oxidative stress that activated the PKR-like endoplasmic reticulum kinase (PERK, or eIF2α kinase 3).

To further explore the regulation of gene expression in response to amino acid deprivation, we deprived cells of different single amino acids and then looked at the effect of

individual amino acid deprivation on ATF4-induced changes in mRNA levels of CARE-containing genes. We demonstrate the presence of a novel GCN2/eIF2α phosphorylation-independent signaling pathway that upregulates expression of CARE-containing genes and that is particularly sensitive to methionine deprivation.

Experimental Procedures

Cell Culture

Wild-type (GCN2+/+) and GCN2(-/-) MEFs immortalized with SV40 Large T antigen were provided by Dr. David Ron (Cambridge University, Cambridge, UK). Knock-in loss-of-function eIF2α mutant (eIF2α (A/A)) and isogenic wild-type (eIF2α (S/S)) cells were supplied by Dr. Randal Kaufman (Sanford-Burnham Medical Research Institute, La Jolla, CA, USA). Wild-type, GCN2(-/-) and eIF2α(A/A) MEFs were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM), pH 7.4, containing 1 mM pyruvate and 4 mM glutamine and supplemented with 1X non-essential amino acid mix (Gibco/Invitrogen), 100 units/mL penicillin and 100 µg/mL streptomycin (pen/strep; Gibco/Invitrogen) and 10% (v/v) fetal bovine serum (FBS, Hyclone). Cells were maintained at 37°C in an atmosphere of 95% air and 5% CO_2. Experimental control medium was the same as normal growth medium with the following changes: medium was prepared using custom DMEM that lacked sulfur amino acids, histidine, leucine and arginine (prepared by Gibco/Invitrogen); medium was supplemented by us with 0.4 mM L-cysteine, 0.2 mM L-methionine, 0.2 mM L-histidine, 0.8 mM L-leucine and 0.4 mM L-arginine; and 10% dialyzed FBS (Hyclone) was used in place of standard FBS. Histidine-deficient (–His), methionine-deficient (–Met), leucine-deficient (–Leu) and arginine-deficient (–Arg) media were prepared similarly except the respective amino acids were not added back to the deficient DMEM. Cells were grown to 60-70% confluence before exposure to treatment

media in order to ensure logarithmic growth throughout the experiment. Cells were collected for qRT-PCR and western blotting as described below.

RNA Isolation and qRT-PCR

Cells were exposed to amino acid-deficient treatment medium for 12 h. Cells were collected in RNeasy lysis buffer (Qiagen) supplemented with 10% (v/v) β-mercaptoethanol. Cells were lysed by centrifugation through a QIAshredder (Qiagen), and RNA was isolated using an RNeasy isolation column (Qiagen) per the manufacturer's instructions. For cDNA synthesis, 1 µg RNA was reverse transcribed using the High-Capacity Reverse Transcription kit (Applied Biosystems) per the manufacturer's instructions. For qRT-PCR, cDNA was diluted in ultrapure H_2O to a concentration of 4 ng/µL. Then 7.5 µL of the cDNA mixture was combined with 37.5 µL 2X Power SYBR green master mix (Applied Biosystems) and the desired amount of primers and H_2O to make 75 µL of complete PCR mix. Final primer concentrations in the 75 µL complete pCR mix were as follows: 300 nM forward and reverse primers for 4EBP1, ATF4 and β-tubulin; 50 nM forward and 50 nM reverse primers for 4EBP2; 50 nM forward and 300 nM reverse primers for ASNS; and 300 nM forward and 50 nM reverse primers for CHOP. Forward and reverse primer sequences were as follows: 4EBP1 forward 5′-GAAGAGCCTCCCATGCAA-3′ reverse 5′-CCATCTCAAATTGTGACTCTTCA-3′; ATF4 forward 5′-ACAGGAAGCATGCAGTTGG-3′ reverse 5′-AGGTGCACACAGGCTGCT-3′; β-tubulin forward 5′-GCTGGACCGAATCTGTGTGT-3′ reverse 5′-GACCTGAGCGAACGGAGTC-3′; 4EBP2 forward 5′-ACAGGAAGCATGCAGTTGG-3′ reverse 5′-AGGTGCACACAGGCTGCT-3′; ASNS forward 5′-GGCTGTGTGTTCAGAAGCT-3′ reverse 5′-AAGGAAGGGCTCCACTTT-3′; CHOP forward 5′-CATACACCACCACACCTGAAAGCA-3′ reverse 5′-GGTGAAAGGCAGGGACTCAGCT-3′.

A 20 µL aliquot of the complete qPCR mix was added to each well of a Roche 480 96-well PCR plate such that each well contained 8 ng of cDNA, with triplicate wells being run for each sample. qPCR was done in a Roche 480 Lightcycler with polymerase activation at 95°C for 10 min followed by denaturation at 95°C for 15s and annealing/elongation at 60°C for 60s. The denaturation and annealing/elongation steps were repeated for a total of 35 cycles. Fluorescence was measured at the end of each annealing/elongation cycle. Following qPCR, melting curves were acquired by a stepwise increase of the temperature from 60°C to 95°C to confirm that a single product had been amplified in the reaction. Fold differences of 4EBP1, 4EBP2, ATF4, ASNS and CHOP mRNA were determined using the ΔΔCp method. The β-tubulin mRNA level was used to normalize values within an experiment, and differences in mRNA levels were calculated as fold the average normalized value for wild-type cells cultured in complete medium.

Protein Isolation and Western Blotting

Cells were exposed to amino acid-deficient or control treatment medium for 24 h, with medium being replaced with fresh treatment medium at 12 h. Cells were collected in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5%, v/v, Nonidet NP-40) supplemented with Complete protease inhibitor and PhosSTOP phosphatase inhibitor "cocktails" (Roche). Homogenates were then centrifuged at 14,000 x g for 20 min, and the supernatants were collected. Protein concentrations of the sample supernatants were determined by the bicinchonic acid assay (BCA Protein Assay Kit, Thermo Scientific Pierce), and 30 µg protein per lane were resolved by SDS/PAGE using a 12% (w/v) polyacrylamide gel. Proteins were transferred to a 0.45-µm Immobilon PVDF membrane (Millipore Corp.), and the membrane was incubated for 1 h at room temperature with blocking buffer for near infrared fluorescent westerns (Rockland or Odyssey blocking buffer). The membrane was then incubated with antibodies to 4EBP1 (1:1000,

Cell Signaling), ATF4 (1:1000, Cell Signaling) or β-tubulin (1:1000, Santa Cruz Biotech) at 4°C overnight. Membranes were washed for 30 min in TTBS (1x Tris-buffered saline, pH 7.6, + 0.1% ,v/v, Tween 20) and then exposed to secondary antibodies conjugated to AlexaFluor 680 or IRDye 800 (1:20,000, Li-COR Biosciences) for 1 h at room temperature. Membranes were again washed for 30 min with TTBS and rinsed in PBS (pH 7.4), and the proteins were visualized and quantified using an Odyssey infrared imagining system (Li-COR Biosciences) and Odyssey software. The relative intensities of the bands of interest were normalized to β-tubulin.

ATF4 siRNA Knockdown

A siRNA oligonucleotide targeting murine ATF4 mRNA (Flexitube siRNA Mm_ATF4_1) and a nonsense negative control (AllStars Negative Control siRNA) were purchased from Qiagen. Cells were transfected using the reverse transcription method. Cells were grown in standard growth medium prepared without pen/strep until they were 80% confluent. The cells were then trypsinized and diluted in fresh growth medium without pen/strep so they would yield a confluence of 50-60% when plated. The siRNA and Lipofectamine-2000 (Invitrogen) were both diluted in optiMEM (Gibco/Invitrogen) and combined according to the manufacturer's protocol. The cell solution was combined with the siRNA-Lipofectamine-optiMEM solution to produce a final siRNA concentration of 33 nM. The cell/transfection solution was mixed and plated as described by the manufacturer (Invitrogen). After 6 h the transfection medium was removed and replaced with control treatment medium. GCN2(-/-) and eIF2α(A/A) MEFs were allowed to recover in control medium for 12 h and 18 h, respectively, at which time they had returned to a logarithmic growth rate; transfected wild-type (control) MEFs were treated similarly. After recovery, cells were cultured in amino acid-deficient medium and

harvested for qRT-PCR and western blot analysis as described above except that cells were exposed to amino acid deficient medium for 12 h instead of 24 h for protein abundance measurements in order to collect cells during logarithmic growth.

Statistical Analysis

All experiments were repeated at least three times with triplicate wells of cells within each experiment. Results were expressed as fold of the mean for the same cell type cultured in control medium. Data were $\log_{10}$ transformed and analyzed using ANOVA and Student's t-test; differences were accepted at $p \leq 0.05$.

Results

The GCN2(-/-) and eIF2a(A/A) MEFs used in these experiments have been characterized previously (38, 39). We confirmed the lack of eIF2a phosphorylation in response to amino acid deprivation in these cell lines in a preliminary experiment. Culture of cells in leucine-deficient medium for 3 to 10 h resulted in marked phosphorylation of eIF2α Ser51 in MEFs from GCN2(+/+) mice but not in MEFs from GCN2(-/-) mice. Similarly, culture of cells in leucine-deficient medium for 3 to 12 h resulted in marked phosphorylation of eIF2α Ser51 in eIF2α(S/S) cells but not in isogenic eIF2α(A/A) knockin cells. Furthermore, transfection of GCN2(-/-) cells with a GCN2 expression plasmid restored the ability of the GCN2(-/-) cells to increase phosphorylation of eIF2α in response to leucine deprivation. Thus, the cell lines used in the studies reported here exhibit the expected phenotype for cells lacking GCN2 or a phosphorylatable eIF2α.

As the GCN2/eIF2α kinase pathway signals via upregulation of both ATF4 translation and a subsequent increase in *Atf4* gene transcription, we first explored the effect of amino acid

deficiency on ATF4 mRNA levels. As expected, ATF4 mRNA levels were significantly increased in GCN2(+/+) MEFs when these cells were cultured in medium deficient in either histidine, leucine, arginine or methionine (Fig. 2.1a). Also, as expected, arginine and leucine deprivation did not result in an increase in ATF4 mRNA in GCN2(-/-) MEFs (Fig. 2.1b). However, though reduced compared to GCN2(+/+) cells, a significant increase in ATF4 mRNA was still observed in GCN2(-/-) MEFS exposed to histidine and methionine deficient medium compared to cells grown in sufficient medium. GCN2 knockout had the least effect on the response of MEFs to methionine deficient medium such that ATF4 mRNA levels in GCN2(-/-) cells cultured in methionine-deficient medium were still 3-fold those of cells cultured in sufficient medium.

In order to determine if the increase in ATF4 expression in GCN2(-/-) cells subjected to essential amino acid deficiency was the result of activation of an eIF2α kinase other than GCN2, nonphosphorylatable eIF2α(A/A) MEFs were used (Fig. 2.1c, d). As observed in GCN2(+/+) MEFs, ATF4 mRNA levels were also upregulated in control eIF2α(S/S) MEFs under essential amino acid deprivation. Less effect of amino acid deprivation on ATF4 mRNA levels were obtained in the eIF2α(A/A) cells. Though reduced compared to eIF2α(S/S) cells, methionine deprivation still resulted in a relatively large increase in ATF4 mRNA levels, and arginine and histidine deprivation resulted in smaller but significant increases, in the eIF2α(A/A) cells. However, leucine deprivation did not result in a significant increase in ATF4 mRNA levels in eIF2α(A/A) cells. These results suggest that the upregulation of ATF4 expression in MEFs in response to essential amino acid deprivation does not strictly require activation of GCN2 or any other eIF2α kinase. However, as the fold increase of ATF4 mRNA was much lower in GCN2(-/-) and eIF2α(A/A) MEFs than in wild-type MEFs, the GCN2-eIF2α kinase signaling pathway

clearly does play a major role in ATF4 mRNA induction in GCN2(+/+) and eIF2α(S/S) MEFs. Methionine deprivation appears to be the strongest activator of the GCN2-/eIF2α phosphorylation-independent pathway leading to increased ATF4 mRNA, whereas ATF4 mRNA induction due to leucine deprivation appears to be totally dependent on GCN2-catalyzed eIF2α phosphorylation.

Past studies in our lab have demonstrated an increase in 4EBP1 expression (mRNA and protein) in liver of rats when they were fed diets that were limiting in sulfur amino acids (40). Therefore, we were interested in determining if the increases in ATF4 mRNA induction independent of the GCN2-eIF2α kinase signaling pathway resulted in increases in 4EBP1 mRNA and protein. As shown in Figure 2.2a, histidine, methionine, arginine or leucine deprivation each resulted in a significant increase in the 4EBP1 mRNA level in GCN2(+/+) MEFS. Leucine deprivation resulted in significantly less upregulation than did deprivation of histidine, methionine or arginine, which all had similar effects. In GCN2(-/-) MEFs (Fig. 2.2b) arginine deprivation resulted in a minimal increase in 4EBP1 mRNA levels, and histidine or leucine derivation resulted in no increase. In contrast, methionine deprivation resulted in a 2.6-fold increase in 4EBP1 mRNA, which was substantial though lower than the 5.2-fold increase observed in GCN2(+/+) MEFs. The results observed for GCN2(+/+) and GCN2(-/-) MEFs were confirmed with eIF2α(S/S) and eIF2α(A/A) MEFs as shown in Figure 2.2c, d. Histidine, arginine, leucine or methionine deprivation all resulted in increased 4EBP1 mRNA in eIF2α(S/S) MEFs, whereas only methionine deficiency resulted in an increase in 4EBP1 mRNA in eIF2α(A/A) MEFs. These results demonstrate that *4Ebp1* gene expression, like *Atf4* gene expression, is regulated in both a GCN2-/eIF2α phosphorylation-dependent and -independent manner. 4EBP1 protein levels were measured in cells cultured in methionine- or histidine-

deficient medium (Fig. 2.2e, f). In GCN2(+/+) and eIF2α(S/S) control MEFs, both histidine- and methionine-deficient medium resulted in a significant increase in 4EBP1 protein abundance. In GCN2(-/-) and eIF2α(A/A) MEFs, 4EBP1 protein levels were not affected by culture in histidine-deficient medium but were still significantly upregulated by culture in methionine-deficient medium, consistent with observations for 4EBP1 mRNA.

As ATF4 induces the ISR by increasing transcription of genes containing a CARE in their promoter region (14), we next aimed to determine if other genes that are known to contain CAREs and to be upregulated by GCN2 and other eIF2α kinase pathways would also be upregulated in an amino acid-specific and GCN2/eIF2α-independent manner. Therefore, we measured the changes in the mRNA levels for two other CARE-containing genes, CHOP and ASNS (Fig. 2.3a, b, c, d). CHOP and ASNS mRNAs were significantly upregulated in GCN2(+/+) and eIF2α(S/S) MEFs exposed to histidine- or methionine-deficient medium. In GCN2(-/-) and in eIF2α(A/A) MEFs, CHOP and ASNS mRNAs still showed a large, through reduced compared to results for GCN2(+/+) and eIF2α(S/S) MEFs, upregulation in response to methionine-deficient medium. GCN2(-/-) and in eIF2α(A/A) cells grown in histidine-deficient medium showed little to no upregulation of ASNS or CHOP mRNA. Thus, the GCN2-/eIF2α-phosphorylation-independent response appears to upregulate CARE-containing genes in addition to 4EBP1 & ATF4, suggesting the response might also target CARE-containing genes.

4EBP1 and 4EBP2 are homologous proteins with their genes having similar intron/exon structures with 3 exons, and 4EBP2, like 4EBP1, is expressed in most tissues (41). Whereas the *4Ebp1* promoter region, as discussed above, contains two CARE elements located 873 and 899 base pairs upstream of the transcription start site (14), a search of the *4Ebp2* gene from 1000 base pairs upstream to 1000 base pairs downstream of the transcription start site did not reveal

the presence of a CARE. Therefore, we would not expect 4EBP2 abundance to be upregulated by the GCN2/ATF4 pathway. This hypothesis was confirmed in Figure 2.3a, b, c, d. In GCN2(+/+) MEFs both methionine and histidine deprivation resulted in a minimal increase in 4EBP2 mRNA. In all other cell types only methionine deprivation resulted in a small increase in 4EBP2 mRNA. The increase in 4EBP2 mRNA in GCN2(+/+) or eIF2α(S/S) cells was not on the scale of those observed for CHOP, ASNS or 4EBP1 mRNA levels, leading us to conclude that amino acid deprivation has a minimal effect on 4EBP2 mRNA induction. Therefore, 4EBP2 mRNA levels were used as a representative mRNA for non-CARE-containing genes for the remaining experiments

Because the accepted GCN2 signaling pathway, when activated, results in an increase in ATF4 mRNA and because both methionine and histidine deprivation in GCN2(-/-) and eIF2α(A/A) MEFs also resulted in a significant upregulation of ATF4 mRNA, we hypothesized that the GCN2/eIF2α kinase pathway and the GCN2-/eIF2α phosphorylation-independent pathway merge at the level of ATF4. In order to determine if ATF4 induction is essential for the GCN2-/eIF2α phosphorylation-independent upregulation of CARE containing gene expression, ATF4 was knocked down using ATF4-specific siRNA. The ATF4 siRNA knockdown was successful with ATF4 mRNA levels being significantly lower in cells treated with ATF4 siRNA compared to the same cell type treated with nonsense (control) siRNA (Fig. 2.4 a, b, c, d). Because ATF4 is an essential transcription factor for other cellular processes, we did not attempt to knockdown ATF4 completely but to prevent an increase in ATF4 expression to a level that would inhibit initiation the ISR. As can be seen in Figure 2.4, MEFs transfected with nonsense siRNA and hence expressing ATF4 responded normally to either histidine- or methionine-deficiency with an increase in ATF4 mRNA. Consistent with the results shown in Figure 2.1,

amino acid deficiency was less effective at inducing ATF4 mRNA in the GCN2(-/-) and eIF2α(A/A) MEFs treated with nonsense siRNA than in the GCN2(+/+) and eIF2α(S/S) MEFs. Also consistent with the results shown in Figure 2.1, methionine deprivation resulted in a much greater increase in ATF4 mRNA than did histidine deprivation in the GCN2(-/-) and eIF2α(A/A) MEFs treated with nonsense siRNA. Thus, the control nonsense siRNA transfected cells for this experiment behaved similarly to cells that were not transfected with siRNA.

In contrast, when ATF4 was knocked down using siRNA, the upregulation in ATF4 mRNA observed during histidine or methionine deprivation was dramatically reduced in all cell types. As can be seen in Figure 2.4, ATF4 knockdown effectively blocked the increase in ATF4 mRNA levels that occurred in cells treated with nonsense siRNA. This was true for all four cell types: GCN2(+/+), GCN2(-/-), eIF2α(S/S) and eIF2α(A/A) MEFs. In fact, the ATF4 mRNA levels in GCN2(-/-), eIF2α(S/S) and eIF2α(A/A) MEFs transfected with ATF4 siRNA and cultured in amino acid deficient medium were the same or lower than levels in cells transfected with nonsense siRNA and cultured in sufficient medium (Fig 2.4 b,c,d), and those in GCN2(+/+) cells cultured in amino acid deficient medium were only slightly higher than the control levels (Fig 2.4a). Therefore, we can conclude that the ATF4 siRNA knockdown was effective at reducing both the GCN2-/eIF2α phosphorylation-dependent and -independent ATF4 mRNA induction due to amino acid deprivation.

4EBP1 and ASNS mRNA levels were measured to determine if expression of CARE-containing genes responds to knockdown of ATF4 (Fig. 2.4 a, b, c, d). ATF4 knockdown in cells exposed to sufficient medium had no effect on 4EBP1 mRNA levels, regardless of cell type, suggesting that basal ATF4 levels were not high enough to affect CARE-containing gene transcription under amino acid-sufficient conditions. ASNS mRNA however was slightly

reduced by ATF4 knockdown in GCN2(-/-) and eIF2α(S/S) MEFs grown in sufficient medium, suggesting some ATF4-mediated gene transcription in these mutant cells even under amino acid sufficient conditions. However, in GCN2(+/+) and eIF2α(S/S) MEFs subjected to histidine or methionine deprivation, the upregulation of 4EBP1 and ASNS mRNA were significantly reduced when ATF4 was knocked down, confirming the necessity of ATF4 for the induction of CARE-containing genes in response to amino acid deprivation. In GCN2(-/-) and eIF2α(A/A) MEFs the knockdown of ATF4 also significantly reduced the upregulation of 4EBP1 and ASNS under methionine deprivation, as well as reducing the smaller increase of ASNS mRNA levels in GCN2(-/-) MEFs under histidine deprivation. These results confirm that the GCN2-/eIF2α phosphorylation-independent pathway, like the GCN2-/eIF2α kinase-dependent pathway, is dependent on ATF4 for the induction of CARE-containing genes

4EBP2 mRNA levels were also measured to determine the effect ATF4 knockdown had on non-CARE containing genes (Fig. 2.4a, b, c, d). Consistent with the results shown in Figure 2.3, histidine and methionine deprivation resulted in minimal changes in 4EBP2 mRNA levels in all cell types. Though significantly different than control cells, these changes were not on the scale of the changes observed for 4EBP1 and ASNS mRNAs. As expected due to the absence of a CARE, ATF4 knockdown had minimal effect on 4EBP2 induction. Therefore, the small increases in 4EBP2 mRNA levels might be due to activation of other stress signaling pathways under amino acid deprivation conditions.

In total these results demonstrate that upregulation of ATF4 is an essential component of both the GCN2/eIF2α kinase-dependent pathway and the GCN2-/eIF2α phosphorylation-independent pathway for upregulation of expression of CARE-containing genes.

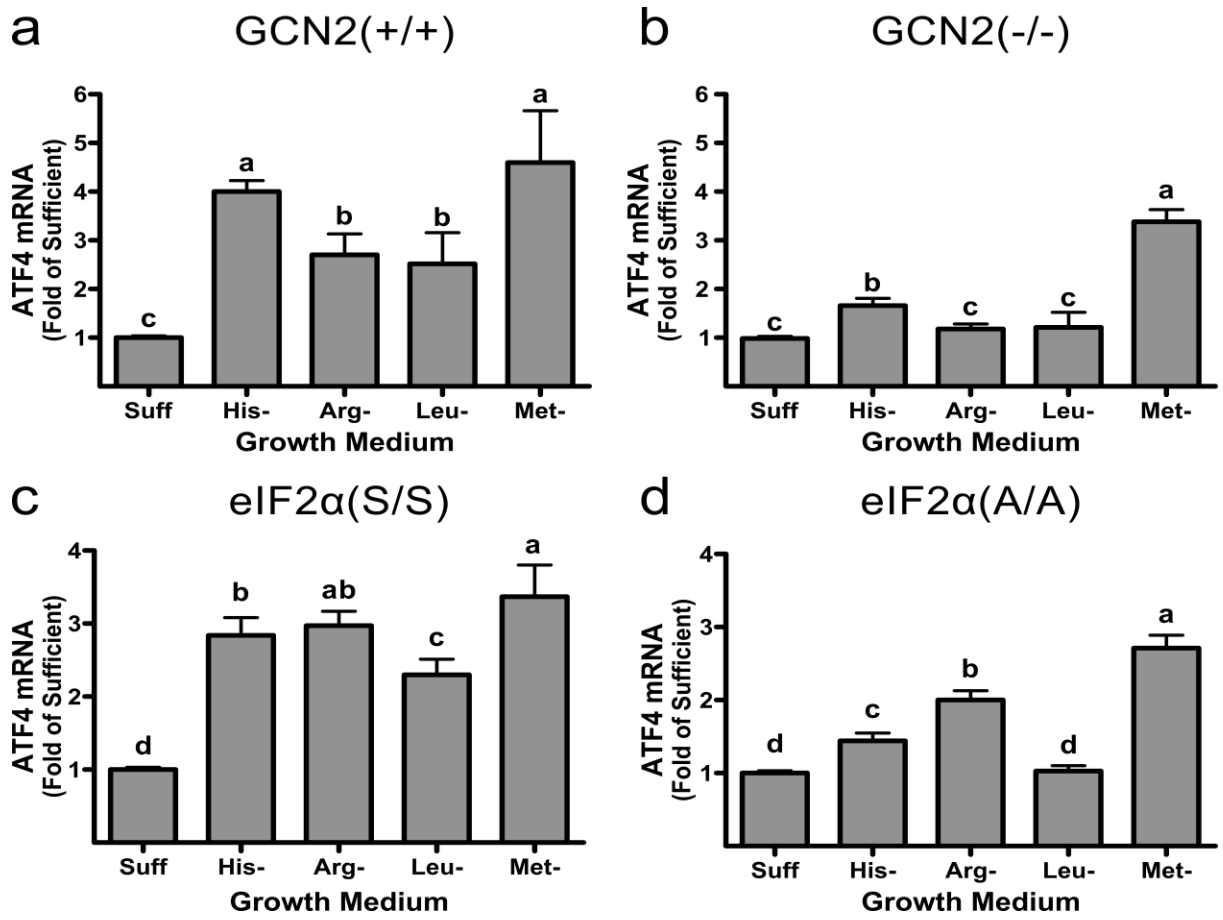

Figure 2.1. Upregulation of ATF4 mRNA in GCN2(+/+), GCN2(-/-), eIF2α(S/S) and eIF2α(A/A) MEFs in response to essential amino acid deficiency. Cells were grown in sufficient medium (Suff) or in medium deficient in histidine (His-), methionine (Met-), leucine (Leu-) or Arginine (Arg-). ATF4 mRNA levels after 12 h of culture in deficient medium, expressed as fold the mean value for cells cultured in sufficient medium, are shown for a) GCN2(+/+), b) GCN2(-/-), c) eIF2α(S/S) and d) eIF2α(A/A) MEFs. Values are means ± SEM for 3 separate experiments, with each experiment having 3 replicates. Bars not denoted by the same letter are significantly different by Student's t-test comparison at $p \leq 0.05$.

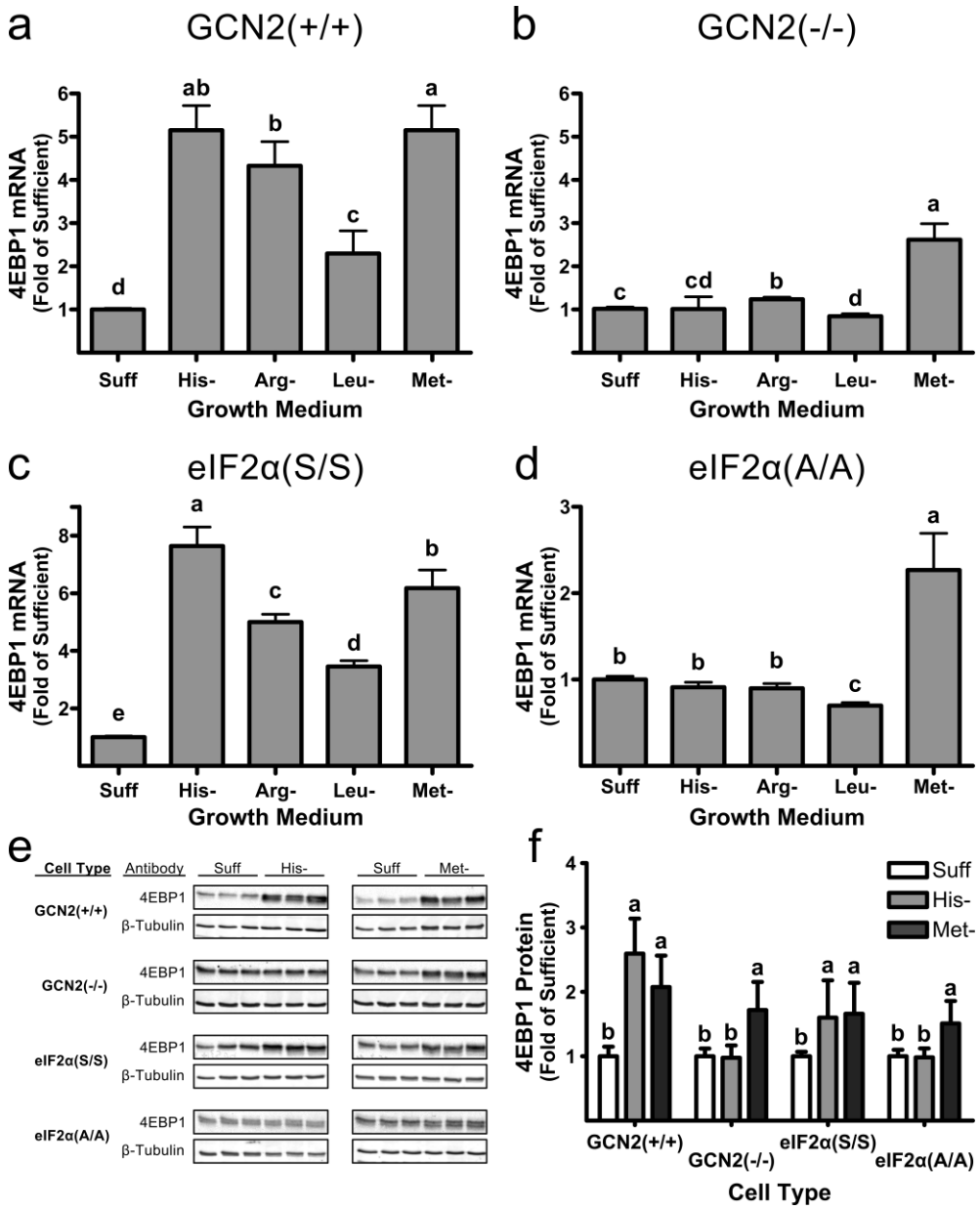

Figure 2.2. Upregulation of 4EBP1 mRNA and protein levels in GCN2(+/+), GCN2(-/-), eIF2α(S/S) and eIF2α(A/A) MEFs in response to essential amino acid deficiency. Cells were grown in sufficient medium (Suff) or in medium deficient in histidine (His-) methionine (Met-), leucine (Leu-) or arginine (Arg-). a-d) ATF4 mRNA levels after 12 h of culture in deficient medium, expressed as fold the mean value for cells cultured in complete medium, are shown for experiments in a) GCN2(+/+), b) GCN2(-/-), c) eIF2α(S/S) and d) eIF2α(A/A) MEFs. e,f) 4EBP1 protein abundance in cells after 24 h of culture, shown as e) representative western blots and f) bar graph of the abundance of 4EBP1 protein, expressed as fold of the level in the same cell type cultured in sufficient medium after normalizing values to β-tubulin. All values for mRNA and protein are means ± SEM for 3 separate experiments, with each experiment having 3 replicates. Bars not denoted by the same letter are significantly different by Student's t-test comparison at $p \leq 0.05$.

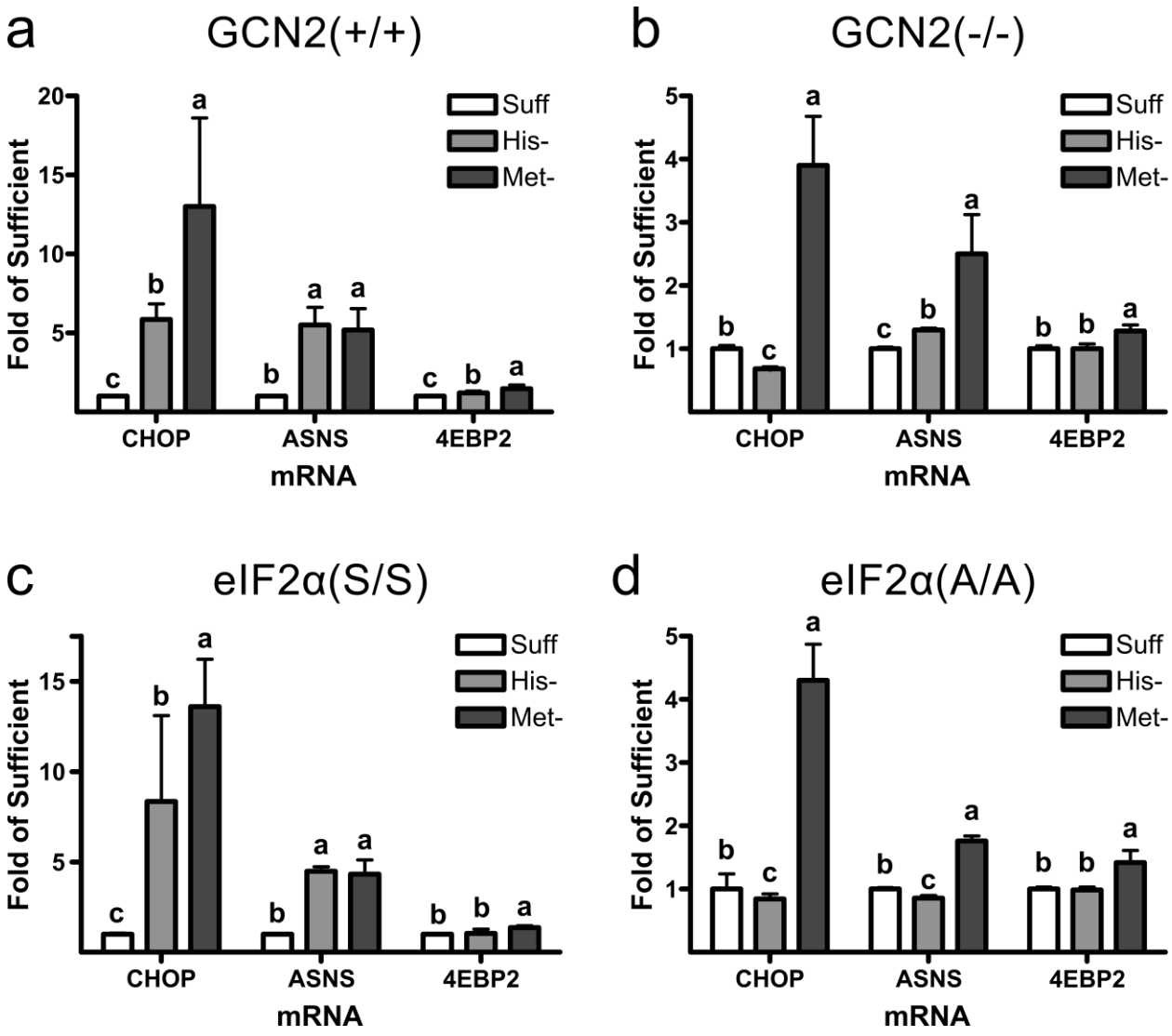

Figure 2.3. Effect of deprivation of histidine (-His) or methionine (-Met) on expression of genes with or without a C/EBP-ATF4 response element (CARE). GCN2(+/+), GCN2(-/-), eIF2α(S/S) and eIF2α(A/A) MEFs were grown in sufficient medium (Suff) or in medium deficient in histidine (His-) or methionine (Met-). CHOP, ASNS or 4EBP2 mRNA levels after 12 h of culture in deficient medium, expressed as fold the mean value for cells cultured in sufficient medium, are shown for a) GCN2(+/+), b) GCN2(-/-), c) eIF2α(S/S) and d) eIF2α(A/A) MEFs. Values are means ± SEM for 3 separate experiments, with each experiment having 3 replicates. Bars not denoted by the same letter are significantly different by Student's t-test comparison at $p \leq 0.05$.

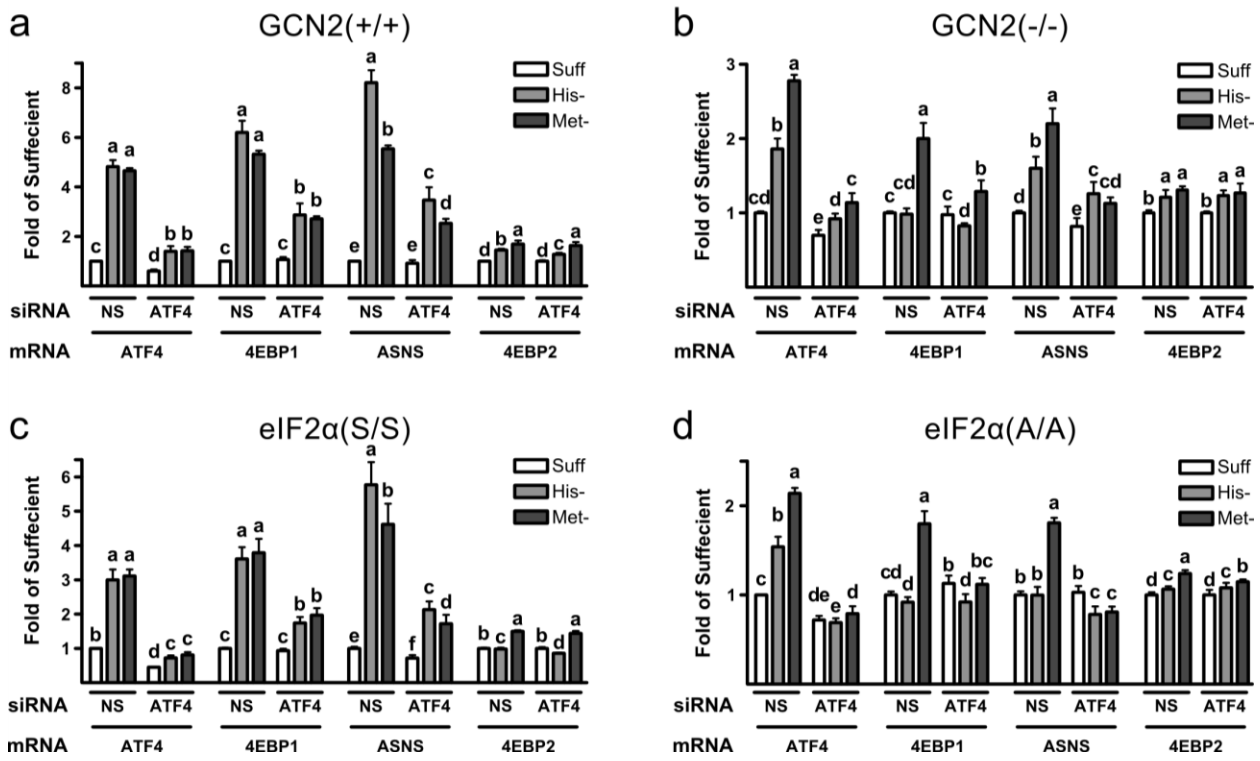

Figure 2.4. Effect of knockdown of ATF4 in GCN2(+/+), GCN2(-/-), eIF2α(S/S) and eIF2α(A/A) MEFs on expression of ATF4, 4EBP1, ASNS and 4EBP2. Nonsense (NS, control) or ATF4 siRNA was transfected into GCN2(+/+), GCN2(-/-), eIF2α(S/S) and eIF2α(A/A) MEFs. After siRNA transfection, MEFs were cultured in sufficient medium (Suff) or in medium deficient in histidine (His-) or methionine (Met-) for 12h. ATF4, 4EBP1, ASNS and 4EBP2 mRNA levels after 12 h of culture in deficient medium after transfection of NS or ATF4 siRNA, expressed as fold the mean value for cells transfected with NS siRNA and cultured in sufficient medium, are shown for a) GCN2(+/+), b) GCN2(-/-), c) eIF2α(S/S) and d) eIF2α(A/A) MEFs. Values are means ± SEM for 3 separate experiments, with each experiment having 3 replicates. Bars not denoted by the same letter are significantly different by Student's t-test comparison at $p \leq 0.05$.

Discussion

Our results confirm that transcriptional responses to amino acid deprivation occur predominantly, but not totally, by the canonical GCN2/eIF2α/ATF4 pathway. GCN2 is well-known to be a sensor of amino acid deficient conditions in cells via the binding of uncharged tRNAs to a regulatory domain of GCN2, causing activation of GCN2's kinase domain. In turn, GCN2 phosphorylates the alpha subunit of eIF2 at Ser51, and this leads to depletion of eIF2-GTP and a lack of ternary complex formation (1). Although global protein synthesis is negatively impacted by the lack of ternary complex, the translation of ATF4 is promoted. ATF4, in turn, upregulates the transcription of various stress response genes that contain elements that bind C/EBP-ATF4 heterodimers, resulting in elevated expression of particular proteins that facilitate the cell's response to lack of amino acids or other stresses.

The mRNA levels for *4EBP1*, *ASNS*, *CHOP* and *ATF4* itself, all of which contain CAREs in their promoter regions, were upregulated in wild-type control cells by deprivation of any one of the four amino acids tested (histidine, arginine, leucine or methionine). Furthermore, the upregulation of mRNAs for the four genes was markedly reduced and in many cases eliminated by knockout of GCN2, mutation of the Ser51 residue of eIF2α to Ala, or knockdown of ATF4. The effects of GCN2 knockout and of eIF2α mutation were generally very similar, indicating that amino acid starvation induces eIF2α phosphorylation almost entirely via activation of GCN2 and not via activation of another eIF2α kinase. These results support the predominant role of the GCN2/eIF2α kinase/ATF4 pathway in mediating the cell's transcriptional responses to amino acid insufficiency, even of one amino acid. However, our results also clearly show that knockout of GCN2 or mutation of Ser51 of eIF2α to a residue that cannot undergo phosphorylation does not totally eliminate the response of cells to amino acid

deficiency, indicating the presence of a GCN2- and eIF2α phosphorylation-independent pathway.

Histidine, arginine, leucine and methionine starvation resulted in somewhat different degrees of transcriptional upregulation of the genes we studied. In general, methionine starvation gave the largest fold changes in mRNA levels, leucine starvation gave the lowest fold changes in mRNA levels, and histidine and arginine gave intermediate fold changes. Patterns were not identical for the different CARE-containing mRNAs analyzed, consistent with the presence of additional minor regulatory mechanisms that differ depending on which amino acid is deficient.

Methionine, and to a lesser extent histidine and arginine, activates a GCN2- and eIF2α phosphorylation-independent but ATF4-dependent pathway that leads to transcriptional upregulation of CARE-containing genes. The transcriptional upregulation of CARE-containing genes is especially sensitive to methionine deficiency in both control cells and in GCN2(-/-) or eIF2α (A/A) cells. The residual upregulation of CARE-containing genes in GCN2(-/-) or eIF2α (A/A) cells was most robust for the case of methionine starvation. A smaller degree of residual upregulation was observed for histidine and arginine-starved cells, but none was observed for leucine-starved cells. The more robust responses to methionine starvation in control cells and in GCN2(-/-) or eIF2α (A/A) cells than to starvation of leucine, histidine or arginine suggests that methionine starvation is being sensed by an additional mechanism that is not dependent upon GCN2 or eIF2α phosphorylation. The requirement for ATF4, however, appears to be common to both the GCN2/eIF2α kinase-dependent and the GCN2-/eIF2α phosphorylation-independent pathways of transcriptional upregulation of CARE-containing genes based on the suppression of both responses by knockdown of ATF4. It should be noted that the PERK-mediated response to sulfur amino acid deficiency reported by Wanders et al. (37) cannot explain the eIF2α

phosphorylation-independent, ATF4-dependent response to methionine that we observed because we included cysteine in our cell culture medium so that cells are not deficient in glutathione and also because the effect was still observed in eIF2α(A/A) cells that cannot be phosphorylated by PERK.

4EBP1 but not 4EBP2 is a CARE-containing gene, and only 4EBP1 is upregulated by GCN2 activation, eIF2α phosphorylation or ATF4 upregulation. Although *ATF4*, *CHOP* and *ASNS* are well-established targets for transcriptional upregulation following activation of eIF2α kinases, *4EBP1* is a less-well established target (27, 30, 33, 40). We were especially interested in 4EBP1 because of its involvement in the regulation of cap-dependent mRNA translation and its regulation by mTORC1 which is also responsive to amino acid availability. Our results confirm that 4EBP1 expression is upregulated in response to essential amino acid deficiency and that this occurs by the canonical GCN2/eIF2α kinase/ATF4 pathway as well as by the methionine-sensitive ATF4-dependent pathway that is independent of GCN2 and eIF2α phosphorylation. In the case of 4EBP1 mRNA upregulation, we also show that this results in an increase in 4EBP1 protein abundance.

Because 4EBP2 is a close homolog of 4EBP1 that differs from 4EBP1 in that its promoter region does not contain a CARE, we also looked at the transcriptional regulation of 4EBP2 in response to amino acid starvation. Although 4EBP2, like 4EBP1, is expressed in most tissues, the physiological role of 4EBP2 and the regulation of 4EBP2 expression and activity have been much less studied than those of 4EBP1. Grolleau et al. (42) reported a differential regulation of 4EBP1 and 4EBP2 expression in a promyelocytic leukemia cell line (HL-60) during granulocytic differentiation, and Tsukiyama-Kohara et al. (43) observed a stronger phosphorylation of 4EBP1 than of 4EBP2 in response to insulin treatment. These results

suggested the possibility that the two homologs may have different functions and be regulated by different signals. This was confirmed in the current study where expression of 4EBP2, in contrast to 4EBP1 and other CARE-containing genes, was only minimally upregulated in response to histidine or methionine deprivation and this upregulation was not reduced by knockout of GCN2, mutation of Ser51 in eIF2α or knockdown of ATF4. Thus, 4EBP1, but not 4EBP2, appears to be a canonical target of GCN2/eIF2α kinase/ATF4 stress response pathways.

A possible mechanism for the methionine-sensitive, GCN2- and eIF2α phosphorylation-independent, ATF4-dependent pathway that leads to transcriptional upregulation of CARE-containing genes is a lack of ternary complex due to a decrease in the concentration of Met-charged $tRNA_i^{Met}$. The ISR has been shown to be highly dependent on the increased translation of ATF4 by a mechanism that depends on a low abundance of ternary complex (eIF2-GTP-Met-$tRNA_i^{Met}$) due to eIF2α phosphorylation blocking the regeneration of eIF2-GTP for new ternary complex formation. Additionally, after ATF4 is translated, it in turn induces its own gene transcription, further increasing ATF4 abundance and initiating the ISR. Our finding that methionine deprivation uniquely induced ATF4 and the ISR in MEFs that did not express GCN2 or a phosphorylatable eIF2α suggests that methionine deprivation maybe able to reduce the available amount of ternary complex by a mechanism not dependent upon GCN2 signaling or Ser51 phosphorylation of eIF2α. A likely alternative mechanism by which a deficiency of methionine might uniquely decrease the amount of ternary complex is that methionine deficiency could lead to a lack of methionine-charged initiator tRNA that is necessary for ternary complex formation. In eukaryotes, both the initiator and elongater $tRNA^{Met}$ are aminoacylated with methionine by methionyl-tRNA synthetase with no unique specificity (44). Structural differences between the initiator and elongator $tRNA^{Met}$ prevent the initiator $tRNA^{Met}$ from participating in

translation elongation, and promote its binding to eIF2 (45, 46). Therefore, under methionine deprivation we would expect the amounts of both charged initiator and elongator $tRNA^{Met}$ to decrease. In addition Met-$tRNA_i^{Met}$ binding to eIF2-GTP is facilitated by the presence of methionine on the $tRNA_i^{Met}$ as non-acylated $tRNA_i^{Met}$ has a K_d value of roughly 130 nM for eIF2-GTP while Met-$tRNA_i^{Met}$ binding to eIF2-GTP has a K_d value of 9 nM (47). Thus, because they are both essential components of the ternary complex, a lack of methionine-charged initiator tRNA would be expected to act somewhat similarly to a lack of eIF2α-GTP in reducing formation of ternary complex.

This mechanism would be consistent with our observation that methionine deficiency was especially robust in inducing ATF4 expression and the ISR in wild-type cells, and that methionine deficiency could still induce ATF4 expression and the ISR in GCN2 knockout cells and in cells with nonphosphorylatable eIF2α. A delay in ternary complex formation, regardless of the cause, would be expected to result in a delay in reformation of the 43S preinitiation complex and an increase in ATF4 synthesis. Although efforts to verify this hypothesis by overexpression of the initiator tRNA versus elongator tRNA for methionine were unsuccessful, due to the high efficiency at which cells charge these tRNAs with methionine, our results clearly demonstrate the existence of GCN2- and eIF2α-phosphorylation-independent mechanisms for upregulation of ATF4 that are highly responsive to methionine deprivation. Furthermore, the hypothesis that initiator tRNA plays a critical role in regulating the abundance of ternary complex is supported by previous studies in yeast. Dever et al. (48) demonstrated that a reduction in the copy number of the initiator tRNA genes resulted in an increase in GCN4, the yeast homolog of ATF4, in *GCN2*-knockout yeast. This induction of GCN4 in yeast with reduced expression of the initiator $tRNA^{Met}$ was further shown to be dependent on the uORF in

the GCN4 mRNA. It is reasonable to hypothesize that both the initiator $tRNA^{Met}$ and methionine-charging to the initiator $tRNA^{Met}$ are necessary for formation of functional ternary complex. Our results are the first to demonstrate the existence of a regulatory mechanism controlling mRNA translation that is uniquely sensitive to methionine status and suggest that there may be a tighter control of protein synthesis and expression of stress-responsive genes in response to methionine deprivation than in response to other amino acid deficiencies.

In summary, we demonstrate the existence of a GCN2- and eIF2α-phosphorylation-independent, but ATF4-dependent, pathway that is highly sensitive to methionine deprivation. We hypothesize this new pathway is due to reduced levels of Met-$tRNA_i^{Met}$ for ternary complex formation and, thus, supplements the canonical pathway whereby eIF2α Ser51 phosphorylation regulates ternary complex formation and availability. CARE-containing genes are upregulated in response to amino acid deprivation by the canonical GCN2/eIF2α phosphorylation/ATF4 pathway and, in addition, by the newly demonstrated GCN2- and eIF2α-phosphorylation-independent pathway. In addition, we demonstrate that 4EBP1, a major cap-binding protein thought to promote mRNA translation, but not its 4EBP2 homolog, is regulated by the ATF4/ISR pathway.

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CHAPTER 3

4EBP1-INDEPENDENT INHIBITION OF MRNA TRANSLATION BY SINGLE AMINO ACID DEPRIVATION

Introduction

The effects of amino acid availability on the regulation of protein synthesis can be mediated through the mechanistic complex of rapamycin complex 1 (mTORC1) pathway, which appears to be especially sensitive to leucine availability (1, 2), and through regulation of the phosphorylation status of the eukaryotic initiation factor 2 alpha subunit (eIF2α) (3).

A major downstream target of mTORC1 signaling is eukaryotic initiation factor 4E binding protein 1 (4EBP1), and effects of mTORC1 signaling on translation initiation are usually attributed to its phosphorylation of 4EBP1. The 4EBPs are a family of small, largely unstructured proteins (4–6) that regulate cap-dependent translation initiation by binding to eIF4E, and 4EBP1 is the most ubiquitous member of this family (4, 7–9). 4EBP1 competes with eIF4G for the same binding region on eIF4E; therefore, with 4EBP1 bound, eIF4E recognizes the 5' cap but cannot recruit eIF4G (7, 9–11). This inhibition of eIF4G-eIF4E binding has been shown to inhibit cap-dependent mRNA translation in experiments conducted in vitro (7, 12). Under nutrient sufficient conditions mTORC1 phosphorylates 4EBP1 at several sites, which inhibits 4EBP1 binding to eIF4E and promotes cap-dependent translation. Conversely, under conditions such as low insulin or low amino acids mTORC1 is inhibited and 4EBP1 becomes hypophosphorylated and bound to eIF4E, thus inhibiting translation (7, 12–16).

The other mechanism by which translation initiation is regulated in response to amino acids involves the phosphorylation of the alpha subunit of eIF2 (eIF2α) by an eIF2α kinase.

When essential amino acids are limiting, tRNAs cannot be fully aminoacylated, and this leads to a consequent increase in non-aminoacylated ("uncharged") tRNAs. Uncharged tRNAs bind to the histidyl-tRNA synthetase−related regulatory domain of the protein GCN2 (i.e. eIF2α kinase 4), causing it to dimerize and auto-phosphorylate, thus becoming activated (17–21). Active GCN2 then phosphorylates eIF2α (22–24). eIF2 is a GTPase that functions during translation initiation to form the 43S preinitiation complex with initiator methionine tRNA (Met-$tRNA_i^{Met}$), the 40S ribosomal subunit, eukaryotic initiation factor 3 (eIF3) and eukaryotic initiation factor 1A (eIF1A). The engagement of the complex with a start codon results in hydrolysis of eIF2-GTP to eIF2-GDP. When eIF2α is phosphorylated, the exchange of GDP with GTP to reform eIF2-GTP is inhibited because phosphorylated eIF2α inhibits eIF2B, which is the guanine nucleotide exchange factor. Without eIF2α being in the GTP-bound form, eIF2 cannot bind to Met-$tRNA_i^{Met}$ and the active 43S preinitiation complex cannot form, resulting in inhibition of mRNA translation initiation (25, 26).

Despite a number of studies looking at the effects of starvation of total amino acids, few studies have compared the effects of deficiencies of different single essential amino acids on protein synthesis. In rats and mice, single essential amino acid deprivation has been shown to have effects on hepatic polysome profiles (27–30) and rates of hepatic protein synthesis (30, 31) as well as on the phosphorylation status of 4EBP1(27, 30, 31) and eIF2α (29–32). Studies in hepatocytes have shown effects of single essential amino acid starvation on rates of protein synthesis, polysome formation, and preinitiation complex formation (33). A study using HeLa cells also showed differences between individual essential amino acids on polysome formation (34). However studies with hepatocytes and Hela cells used short periods of amino acid deprivation of 15 min (33) or 2 h (34) respectively, and did not look at the mechanisms behind

inhibition of translation.

The current study aims to determine the mechanisms involved in the inhibition of translation when single essential amino acids are deprived in human cells. We therefore compared the effects of 12 h leucine, histidine, arginine and methionine starvation in HEK 293T cells on translation, as well as 4EBP1 and eIF2α phosphorylation status. We observed that each essential amino acid tested, when deprived, inhibited cell growth to a similar extent, but each had dissimilar effects on the polysome profile. The effects on cell growth and the polysome profile were not replicated when cells were transfected with non-phosprylatable-4EBP1 and phosphomimic eIF2α, with non-phosprylatable-4EBP1 having little effect on cell growth or translation. We also show that methionine or leucine, when deprived, had great inhibitory effects on the polysome profile however had very different effects on mTORC1 inhibition. These results led us to hypothesize that methionine and leucine depuration inhibit translation through different mechanisms with leucine deprivation inhibiting translation through a leucine-dependent mechanism, perhaps a downstream target of mTORC1 other than 4EBP,1 and with methionine deprivation inhibiting translation through a decrease in charged initiator tRNA and inhibition of cellular methylation reactions.

Experimental Procedures

Cell Culture

Wild-type human embryonic kidney (HEK) 293T cells immortalized with SV40 Large T antigen were cultured in high glucose Dulbecco's modified Eagle’s medium (DMEM) (pH 7.4) containing 1 mM pyruvate and 4 mM glutamine and supplemented with 1X non-essential amino acid mix (Gibco/Invitrogen), 100 units/mL penicillin and 100 µg/mL streptomycin (pen/strep; Gibco /Invitrogen), and 10% (v/v) fetal bovine serum (FBS, Hyclone). Cells were maintained at

37 °C in an atmosphere of 95% air and 5% CO_2.

Amino Acid Deprivation

Experimental control medium was the same as normal growth medium with the following changes: medium was prepared using DMEM that lacked sulfur amino acids, histidine, leucine and arginine (custom prepared by Gibco/Invitrogen) and was supplemented with 0.4 mM L-cysteine, 0.2 mM L-methionine, 0.2 mM L-histidine, 0.8 mM L-leucine and 0.4 mM L-arginine. To eliminate amino acids in serum, 10% dialyzed FBS (Hyclone) was used in place of standard FBS. Histidine-deficient (His-), methionine-deficient (Met-), leucine-deficient (Leu-) and arginine-deficient (Arg-) media were prepared similarly except the respective amino acids were not added back to the deficient DMEM.

Cells were plated at a concentration of 4.0×10^4 cells/cm^2 in 150 mm plates and allowed to grow overnight. For experiments, growth medium was replaced with experimental control or amino acid-deficient medium. After 12 h, cells were collected for polysome profiling or cap-binding assay as described below.

Plasmid generation and transfection

Plasmid pCMV6-Kan/Neo containing cDNA clones of mouse *4ebp1* cDNA or Myc-DDk-tagged human eIF2S1 (eIF2α) were purchased from Origene. The mouse transcript of the 4EBP1 protein is conserved between mice and humans. A non-phosphorylatable 4EBP1 mutant was generated using the Q5® Site-Directed Mutagenesis Kit (New England Biosystems) in order to convert the threonine 37 and threonine 46 residues into alanines ((4EBP1(T37A/T46A)). The eIF2α cDNA clone was mutated in order to generate a phosphomimic mutant by mutating serine 51 to aspartate ((eIF2α(S51D)).

For 4EBP1 transfection, HEK 293T cells were transfected using the Lonza

Nucleofector®-2b device as described by the manufacturer. Cells were grown in standard growth medium until they were 80% confluent. The cells were then trypsinized, counted using a hemocytometer and centrifuged at 400 g for 10 min at room temp. Then 1.0 x 10^6 cells were combined with 5 µg of 4EBP1(T37A/T46A) plasmid, 4EBP1 wild-type (WT) plasmid or empty vector in Ingenio® Electroporation solution (Mirus Bio). The cell-plasmid mixture was then electroporated using the recommended program Q-001. Six transfection reactions were combined for each 150 mm plate so that final cell concentrations would be 4.0 x 10^4 cells/cm^2. Cells were plated in normal growth medium and were collected 24 h post-transfection for polysome profiling or eIF4E pull-down as described below.

4EBP1(T37A/T46A) and eIF2α(S51D) cotransfection

For 4EBP1(T37A/T46A) and eIF2α(S51D) cotransfection, plasmids were cotransfected using Lipofectamine 3000 (Invitrogen) and the reverse transcription method. Cells were grown in standard growth medium prepared without pen/strep until they were 80% confluent. The cells were then trypsinized and diluted in fresh growth medium without pen/strep so they would yield 1.0 x 10^5 cells/mm^2 when plated on 150 mm plates. To prepare plasmids for transfection, 46 µg of empty vector, 4EBP1(T37A/T46A) and eIF2α(S51-D) were each diluted in 1500 µL Opti-MEM (Gibco/Invitrogen) supplemented with 67.5 µL P3000 reagent. For the cotransfection, 23 µg of 4EBP1(T37A/T46A) and 23 µg of eIF2α(S51D) plasmids were diluted in 1500 µL Opti-MEM supplemented with 67.5 µL P3000 reagent. For transfections, 90 µL Lipofectamine 3000 was diluted in 1500 µL Opti-MEM and then combined with the diluted plasmids according to the manufacturer's protocol. The diluted cell suspension was combined with the plasmid-Lipofectamine solution, mixed and plated. After 6 h the transfection medium was removed and replaced with equilibrated growth medium and cells were allowed to grow for 6 h after which

growth medium was replaced with equilibrated control, Met– or Leu– medium. After 12 h in the treatment medium cells were collected for polysome profiling as described below.

Polysome profiling and western blotting

At 5 min before cell collection, cycloheximide (CHX) was added to the medium at a concentration of 100 µg/mL. Cells were then washed once with ice-cold PBS containing CHX (100 µg/mL) and harvested in 300 µL ice-cold polysome buffer (10 mM HEPES (pH 7.4), 100 mM KCl, 5 mM $MgCl_2$) containing CHX (100 µg/mL) and Triton-100 (1% (v/v)). Cells were then disrupted by vortexing followed by freezing and thawing. Lysates were cleared by centrifugation at 4 °C and 12,000 g for 15 min. 50 µL of supernatant was removed and frozen at -80 °C for western blotting. The remaining cell supernatant was used for polysome profiling.

Sucrose solutions were prepared in polysome buffer. Sucrose density gradients (15–45% (w/v)) were freshly made in SW41 ultracentrifuge tubes (Beckman Coulter) using a BioComp Gradient Master (BioComp) according to the manufacturer's instructions. Cell lysates were loaded onto the sucrose gradients, followed by centrifugation for 150 min at 32,000 rpm at 4 °C in an SW41 rotor. After centrifugation, fractions were pushed through the fractionation system (Icso) with 60% sucrose at 0.375 mL/min and OD254 values were continuously monitored and recorded. OD254 profiles were scanned into the computer and traced (Inkscape). Polysome/monosome (P/M) ratios of the traced profiles were determined by aligning profiles to the bottom of the monosome peak and measuring the areas below the monosome and polysome peaks to a line equal to the lowest point on each analysis set.

For western blotting, protein concentrations of the sample supernatants were determined by the bicinchonic acid assay (BCA Protein Assay Kit, Thermo Scientific Pierce), and 50 µg protein per lane were resolved by SDS/PAGE using a 12% (w/v) polyacrylamide gel. Proteins

were transferred to a 0.45-µm Immobilon PVDF membrane (Millipore Corp.) and the membrane was incubated for 1 h at room temperature with blocking buffer for near infrared fluorescent westerns (Rockland or Odyssey blocking buffer). The membrane was then incubated with antibodies to 4EBP1 (1:1000, Cell Signaling), T37/T46-P 4EBP1 (1:1000, Cell Signaling), eIF2α (1:1000, Cell Signaling) eIF2α Ser51-P (1:1000, Cell Signaling), cleaved caspase-3 (1:1000, Cell Signaling), total caspase-3 (1:1000, Cell Signaling) or β-actin (1:1000, Cell Signaling) at 4°C overnight. Membranes were washed for 30 min in TTBS [50 mM Tris-Cl (pH 7.6), 150 mM NaCl, 0.1% ,v/v, Tween 20] and exposed to secondary antibodies conjugated to AlexaFluor 680 or IRDye 800 (1:20,000, Li-COR Biosciences) for 1 h at room temperature. Membranes were again washed for 30 min with TTBS and rinsed in PBS (pH 7.4), and the proteins were visualized and quantified using an Odyssey infrared imagining system (Li-COR Biosciences) and Odyssey software. The relative intensities of the bands of interest were normalized to β-actin.

eIF4E pull-down

Cells were plated at a concentration of 4.0×10^4 cells/cm^2 in 150 mm plates and allowed to begin growth overnight. Growth medium was replaced with experimental control or amino acid-deficient medium. After 12 h, cells were washed 2X with PBS and then lysed in pulldown buffer [50 mM HEPES (pH 7.4), 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM DTT, complete protease inhibitor and PhosSTOP phosphatase inhibitor "cocktails" (Roche)] supplemented with 0.4% NP-40 (Fluka). Cell lysates were then centrifuged at 10,000 g for 15 min at 4 °C, and the supernatant was removed. Protein concentration of the lysis supernatant was determined by the BCA assay.

For eIF4E-pulldown, 50 µL of m7-GTP-agarose (Jenna Biosystems) was washed 2X with

500 µL of pulldown buffer without NP-40. After each wash the m7-GTP-agaraose was centrifuged at 2,500 g for 1 min to pellet the beads and the supernatant was removed. A volume of lysate containing 1 mg of protein was diluted to 500 µL in pulldown buffer and added to the washed m7-GTP-agarose. Samples were rotated at 4°C for 90 min. After incubation, supernatant was removed and beads were washed 2X in 500 µL of pulldown buffer, followed by 500 µL of 0.5 mM GTP in pulldown buffer in order to reduce non-specific binding. Protein was eluted from the beads with the addition of 50 µL 0.5 mM m7-GTP in pulldown buffer and rotation at room temperature for 30 min. Incubation mixtures were then centrifuged at 2,500 g for 1 min to pellet the beads, and supernatant containing the eluted proteins was removed. Proteins eluted from the beads were resolved by SDS/PAGE using a 10% (w/v) polyacrylamide gel. Western blotting was done as described above with antibodies to 4EBP1 (1:1000, Cell Signaling), eIF4G (1:1000, Cell Signaling) or eIF4E (1:1000, Cell Signaling). The relative intensities of the bands of interest were normalized to those for eIF4E.

Statistical Analysis

All experiments were repeated at least three times with triplicate wells of cells within each experiment. Results were expressed as fold of the mean for the same cell type cultured in control medium. Western band intensities, total protein concentrations and P/M ratios were $\log_{10}$ transformed and analyzed using ANOVA and Student's t-test. Differences were accepted at $p \leq 0.05$.

Results

Essential Amino Acid deprivation

Deprivation of arginine, histidine, leucine or methionine each resulted in a decrease in cell growth/proliferation as measured by total protein content of cell cultures. The extent of

reduction in protein content of cell cultures was not different among the four essential amino acids studied (Fig. 3.1a). To assess the effect of the deficiency of a single essential amino acid on mRNA translation (i.e., protein synthesis), we examined the effect of essential amino acid deficiency on polysome profiles. Figure 3.1b shows the results of a representative experiment, with the polysome profiles for cells cultured in each of the four essential amino acid-deficient media overlaid along with that for cells cultured in sufficient control medium. In contrast to the similar effects of arginine, histidine, leucine and methionine deficiency on the growth of cultured HEK 293T cells, a deficiency of each of these amino acids had different effects on the polysome profile. Histidine deprivation did little to change the polysome profile. Arginine deprivation resulted in an increase in the monosome peak and a slight decrease in the polysome fraction. Leucine deprivation resulted in an increase of the monosome peak and an even larger reduction in the polysome fraction compared to histidine or arginine deprivation. Methionine deprivation resulted in an increase in the monosome peak and the most extensive decrease in polysome fractions of the four amino acids tested. The shift in polysome profiles was also quantified as the ratio of the area of the polysome peaks (P) to the area of the monosome peak (M). As shown in Figure 3.1c, the P/M ratio was not significantly different for cells cultured in histidine-deprived medium. Arginine significantly reduced the P/M ratio compared to that for cells grown in sufficient medium or for cells deprived of histidine. Leucine and methionine deprivation had the most drastic effects on the P/M ratio, with the P/M ratio being significantly lower than those in cells grown in histidine- or arginine-deficient medium ($p<0.05$). The observed changes in the P/M ratios were mimicked by the number of peaks observed in the polysome fractions. As can be seen in Figure 3.1d, leucine and arginine deprivation resulted in a significant decrease in polysome peak number, whereas histidine had no effect. In contrast to the results observed for

the P/M ratio, methionine deprivation further reduced the number of polysome peaks compared to arginine and leucine deprivation.

Because each amino acid tested, when deprived, resulted in differences in the polysome profiles and P/M ratios, we aimed to determine if deprivation of any one amino acid was more detrimental to the cells than that of other amino acids. To assess this, we analyzed the cells for the presence of cleaved caspase-3 as an indicator of apoptosis. As can be seen in Figure 3.1e there was no increase in cleaved caspase-3 as a result of a deprivation of any amino acid tested. This demonstrates that the differences in cell growth, polysome profiles and P/M ratios observed in Figures 3.1a,b,c are not a result of changes in apoptosis.

Because 4EBP1 phosphorylation status is thought to regulate the rate of cap-dependent mRNA translation in response to mTORC1 signaling, we wanted to see if increases in 4EBP1 binding to cap-associated eIF4E occurred with amino acid deprivation and, if so, if these increases would correspond with the decreases in P/M ratios. eIf4E was pulled down using a cap analog, and the relative amounts of 4EBP1 and eIF4G bound to eIF4E were determined. As shown in Figures 3.2a,b, similar amounts of eIF4E were bound under all conditions, as would be expected, but the relative amounts of 4EBP1 bound to eIF4E were higher for cells cultured in amino acid-deficient medium than for cells grown in sufficient medium. Leucine deprivation had the strongest effect on the association of 4EBP1 with eIF4E, resulting in the greatest reduction in eIF4G association. Methionine deprivation did not result in a significant increase in 4EBP1 association or a significant decrease in eIF4G association with eIF4E. Therefore, although leucine deprivation and methionine derivation were equally effective at inhibiting translation, leucine was more effective than methionine deprivation at inhibiting mTORC1 activity.

Amino acid deprivation inhibits translation independent of mTORC1 through the

phosphorylation of eIF2α. Therefore, we next aimed to determine if differences in eIF2α-phosphorylation would correspond to differences observed in the P/M ratios due to essential amino acid deprivation. As can be seen in Figure 3.2c,d, deprivation of any amino acid tended to result in an increase in eIF2α-phosphorylation, although only methionine deprivation resulted in a statistically significant increase compared to cells grown in sufficient medium. However, the levels of eIF2α-phosphorylation resulting from leucine, histidine, and arginine-deprivation were not significantly different than those produced by methionine deprivation.

Thus, although deprivation of any one of the four amino acids tested had a similar inhibitory effect on cell growth/proliferation, deprivation of the four essential amino acids had varying effects on the polysome profile. In particular, the effectiveness of methionine or leucine deprivation in suppressing polysome formation was striking. Changes in cell growth and polysome formation were not correlated with changes in 4EBP1 or eIF4G association with eIF4E. In addition, the relative effectiveness of a lack of histidine, arginine, leucine or methionine on polysome formation did not parallel the changes in the ratio of phosphorylated eIF2α to total eIF2α.

Mutant 4EBP1 transfection

To elucidate the effect that 4EBP1 binding to cap-associated eIF4E has on changes in the polysome profile, we developed a 4EBP1 mutant that cannot be phosphorylated by mTORC1 by mutating two key phosphorylatable threonine residues (Thr 37 and Thr 46) to alanines (4EBP1 T37A/T46A). Transfection of HEK 293T cells with wild-type 4EBP1 or with mutant nonphosphorylatable 4EBP1 T37A/T46A resulted in higher cellular levels of total 4EBP1 protein compared to control cells transfected with vector alone, as shown in Figure 3.3a, b. However, the amount of phosphorylated 4EBP1 detected by an antibody specific for the

phosphorylated Thr37 and Thr46 sites of 4EBP1 indicated an increase in phosphorylated 4EBP1 only in cells transfected with the wild-type 4EBP1. Because phosphorylation of 4EBP1 by mTORC1 blocks its association with eIF4E, the greater amount of nonphosphorylated 4EBP1 in cells transfected with 4EBP1 T37A/T46A would be expected to result in more association of 4EBP1 with eIF4E. This was indeed the case, as shown in Figure 3.3c,d. Transfection of cells with wild-type 4EBP1 had no effect on the amount of eIF4E-associated 4EBP1 compared to empty vector-transfected control cells, but the eIF4E pulled down from cells transfected with mutant 4EBP1 T37A/T46A had 4-times as much associated 4EBP1. This confirms that the mutant nonphosphorylatable 4EBP1 T37A/T46A bound eIF4E efficiently even when cells were grown in complete medium.

After determining that the mutant 4EBP1 was indeed associating with eIF4E under normal growth conditions, the effect of overexpression of wild-type and mutant nonphosphorylatable 4EBP1 on polysome formation was determined, again in cells cultured in complete medium. At 24 h after transfection, there was no significant difference in the total amount of protein in the various cultures, suggesting little effect of increased 4EBP1 binding (i.e., nonphosphorylated 4EBP1 binding) to eIF4E on the rate of cell growth/proliferation (Fig. 3.3e). On the other hand, the overlay of polysome profiles for cells transfected with empty vector, wild-type 4EBP1, or mutant nonphosphorylatable 4EBP1 T37A/T46A demonstrated a slight increase in the number of monosome peaks in cells transfected with the mutant nonphosphorylatable 4EBP1 (Fig. 3.3f). Calculation of P/M ratios, however, demonstrated no significant reduction of the P/M ratio for cells transfected with 4EBP1(T37A/T46A) compared to those for cells transfected with the empty vector (Fig. 3.3g), and the number of polysome peaks was not significantly different from those for cells transfected with either empty vector or with

wild-type 4EBP1 (Fig. 3.3h).

As in the experiments shown in Figure 3.1, these studies with nonphosphorylatable 4EBP1 suggest that an increase in 4EBP1 association with eIF4E by itself does not inhibit cell growth and has relatively little effect on the formation of polysomes. This implies that leucine deprivation does not inhibit translation by decreasing 4EBP1 phosphorylation. These results support the hypothesis that the different effects of single essential amino acid deficiency on cell growth and on polysome formation are not mediated by 4EBP1 binding to eIF4E to block translation initiation.

Mutant 4EBP1 and eIF2α phosphomimic cotransfection

Under amino acid deprivation it has been shown that 4EBP1 is dephosphorylated and eIF2α is phosphorylated, and both processes have been associated with decreased translation of mRNA into protein (27, 28, 30). Therefore, we investigated the effect that transfecting HEK 393T cells with both non-phosphorylatable mutant 4EBP1 T37A/T46A and phosphomimetic eIF2α (S51D) would have on cell growth and the polysome profile. Western blotting as shown in Figure 3.4a demonstrated that co-transfection was successful as cells transfected with both 4EBP1 T37A/T46A and eIF2α S51D had an increase both in total 4EBP1 and eIF2α S51D. As can be seen in Figure 3.4b, the cotransfection did suffer from competition as the 4EBP1 induction was not as great as seen when 4EBP1 T37A/T46A was transfected alone, but eIF2α S51D expression was not affected by 4EBP1 T37A/T46A coexpression. Expression of eIF2α S51D had no effect on the amount of endogenous eIF2α that was present in phosphorylated form but did result in a higher level of phospho- + phosphomimetic-eIF2α. Therefore, cotransfected cells had higher levels of unphosphorylated 4EBP1 and higher levels of phospho- + phosphomimetic- eIF2α compared to control cells transfected with the vectors alone.

Although the higher levels of unphosphorylated 4EBP1 and phosphorylated eIF2α would both be expected to suppress translation initiation, transfection of either 4EBP1 T37A/T46A or eIF2α S51D or cotransfection of both had no effect on cell growth, only a modest effect on the P/M ratio, and no effect on the number of polysome peaks compared to empty vector-transfected control cells. In contrast, leucine or methionine deprivation had significant effects on cell growth (Fig. 3.4c) and polysome formation (Fig. 3.4 d,e, f). In this series of experiments, methionine or leucine deprivation in cells that had been transfected with empty vector resulted in a borderline significant ($p<0.10$) decrease in total protein. Because the reduction in total protein was of a similar magnitude to that observed in the series of experiments shown in Figure 3.1, we therefore conclude that this reduction was real despite the borderline statistical significance. Methionine or leucine deprivation had a much greater effect on the P/M ratio than did cotransfection of both 4EBP1T37A/T46A and eIF2α S51D (Fig. 3.4d,e). Leucine deprivation resulted in a borderline significant ($p<0.10$) reduction in polysome peak number while methionine deprivation reduced the number of polysome peaks significantly compared to vector control, 4EBP1T37A/T46A and eIF2α S51D transfections and leucine deprivation (Fig. 3.4f).

Based on these results we can conclude that for cells grown in complete medium, increases in both non-phosphorylated 4EBP1 and phosphorylated eIF2α are not sufficient to bring about significant reductions in cell growth or in P/M ratios. These results are consistent with the idea that deprivation of essential amino acids, at least of leucine or methionine, may affect the polysome profile, protein synthesis and cell growth by mechanisms that are independent of 4EBP1 and eIF2α phosphorylation/ dephosphorylation pathways.

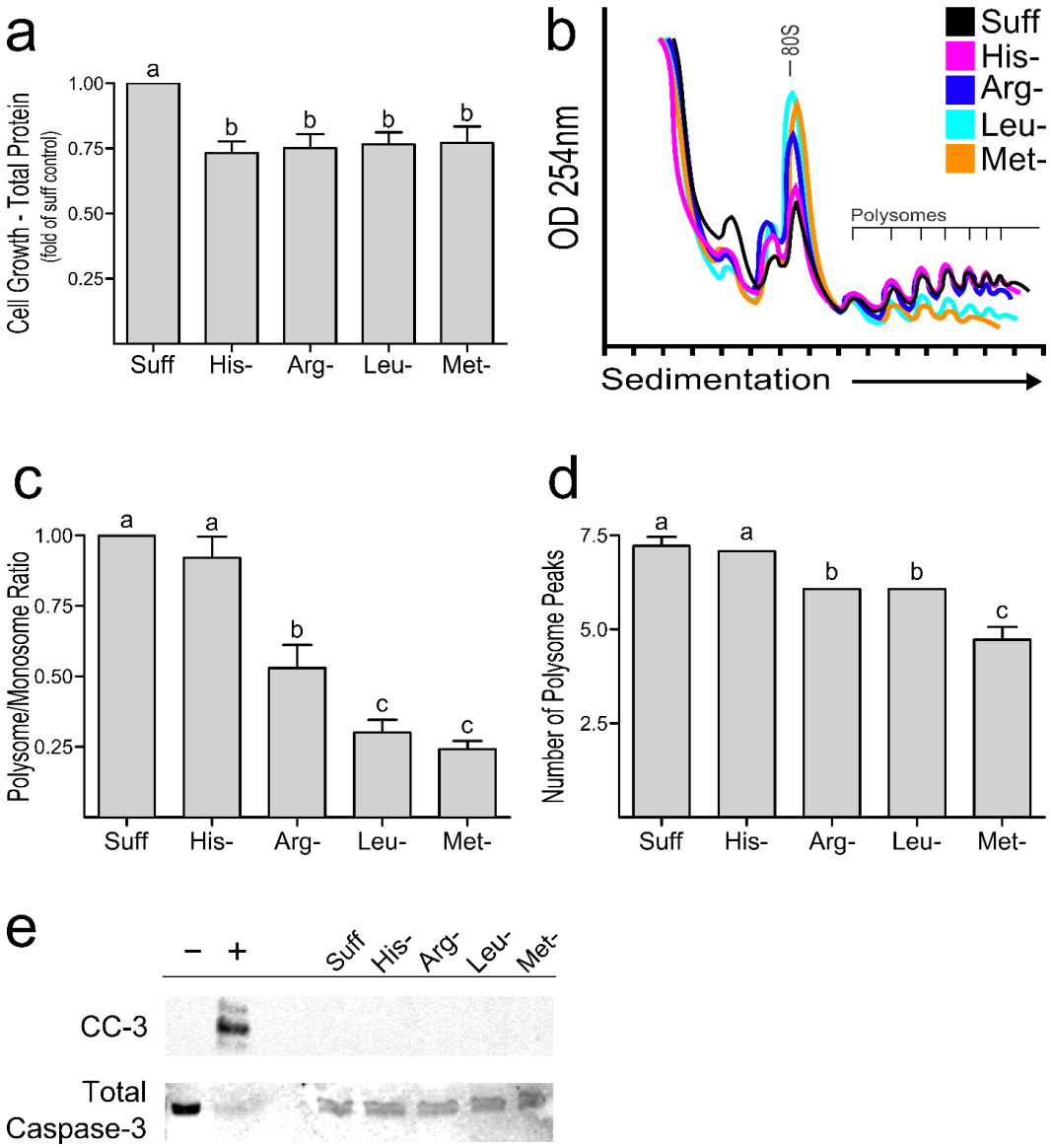


Figure 3.1. Changes in the growth and polysome profile of HEK 293T cells in response to deficiency of a single essential amino acid. Cells were grown in complete medium (Suff) or in medium deficient in histidine (His–), arginine (Arg–), leucine (Leu–) or methionine (Met–). a) Final protein content of HEK 293T cells grown in amino acid sufficient or deficient medium for 12 h, expressed as a fraction of the final protein content of cells grown in sufficient medium. b,c,d) Polysome profiles of cells grown in amino acid sufficient or deficient medium for 12 h. b) Image of overlaid polysome profiles from a representative experiment, demonstrating differences observed in polysome profiles. c) Ratios of polysome area to monosome area of polysome profiles. d) Number of peaks in the polysome fraction of the polysome profiles. e) Cleaved caspase-3 assay on 293T cells. A representative western blot for the cleaved caspase-3 assay. Positive (+) and negative (–) control extracts are from control Jurkat cells and Jurkat cells treated with cytochrome c, respectively. a, c, d) Values are means ± SEM for 3 separate experiments. Bars labeled with different letters are significantly different by Student t-test at $p \leq 0.05$.

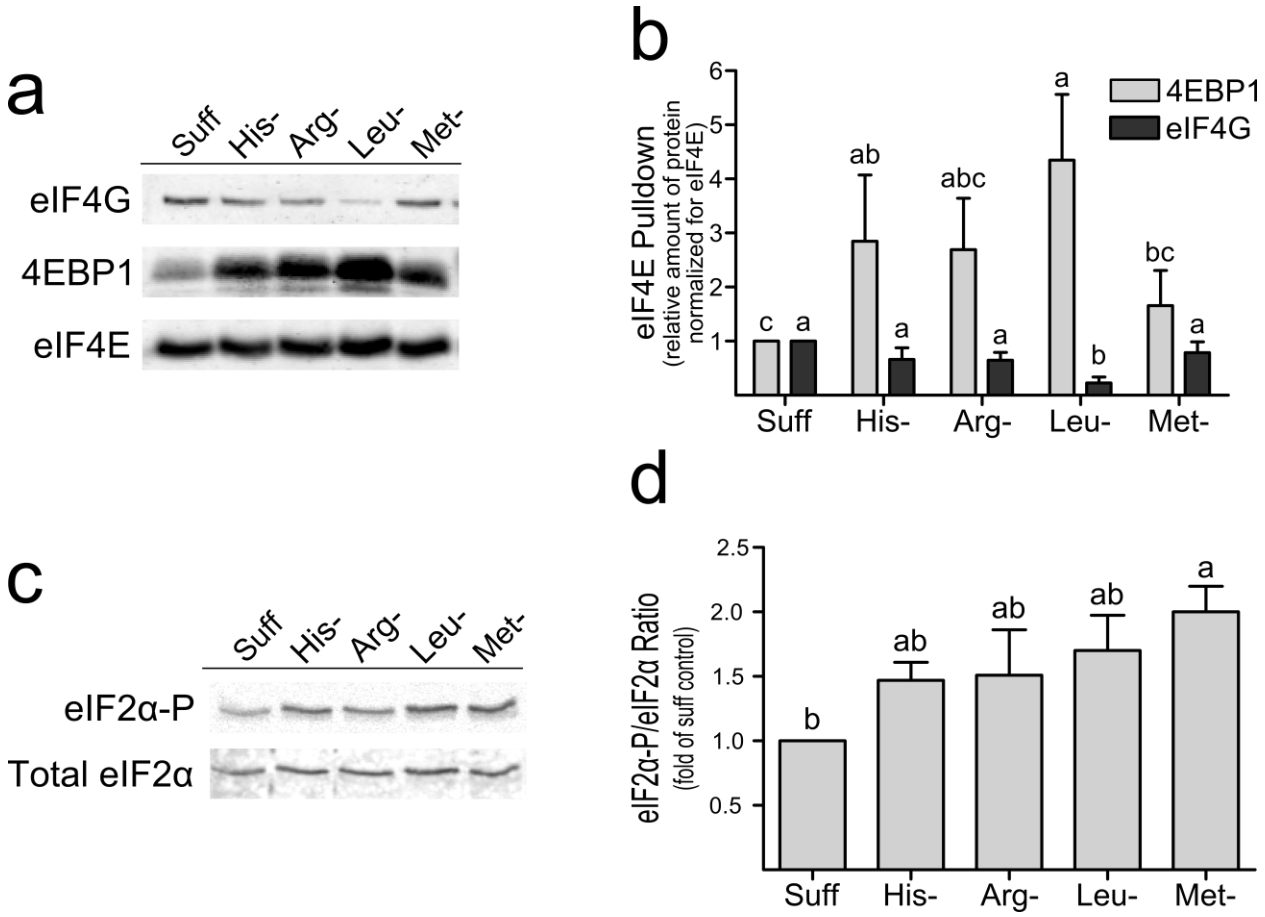


Figure 3.2. Differences in 4EBP1 and eIF2α phosphorylation in HEK 293T cells in response to deficiency of a single essential amino acid. a,b) m7 cap-analog pulldown of eIF4E and associated 4EBP1 and eIF4G. a) A representative western blot for the cap pulldown assays. b) Amount of 4EBP1 and eIF4G associated with eIF4E expressed as fold the mean value for cells cultured in complete medium after normalizing to eIF4E. c,d) Amount of phosphorylated-eIF2α in HEK 293T cells. c) A representative western blot for phosphorylated and total eIF2α. c) Ratio of phosphorylated-eIF2α to total eIF2α expressed as fold the mean value for cells cultured in complete medium. b, d) Values are means ± SEM for 3 separate experiments. Bars labeled with different letters are significantly different by Student t-test at $p \leq 0.05$.

Figure 3.3. Effect of transfection of HEK 293T cells with wild-type versus non-phosphorylatable-4EBP1. Cells were transfected with empty vector, wildtype 4EBP1, or non-phosphorylatable mutant (T37A/T46A) 4EBP1 and grown in sufficient control medium. a,b) Abundance of total and Thr 37,46-phosphorylated 4EBP1 in transfected cells at 24 h post-transfection. a) Representative western blot of the results of the 4EBP1 transfection experiment. b) Abundance of total and Thr 37,46-phosphorylated 4EBP1, normalized for actin and expressed as fold the mean values for cells transfected with empty vector. c,d) m7 cap-analog pulldown of eIF4E and associated 4EBP1 from transfected cells at 24 h post-transfection. c) A representative western blot for the cap pulldown assays. d) Amount of 4EBP1associated with eIF4E, expressed as fold the mean value for cells transfected with empty vector after normalizing to eIF4E. e) Cell growth assessed by total protein content at 24 h post-transfection, expressed as a percentage of total protein in cells transfected with the empty vector. f,g,h) Polysome profiles of transfected cells at 24 h post-transfection. f) Representative image of polysome profiles from one experiment. g) Ratios of polysome to monosome areas of polysome profiles. h) Number of peaks in the polysome fraction of the polysome profiles. b,d,e,g,h) Values are means ± SEM for 3 separate experiments. Bars labeled with different letters are significantly different by Student t-test at $p \leq 0.05$.

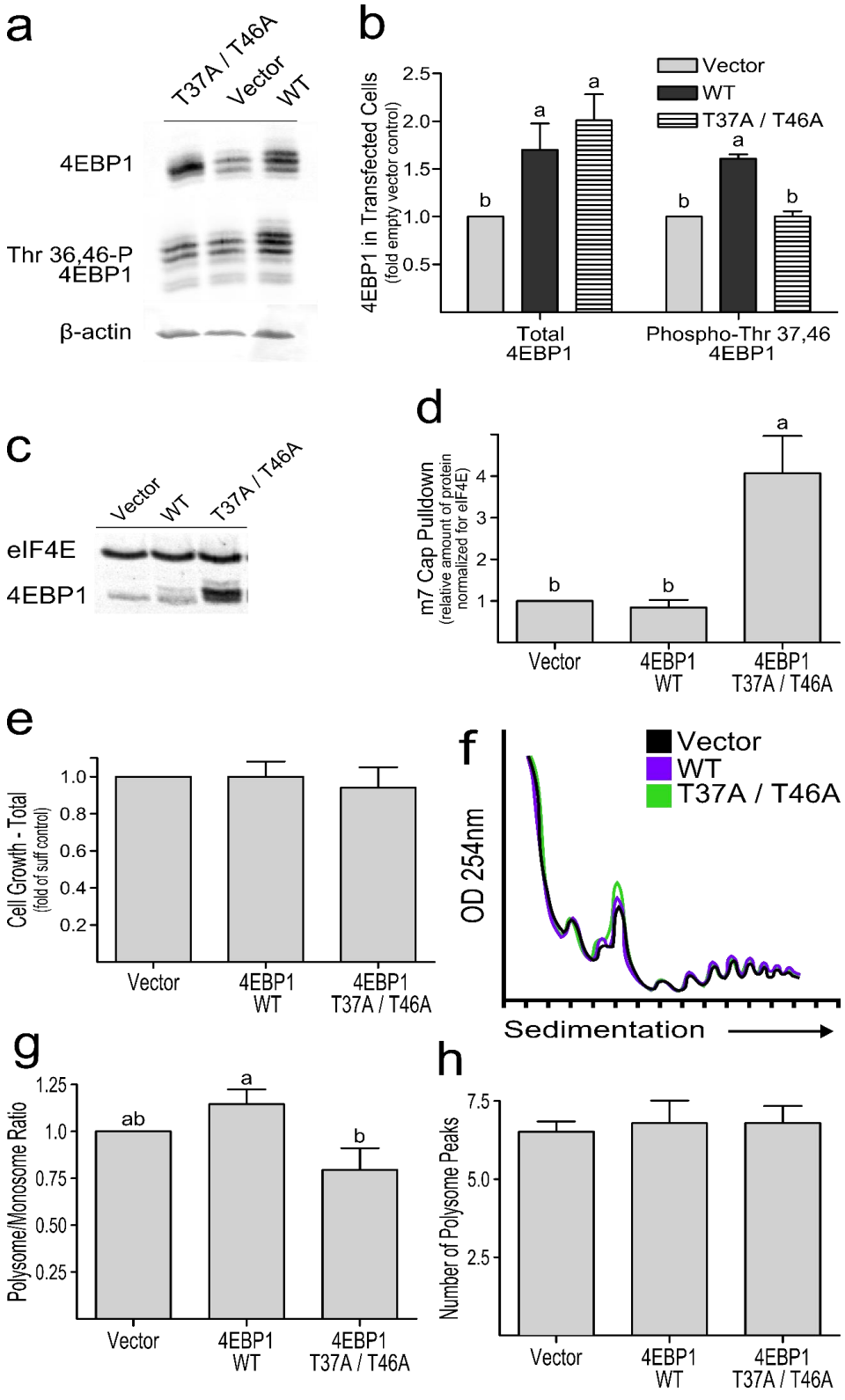
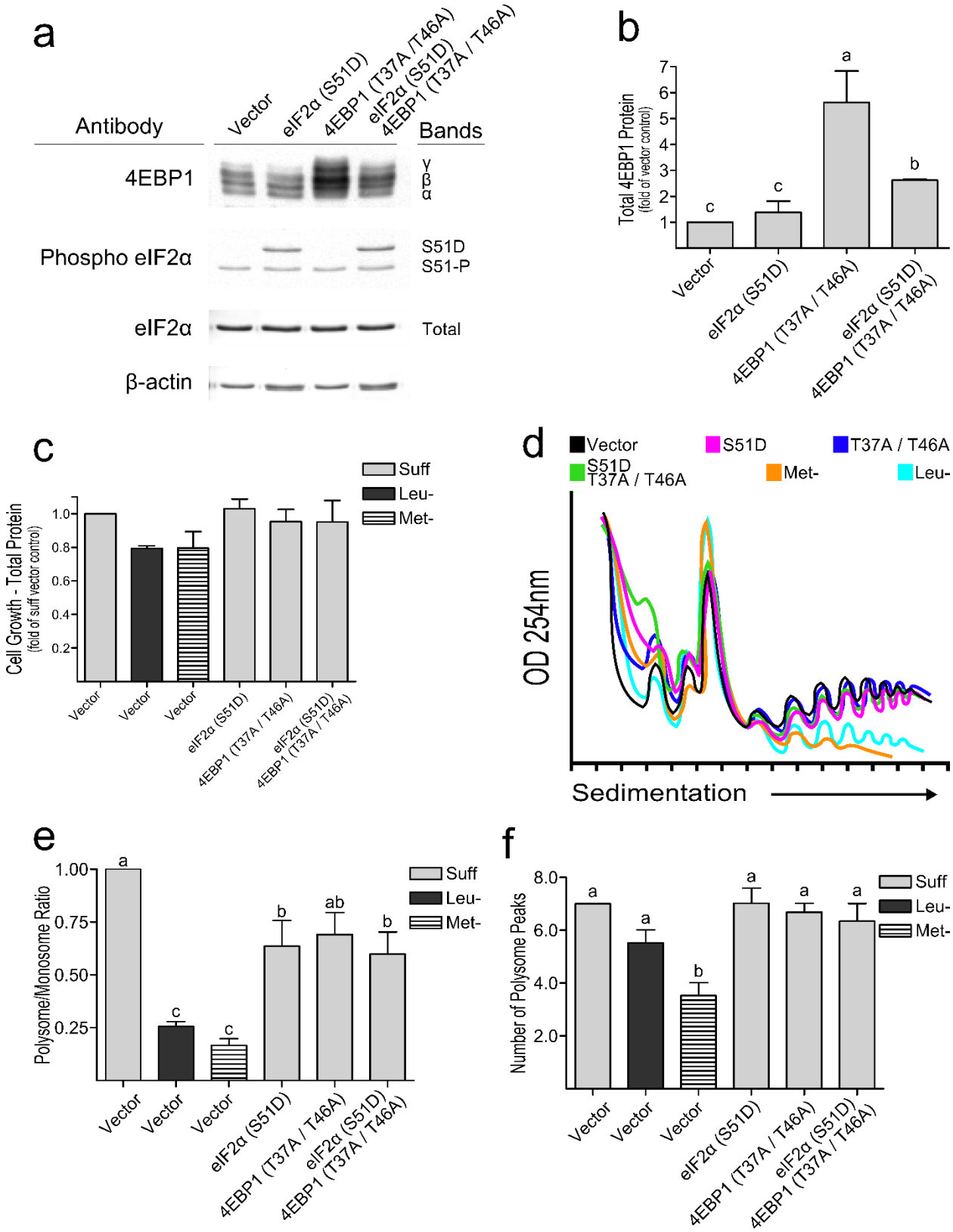

a
T37A / T46A
Vector
WT
4EBP1
Thr 36,46-P 4EBP1
β-actin
b
Vector
WT
T37A / T46A
4EBP1 in Transfected Cells (fold empty vector control)
2.5
2.0
1.5
1.0
0.5
a
a
a
b
b
b
Total 4EBP1
Phospho-Thr 37,46 4EBP1
c
Vector
WT
T37A / T46A
eIF4E
4EBP1
d
m7 Cap Pulldown (relative amount of protein normalized for eIF4E)
4
3
2
1
a
b
b
Vector
4EBP1 WT
4EBP1 T37A / T46A
e
Cell Growth - Total (fold of suff control)
1.0
0.8
0.6
0.4
0.2
Vector
4EBP1 WT
4EBP1 T37A / T46A
f
Vector
WT
T37A / T46A
OD 254nm
Sedimentation
g
Polysome/Monosome Ratio
1.25
1.0
0.75
0.50
0.25
ab
a
b
Vector
4EBP1 WT
4EBP1 T37A / T46A
h
Number of Polysome Peaks
7.5
5.0
2.5
Vector
4EBP1 WT
4EBP1 T37A / T46A

Figure 3.4. Transfection of HEK 293T cells with non-phosphorylatable-4EBP1 and with phosphomimetic eIF2α. Cells were transfected with 4EBP1(T37A/T46A) only, eIF2α(S51D) only, both 4EBP1(T37A/T46A) and eIF2α(S51D), or empty vector. Both plasmids were synthesized using the same vector, so only one vector was transfected as a control. a,b) Relative expression levels of eIF2α and 4EBP1 in transfected cells cultured in sufficient medium at 24 h post-transfection. a) Representative western blot of lysates of transfected cells. The eIF2α(S51D) plasmid contained a Myc-DDk tag and therefore migrates separately than phosphorylated endogenous eIF2α (i.e., eIF2α S51-P) b) Total 4EBP1 abundance in lysates of transfected cells cultured in sufficient medium, measured at 24 h post-transfection, expressed as fold the mean value for cells transfected with empty vector after normalization by β-actin. c) Cell growth assessed by total protein content of cells transfected with empty vector, 4EBP1 (T37A/T46A), eIF2α(S51D), or 4EBP1(T37A/T46A) plus eIF2α(S51D) at 24 h post-transfection and expressed as fold the protein content of cells transfected with empty vector and grown in sufficient medium. For comparison, results for empty vector-transfected cells grown in either leucine-deficient or methionine-deficient medium for the 12 h before harvest are also shown. d,e,f) Polysome profiles for transfected cells at 24 h post-transfection with empty vector, 4EBP1 (T37A/T46A), eIF2α(S51D), or 4EBP1(T37A/T46A) + eIF2α(S51D), as well as for cells transfected with empty vectors and then exposed to leucine- or methionine-deficient medium for 12 h. d) Representative image of overlaid polysome profiles from one experiment. e) Ratios of polysome area to monosome area of the polysome profiles. f) Number of peaks in the polysome fraction of the polysome profiles. b,c,e,f) Values are means ± SEM for 3 separate experiments. Bars labeled with different letters are significantly different by Student t-test at $p \leq 0.05$.


a
Antibody
Vector
eIF2α (S51D)
4EBP1 (T37A /T46A)
eIF2α (S51D)
4EBP1 (T37A / T46A)
Bands
4EBP1
γ
β
α
Phospho eIF2α
S51D
S51-P
eIF2α
Total
β-actin
b
Total 4EBP1 Protein
(fold of vector control)
c
Cell Growth - Total Protein
(fold of suff vector control)
Suff
Leu-
Met-
d
Vector
S51D
T37A / T46A
S51D
T37A / T46A
Met-
Leu-
OD 254nm
Sedimentation
e
Polysome/Monosome Ratio
f
Number of Polysome Peaks

Discussion

Essential amino acid deficiency restricts cell growth. HEK 293T cell proliferation, as assessed by total protein content, was similarly suppressed by the absence of histidine, arginine, leucine or methionine from the culture medium. The absence of cleaved caspase-3 indicates that the reduction in total protein content was not due to cell death by apoptosis but rather to a restriction of growth/proliferation. It is not surprising that lack of an essential nutrient restricts cell growth, but our finding that these effects on cell growth were not closely correlated with any of the measures of mRNA translation (P/M ratio, number of polysome peaks) was not expected. This could suggest that cell proliferation is regulated by mechanisms separate from those regulating the rate of protein synthesis.

4EBP1 dephosphorylation does not correlate with the effects observed under Essential Amino Acid deprivation. Past studies have demonstrated that transfection of cells with a non-phosprylatable-4EBP1mutant inhibit the translation of cap-dependent genes. These studies however measured the reduction of translation through reporter gene constructs containing 5′UTRs from known cap dependent genes. (35–37) Previous studies where non-phosprylatable-4EBP1 was transfected into cells and total protein synthesis was measured call the role of 4EBP1 in regulating total cellular translation into question. When non-phosprylatable-4EBP1 was expressed in rat Rat1a and TGR cells there was no change in protein synthesis (38), and only the TGR cells demonstrated a change in the polysome profile. In addition Kimball et al (39) demonstrated that eIF2α-phosphorylation and not 4EBP1 activity was the primary mediator of a decrease in protein synthesis under leucine or histidine deprivation. Expression of a mutated non-phosprylatable-4EBP1 resulted in markedly greater association of 4EBP1 with eIF4E, but no significant effect on polysome formation or cell growth supports the idea that 4EBP1 alone does

not effectively prevent the initiation of mRNA translation at a global level. These results suggest that 4EBP1 dephosphorylation under mTORC1 inhibition is not sufficient to inhibit overall mRNA translation. In particular, our results show that 4EBP1 dephosphorylation and binding to cap-associated eIF4E is not the mechanism by which methionine deficiency exerts its marked effects on polysome formation.

eIF2α phosphorylation does not correlate with the different effects of deprivation of various amino acids on polysome profiles. The phosphorylation of eIF2α in response to GCN2 activation by single essential amino acid starvation is another well-documented mechanism for regulation of mRNA translation (40). In fact, the ratio of phosphorylated eIF2α to total eIF2α tended to increase with essential amino acid deprivation in our studies and was significantly elevated in cells grown under methionine deficient conditions, suggesting it might be involved in the observed effects of essential amino acid deficiency on polysome formation. However, the degree of eIF2α phosphorylation was not significantly different in cells deprived of leucine or methionine compared to cells deprived of arginine or histidine, suggesting that this could not account for the much greater suppression of polysome formation by deprivation of methionine or leucine verses arginine or histidine. Transfection of an eIF2α phosphomimic also was effective at decreasing the P/M ratio, but the phosphomimic eIF2α was much less effective than methionine or leucine deprivation in suppressing polysome formation despite the higher cellular level of phosphorylated eIF2α plus phosphomimic eIF2α. Simultaneous expression of both phosphomimic eIF2α and mutated nonphosphorylatable 4EBP1 did not yield any effects of polysome ratio beyond those produced by phosphomimic eIF2α alone. Hence, eIF2α phosphorylation does not appear to fully explain the strong and differential effects of essential amino acid deficiency on mRNA translation.

Leucine and Methionine deprivation strongly inhibit mRNA translation through different possible mechanisms. Our most striking observation is the much stronger suppression of polysome formation by deprivation of leucine or methionine compared to deprivation of histidine or arginine. These effects of leucine or methionine could not be accounted for by changes in eIF2α phosphorylation or 4EBP1 association with eIF4E. The differences in the polysome peak number and the shape of the polysome fractions also suggest that leucine and methionine inhibit translation through different mechanisms. Leucine deprivation results in a decrease in polysome area, but did not reduce the number of polysome peaks equal to that of methionine deprivation. This suggests that leucine deprivation results in an overall decrease in the number of ribosomes on the mRNA, but does not inhibit the formation of large polysomes. In contrast, methionine deprivation significantly reduces the number of polysome peaks as well as polysome area, suggesting under methionine deprivation global translation initiation is inhibited resulting in the loss of downstream ribosomes.

Our results do not support a role of 4EBP1 binding to eIF4E in suppressing global mRNA translation in response to amino acid deficiency. Although leucine deprivation clearly strongly inactivated mTORC1's kinase activity as judged by 4EBP1 dephosphorylation, the resulting increase in 4EBP1's binding affinity for eIF4E does not seem to explain the effect of leucine deprivation on polysome formation because the effect could not be reproduced by expression and binding of mutant 4EBP1 T37A/T46A to eIF4E. Methionine deprivation resulted in no significant increase in 4EBP1-eIF4E association and no reduction in eIF4E-eIF4G association indicating mTORC1/4EBP1 was unlikely to be involved in mediating the effects of methionine deprivation. Although leucine-specific effects might still depend on 4EBP1 phosphorylation status, the observation that ectopic expression of a non-phosphorylatable-4EBP1

had no effect, suggests this was unlikely.

The observation that leucine deprivation had a much larger effect on 4EBP1-eIF4G association than did methionine, histidine or arginine deprivation is consistent with leucine having a greater effect on mTORC1 kinase activity and possibly affecting mRNA translation/polysome formation through some other mTORC1 target (e.g. S6K1) (41) or downstream S6K1 target (eIF3, eIF4B) (42, 43). However, a global effect of these targets on mRNA translation has not been shown.

Alternatively, leucine deficiency and its effects on mTORC1/4EBP1 may be selectively inhibiting translation of a subset of mRNAs (e.g TOP-mRNAs), and the decrease in translation of one or more of these selectively affected mRNAs may in turn mediate a more global suppression of mRNA translation to yield the otherwise observed polysome profile. The much larger effect of leucine deficiency compared to other amino acid deficiencies on mTORC1 activity is also consistent with reports of leucine-specific regulation on mTORC1 through the cytosolic proteins Sestrin2 (1) and leucyl-tRNA synthetase (44).

mTORC1 has also been shown to be particularly sensitive to arginine (45). The fact that arginine deprivation was not as effective as leucine deprivation could be a result of the different mechanisms by which mTORC1 senses leucine or arginine concentrations. Arginine has been reported to play a role in retaining mTORC1 at the lysosomal membrane due to interactions with the cationic amino acid transporter SLC38A9 in the lysosomal membrane (46). As with leucine deprivation, our results do not provide insights into possible mechanisms by which a lack of arginine suppresses polysome formation other than indicating that it is unlikely to be mediated by dephosphorylation of 4EBP1.

The ability of methionine to suppress polysome formation suggests that it, like leucine,

plays a unique role in regulating translation. Methionine-specific effects could possibly be mediated by a lack of methionine-charged initiator tRNA or a lack of metabolites of methionine, such as *S*-adenosylmethionine (SAM) or cysteine. SAM is the donor of methyl groups to numerous biological reactions (47). Cysteine is substrate for the synthesis of glutathione, which plays a critical role in maintaining redox balance and preventing oxidative stress. Our methionine-deficient medium contained cysteine so a lack of cysteine/glutathione is not a likely explanation of our findings, but a lack of methionine-charged $tRNA_i^{Met}$ or a lack of methylation reactions are possibilities.

Previous studies have shown that deprivation of a single amino acid results in a decrease in the amount of aminoacylated tRNAs corresponding to that amino acid (23). Therefore, we hypothesize that after 12 hours of exposure to methionine-deficient medium, both methionine initiator and elongator tRNA aminoacylation levels would have decreased, and the decrease in Met-$tRNA_i^{Met}$ levels due to methionine deprivation would inhibit formation of the 43S preinitiation complex as Met-$tRNA_i^{Met}$ binding to eIF2-GTP is facilitated by the presence of methionine on the $tRNA_i^{Met}$. Non-acylated $tRNA_i^{Met}$ has a K_d value of roughly 130 nM for both eIF2-GTP while Met-$tRNA_i^{Met}$ has a K_d of 9 nm to eIF2-GTP (48). This reduction would therefore reduce the number of ribosomes bound to the mRNA, resulting in the observed decrease in the P/M ratio and number of polysome peaks. The hypothesis that mRNA translation initiation is suppressed due to a lack of ternary complex formation is supported by our previous findings that methionine deprivation in murine embryonic fibroblasts resulted in a GCN2- and eIF2α kinase-independent but ATF4 dependent increase in the integrated stress response (Mazor and Stipanuk, submitted). The ability of methionine deprivation to involve an increase in ATF4 level and the integrated stress response in the absence of eIF2α phosphorylation is most easily

explained by an of effect of methionine deficiency that suppresses ternary complex formation. It is also supported by the findings of Dever et al. (49) in *gcn2* knockout yeast. A reduction in the copy number of the initiator tRNA genes in the *gcn2* knockout yeast resulted in an increase in GCN4, the ATF4 analogue, in a manner dependent on the uORF in the GCN4 mRNA. A lack of ternary complex is known to result in an increase in ATF4 translation and subsequently an increase in the expression of genes that are activated by the binding of C/EBP-ATF4 heterodimers to C/EBP-ATF4 response elements on the gene promoter region (50).

Methionine deprivation has been shown to inhibit methylation reactions and as a result reduce the methylation of many macromolecules including DNA. (51, 52). Methylation of DNA and histones has been shown to repress transcription (53), and DNA methylation has been shown to play a role in aging as well as cancer development (54–57). Recent studies have suggested that decreases in methylation due to methionine deprivation can suppress global mRNA translation and activate the integrated stress response independent of GCN2. Working with MCF7 cells, Tang et al (58) demonstrated that methionine deprivation resulted in a transcriptional response that was different from that produced by a lack of other essential amino acids and that was dependent on the decrease in cellular SAM content. Qian and coworkers have recently demonstrated that translation can be initiated independent of the 5′ cap through methylation of adenosine (m^6A) in the 5′UTR (60, 61). They demonstrated that eIF3 binds the m^6A in the 5′UTR and recruits the 43S PIC and can begin translation independent of eIF4E. As methionine deprivation has been shown to reduce methylation reactions, we hypothesize that some of the changes in translation initiation we observed in methionine-deficient HEK293T cells might be attributable to a decrease in this m^6A dependent translation initiation. The lack of response of 4EBP1 phosphorylation status to methionine deprivation along with the large effect methionine

deprivation had on polysome formation would be consistent with a role of methionine-dependent methylation in overall translation initiation.

In summary, the current study demonstrates that the deprivation of leucine or methionine results in a large inhibitory effect on translation initiation and on polysome formation that is not replicated by 4EBP1 dephosphorylation or eIF2α phosphorylation. These results lead us to hypothesize that 4EBP1 alone is not a major regulator of global mRNA translation. It is becoming clear that translation initiation is more complex than previously thought and that much of mRNA translation may depend on cep-independent processes for initiation. Our results also suggest that leucine and methionine each can have major impacts on mRNA translation but act by different mechanisms. Further exploration of the role of leucine and methionine in mRNA translation may help elucidate these mechanisms and extend our understanding of mRNA translation.

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SUMMARY

The overall objective of this work was to increase the understanding on how individual essential amino acids regulate translation and the integrated stress response (ISR). We compared the response of leucine, arginine, methionine and histidine deprivation in MEF cells without a functional GCN2/eIF2α signaling pathway. In wild-type cells, we observed that deprivation of each amino acid resulted in an increase in the mRNA of ATF4, as well as the CARE-containing genes ASNS and 4EBP1. 4EBP2, a non-CARE-containing gene, was not upregulated in response to essential amino acid deprivation. When the GCN2/eIF2α stress response pathway was inhibited in either GCN2 knockout (GCN2(-/-)) or non-phosphorylatable eIF2α(S51A) mutant MEFs, histidine or methionine deprivation resulted in a continued upregulation of CARE-containing genes. Methionine deprivation was particularly effective at increasing CARE-containing genes, independent of GCN2 or eIF2α. The GCN2/eIF2α-independent and dependent pathways were shown to converge at ATF4. When ATF4 was knocked down in GCN2(-/-) or eIF2α(S51A) MEFs, neither methionine nor histidine deprivation resulted in an increase in the mRNA of CARE-containing genes. These results add to our understanding of how individual amino acids regulate the ISR, by demonstrating the presence of a GCN2/eIF2α-independent pathway that resulted in an increase in CARE-containing genes. Additionally, we demonstrated that methionine deprivation is particularly effective at activating this pathway, suggesting a possible mechanism that involves inhibition of translation initiation due to a decrease in initiator tRNA charging.

To further elucidate the effect that single amino acid deprivation has on translation initiation, HEK293T cells were exposed to leucine, arginine, methionine and histidine-deficient media. All amino acids deprived inhibited cell growth equally, but methionine and leucine were

shown to be the most effective at shifting ribosomes from the polysome fraction to the monosome fraction. In addition, methionine and leucine deprivation were shown to have different effects on the phosphorylation status of 4EBP1 and eIF2α. Leucine deprivation did not increase the phosphorylation of eIF2α above control, but did result in a significant increase in 4EBP1 association with eIF4E. By contrast, methionine did not significantly increase 4EBP1-eIF4E association, but did increase eIF2α phosphorylation, suggesting that methionine and leucine inhibit translation through different mechanisms. Overall, these results suggest that methionine and leucine inhibit translation through different mechanisms, with methionine seeming to directly inhibit translation initiation, while leucine inhibits translation through an additional mechanism.

Based on the different effects of methionine and leucine on 4EBP1 and eIF2α phosphorylation, we also aimed to determine what effect these proteins had on translation individually or together in the absence of other signals. To do this we mutated Thr 37 and Thr 46 in 4EBP1 to alanines [4EBP1(T37A/T46A)] to prevent inhibition of 4EBP1-eIF4E binding by mTORC1. We also mutated Ser51 in eIF2α to aspartate [eIF2α(S51D)] to create a phosphomimic protein which functions analogously to phosphorylated eIF2α. 4EBP1(T37A/T46A) when transfected into HEK293T cells was shown to have no effect on either cell growth or translation. eIF2α(S51D) had no effect on cell growth, though it did slightly inhibit the shift of ribosomes from polysomes to monosomes. When 4EBP1(T37A/T46A) and eIF2α(S51D) were co-transfected, there was no additional effect over that observed in cells transfected with eIF2α(S51D) alone, suggesting that eIF2α and 4EBP1 do not work in conjunction to inhibit cell growth and translation. In addition, when HEK293T cells transfected with an empty vector were exposed to leucine or methionine-deficient medium, cell growth was reduced, and there was a

large shift of ribosomes away from the polysome fraction. Together, these results suggest that in the absence of other signals, 4EBP1 binding to eIF4E does not inhibit translation. This calls into question the commonly-believed mechanism that 4EBP1 plays a major role in regulating eukaryotic translation. Although eIF2α phosphorylation was able to inhibit translation in cells grown in sufficient media, it was not to the extent of cells exposed to leucine or methionine-deficient media. This suggests that individual amino acids, when deficient, inhibit translation through a mechanism independent of 4EBP1 and eIF2α.

FUTURE DIRECTIONS

There are several major questions raised by the results in this thesis that warrant further study to be resolved. The first of which is the mechanisms by which methionine deprivation increases ATF4 induction independent of GCN2 and eIF2α. Our hypothesis is that methionine deprivation increases the amount of uncharged initiator tRNA, and as such, decreases the cellular concentration of active ternary complex. We tested this hypothesis by transfecting MEF cells with mutated initiator and elongator tRNAs in order to determine if an increase in initiator tRNA specifically results in the same effects as methionine deprivation in GCN2(-/-) and eIF2α(S51A) MEFs. Our results were inconclusive. However, further attempts could be made with tRNA transfections, or CRISPER to delete genes specifically for initiator tRNA, as was done in yeast.

The second question is: what are the mechanisms that drive the strong inhibition of translation under methionine or leucine deprivation? It could be as simple as differences in cellular concentrations of each amino acid tested, or it could be due to regulation of different pathways. Our hypothesis, that leucine inhibits translation through a mTORC1-dependent mechanism independent of 4EBP1, could be tested by incubating cells with a mTORC1 inhibitor such as torin1 or rapamycin, and then depriving cells of leucine. Measures of translation such as cell growth and polysome profile could be assessed to determine whether leucine deprivation inhibits translation independent of mTORC1. Whether methionine inhibits translation by inhibiting methylation reactions could be assessed by measuring cellular methylation after methionine deprivation, as well as determining if methionine deprivation can inhibit 5’UTR methylation translation initiation.

The third question raised is: what role do 4EBPs play in the regulation of translation? This question could be addressed by mutating all 4EBPs so that they cannot be phosphorylated

and then measuring translation initiation to determine what effect, if any, they have on global translation. The effect that 4EBPs have on the translation of specific mRNAs can be assessed using ribosomal sequencing where changes in the ribosome density of individual mRNAs due to mutation of the 4EBPs could be determined.