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Biochem J. Author manuscript; available in PMC 2013 June 28.

Published in final edited form as:
Biochem J. 2013 January 1; 449(1): 1–10. doi:10.1042/BJ20121098.

Amino acid sensing in dietary-restriction-mediated longevity: roles of signal-transducing kinases GCN2 and TOR

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Abstract

DR (dietary restriction), or reduced food intake without malnutrition, is associated with extended longevity, improved metabolic fitness and increased stress resistance in a wide range of organisms. DR is often referred to as calorie restriction, implying that reduced energy intake is responsible for its widespread and evolutionarily conserved benefits. However, recent data indicate dietary amino acid restriction as a key mediator of DR benefits. In fruitflies, an imbalance in essential amino acid intake is thought to underlie longevity benefits of DR. In mammals, reduced dietary protein or essential amino acid intake can extend longevity, improve metabolic fitness and increase stress resistance. In the present paper we review two evolutionarily conserved signal transduction pathways responsible for sensing amino acid levels. The eIF2α (eukaryotic initiation factor 2α) kinase GCN2 (general amino acid control non-derepressible 2) senses the absence of one or more amino acids by virtue of direct binding to uncharged cognate tRNAs. The presence of certain amino acids, such as leucine, permits activation of the master growth regulating kinase TOR (target of rapamycin). These two signal transduction pathways react to amino acid deprivation by inhibiting general protein translation while at the same time increasing translation of specific mRNAs involved in restoring homoeostasis. Together, these pathways may contribute to the regulation of longevity, metabolic fitness and stress resistance.

Keywords

amino acid; dietary restriction (DR); eukaryotic initiation factor 2α (eIF2α); general amino acid control non-derepressible 2 (GCN2); target of rapamycin (TOR)

INTRODUCTION

Coding amino acids are necessary for protein synthesis, but are also involved in a number of other essential processes. For example, methionine is required for one-carbon transfer reactions, tryptophan is a precursor for NAD and serotonin biosynthesis, glutamate acts as a neurotransmitter, and a number of amino acids can serve as intermediate metabolites in a variety of processes ranging from gluconeogenesis to anaplerosis. Although lower organisms can synthesize all of the coding (and non-coding) amino acids from carbon skeletons and nitrogen, some of these biosynthetic pathways were lost during evolution of multicellular organisms. Thus humans (as well as mice and fruitflies) must acquire the EAAs (essential amino acids) through dietary means.

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DR (dietary restriction), defined loosely as reduced food intake without malnutrition, was first shown to extend longevity in rats by Clive McKay in the 1930s [1]. In the ensuing decades, lifespan extension by DR has been demonstrated in multiple species, along with a number of other benefits, including extended healthspan, improved metabolicfitness and increased stress resistance.A large number of changes have been documented upon DR, including energy metabolism, gene expression, protein turnover, immune function and oxidative stress to name just a few [2]. However, which of these changes are evolutionarily conserved requirements for the benefits of DR remains unclear.

DR is also known as CR (calorie restriction), based, in part, on data in rodents suggesting that the reduction in total calorie intake is more important than the source of those calories (e.g. carbohydrates compared with protein) [3]. Previous data in fruitflies indicate that the source of the calories does matter, and that reduction of protein calories in the form of yeast contributes more to longevity extension than carbohydrate calories [4]. Furthermore, adding back EAAs erases the benefits of DR on longevity extension in fruitflies [5]. In rodents, it has long been known that reduced dietary intake of protein or certain amino acids, namely methionine and tryptophan, can also extend longevity [6,7]. What this has to do with lifespan extension by DR remains unclear, as there are many similarities, but also differences, between rodents on DR and those on methionine restriction [8].

How do cells sense the availability of amino acids, and what role might these mechanisms play in conveying benefits of DR? Cells have evolved different mechanisms to sense both the absence of individual amino acids as well as the presence of some others. Lack of amino acids is generally sensed by a surrogate marker, an uncharged cognate tRNA. Uncharged tRNAs then trigger adaptive responses through a variety of downstream mechanisms to rectify the situation. In bacteria, these involve direct effects on transcriptional control, whereas in eukaryotes uncharged tRNAs trigger a signal transduction pathway via direct interaction with the eIF (eukaryotic initiation factor) 2α kinase GCN(general amino acid control non-derepressible) 2. The presence of certain amino acids, such as leucine, can also be sensed, activating signal transduction pathways that integrate responses to environmental cues, including nutrients, through the TOR (target of rapamycin) kinase. Less is known about upstream amino-acidsensing mechanisms in this pathway. A common output of amino acid deficiency sensed by either mechanism is repressed general translation, but enhanced (or derepressed) translation of particular mRNAs involved in restoring homoeostasis. The mechanisms by which GCN2 activation and TOR repression affect translation are also distinct. In the present review, we will discuss both GCN2-and TOR-based amino-acid-sensing mechanisms, downstream effects on translation and how these pathways may contribute to the benefits of DR, including stress resistance and longevity.

SENSING AMINO ACID DEFICIENCY: FROM UNCHARGED tRNAs TO THE GCN2 KINASE

Uncharged tRNAs activate the stringent response to amino acid deprivation in bacteria

Protein synthesis is universally important to life as we know it, and mechanisms to sense the building blocks of proteins, amino acids, are conserved across evolution. In bacteria, nutrient deficiency activates the stringent response, so-called because of the stringent inhibition of transcription of stable RNAs including rRNA and tRNAs [9]. The stringent response is dependent on unusual nucleotides, ppGpp (guanosine 5′,3′ bispyrophosphate) or pppGpp (guanosine pentaphosphate), collectively referred to here as ppGpp [10,11]. ppGpp binds to the transcriptional regulator RelA and modulates RNA polymerase activity in a promoter-specific fashion, down-regulating some genes and up-regulating others [12]. Although the stringent response can occur upon deprivation of phosphate, carbon, iron or

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fatty acids, it is best understood in response to amino acid deprivation. When intracellular amino acids are low, cognate uncharged tRNAs accumulate and can compete with binding of charged tRNAs to the A-site of ribosomes. When an uncharged tRNA occupies an A-site [13], translation slows and the ribosome-associated ppGpp synthase RelA is activated [11]. ppGpp, in complex with the transcriptional regulator DksA, can then repress stable RNA transcription by binding to RNA polymerase near the active site and inhibiting its activity [14]. At the same time, ppGpp/DksA can activate transcription of amino acid biosynthetic genes.

Uncharged tRNAs trigger the amino acid starvation response in eukaryotes by activating an eIF2α kinase

As in bacteria, intracellular uncharged tRNAs signal amino acid depletion in eukaryotic cells. Unlike bacteria, yeast have a dedicated uncharged tRNA-sensing molecule, the signaltransducing kinase GCN2 [15]. GCN2 has a domain with homology with HisRS (histidyl-tRNA synthetase) that binds to uncharged tRNAs [16,17], resulting in kinase activation through homodimerization and autophosphorylation [15,18]. Besides itself, activated GCN2 has only one other known target, eIF2α. Phosphorylation of eIF2α prevents efficient translational initiation at the starting methionine codon. This slows translation of most mRNAs while at the same time favouring the translation of select mRNAs with specific regulatory elements in their 5′-UTRs (untranslated regions). This phenomenon of selective translational up-regulation of specific mRNAs in the face of overall reduced global translation, known as translational derepression, is described in more detail later in the present review. One such derepressed mRNA encodes GCN4, the main effector of the GAAC (general amino acid control) pathway responsible for activating the transcription of amino acid biosynthetic and transport genes [19,20]. In mammals, the GCN4 orthologue controlling the transcriptional response to amino acid starvation, ATF4 (activating transcription factor 4), is similarly stabilized by translational derepression.

Conditions leading to GCN2 activation

In theory, any uncharged tRNA can bind the HisRS-like domain of GCN2. In yeast, a handful of conditions leading to accumulation of uncharged tRNAs have been experimentally verified to activate GCN2. These conditions include amino acid depletion, mutation of aminoacyl-tRNA synthetase genes or chemical inhibition leading to the accumulation of tRNAs for histidine, tryptophan, lysine, arginine, serine and the BCAAs (branched-chain amino acids) leucine, isoleucine and valine [17]. Interestingly, deprivation of a single amino acid can result in deacylation of non-cognate tRNAs as well. For example, leucine starvation of auxotrophic yeast results in accumulation of uncharged serine and threonine tRNAs in addition to leucine tRNAs [21].

In mammals, GCN2 can be rapidly activated in brain and liver (but apparently not kidney) upon ingestion of a meal lacking EAAs including leucine, histidine, tryptophan or lysine [22–24]. GCN2 can also be activated by non-dietary depletion of amino acids [25]. For example, in the context of an acute stress such as trauma or sepsis, increased NO (nitric oxide) production by NOS (NO synthase) can rapidly deplete the conditional EAA arginine from the blood. Local depletion of the EAA tryptophan in the placenta by the tryptophan catabolizing enzyme IDO (indoleamine 2,3-dioxygenase) can anergize T-cells that might otherwise react against the fetus. This purposeful immunosuppression is dependent on GCN2 expression in T-cells [26]. Other amino-acid-catabolizing enzymes, such as asparaginase, have been co-opted as chemotherapeutic agents against blood-borne cancers; the response to this agent in mice is dependent on GCN2 [27]. Simulated amino acid starvation can also be achieved by blocking the charging of tRNAs with their cognate amino acid. Halofuginone, for example, is a competitive inhibitor of the prolyl-tRNA synthetase

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[28] that can activate the amino acid starvation response *in vitro* and *in vivo* [29,30]. It is used to treat a variety of maladies ranging from psoriasis (an autoimmune disorder) to cancer.

Thus depletion of essential, non-essential or conditionally essential amino acids by dietary, enzymatic or pharmacological means can activate the amino acid starvation response by increasing the concentration of uncharged tRNA species and activating GCN2. Because GCN2 regulates adaptive changes to perceived amino acid deficiency, mice lacking this protein appear normal in the absence of such a challenge. This is not the case for one of the prime effectors of the GCN2 response, ATF4, which is required not only for the response to amino acid insufficiency, but also for the normal anabolic response to insulin, mediated, at least in part, through mTORC [mTOR (mammalian TOR) complex] 1 [31,32]. Cells lacking ATF4 require excess non-EAAs including cysteine (or antioxidants such as glutathione or *N*-acetylcysteine) [33], and ATF4-knockout mice have multiple developmental abnormalities and are smaller than control littermates [34].

Specificity of the GCN2-dependent amino acid starvation response

GCN2 is one of a family of four kinases that share a common target, Ser^{51} of the translation initiation factor eIF2α. In addition to amino acid deficiency, GCN2 can also be activated by glucose deprivation and UV irradiation [35]. The other three eIF2α kinases are activated by diverse signals in different tissues, ranging from haem deficiency in erythrocytes [HRI (haem-regulated eIF2α kinase)] to endoplasmic reticulum stress in pancreatic β-cells {PERK [PKR (double-stranded-RNA-dependent protein kinase]-like endoplasmic reticulum kinase} to infectious or metabolic stress in a variety of tissues (PKR). Global translational suppression and translational derepression of targets such as ATF4 are not unique to GCN2-mediated eIF2α phosphorylation upon amino acid starvation. Nonetheless, some specificity of each response to the appropriate stimulus is maintained, possibly as a result of additional, unique targets [36]. PKR, for example, has other direct targets besides eIF2α, including protein phosphatase 2A [37]. Although GCN2 has no known direct targets besides eIF2α, DNA-PK (DNA-dependent protein kinase) can be phosphorylated in a GCN2-dependent manner upon exposure of cells to UV [38].

SENSING AMINO ACID SUFFICIENCY: ROLE OF THE TOR SIGNAL TRANSDUCTION PATHWAY

Amino acid, energy and growth factor signalling integrated through TOR

Our present understanding of the ability of cells to sense the presence, rather than absence, of specific amino acids is based on genetic and pharmacological perturbation of the signal transduction cascade involving the TOR serine/threonine kinase. TOR is best known for controlling cell size and proliferation in response to the presence of adequate energy, nutrients and growth factors. TOR was first identified as the cellular target of the growth-inhibiting bacterial compound rapamycin, and is conserved from yeast to humans. Yeast has two TOR isoforms: TOR1 and TOR2, which are functionally distinct. Mammalian cells have a single TOR kinase, mTOR, which exists in two structurally and functionally distinct multi-protein complexes: mTORC1 and mTORC2. mTORC1 is acutely inhibited by rapamycin and is sensitive to nutrient levels, whereas mTORC2 is neither readily sensitive to nutrients nor acutely inhibited by rapamycin [39].

The kinase activity of mTORC1 is regulated by its association with the Ras-related GTPase Rheb (Ras homologue enriched in brain). In its GTP-bound form, Rheb directly activates mTORC1. The Rheb-specific GAP (GTPase-activating protein) is the TSC (tuberous sclerosis complex) 2 protein, which functions as a heterotrimer with its binding partners

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TSC1 and TBC1D7 [40,41]. The TSC1–TSC2–TBC1D7 complex is the critical negative regulator of mTORC1 signalling with respect to most of its upstream regulators, such as growth factors, insulin and energy levels [42]. The best-characterized downstream effectors of mTORC1 signalling include 4E-BP1 and 4E-BP2 (eIF4E-Binding proteins 1 and 2), and the p70 ribosomal protein S6K1 and S6K2 (S6 kinases 1 and 2). These proteins are directly phosphorylated by mTORC1, and are thus commonly used as markers of TOR activity [43].

Select amino acids regulate TORC1 signalling

Amino acid levels regulate both protein synthesis and autophagic proteolysis. Evidence supporting the role of mTORC1 in this regulation came from cell culture studies in which phosphorylation of mTORC1 substrates S6K and 4E-BP were associated with translational control and inhibition of autophagy upon amino acid stimulation [44–48]. In rat adipocytes, addition of amino acids to the medium stimulates S6K phosphorylation, which is sensitive to rapamycin treatment [46]. In CHO (Chinesehamster ovary) cells, amino acid withdrawal results in a rapid dephosphorylation of S6K and 4E-BP1 and abolishes the ability of growth factors to stimulate S6K activity. Re-addition of single amino acid dropout mixtures lacking leucine or arginine reduces S6K activity by 90% or 70% respectively [45]. Interestingly, re-addition of either leucine or arginine individually is unable to stimulate S6K activity. Similar studies carried out in different contexts and cell types, including H4IIE hepatoma, L6 muscle and pancreatic β-cells, revealed important details about the regulatory properties of individual amino acids on TOR activity. All agreed on the prominent role of leucine [47,49–51]. However, in certain contexts, arginine stimulates S6K activity [52], whereas in others each of the BCAAs (leucine, isoleucine and valine) has similar potency in stimulating 4E-BP1 phosphorylation [47]. The non-EAA glutamine can also modulate leucine- and argininestimulated mTORC1 signalling, although in different directions in different cell types. In rat intestinal epithelial cells and myogenic C2C12 cells, glutamine antagonizes arginine- and leucinemediated mTORC1 activation respectively [52–54]. In isolated rat hepatocytes, leucine and glutamine work additively to increase mTORC1 signaling [50]. In HeLa cells, the interaction between glutamine and leucine has been ascribed to a heterodimeric bidirectional amino acid antiporter consisting of SLC7A5 (solute carrier family 7 member 5)/SLC3A2 (solute carrier family 3 member 2) that regulates the simultaneous efflux of glutamine and influx of leucine and other EAAs [55]. Interestingly, in a previous study, carried out in a variety of cell types, it was shown that leucine and glutamine co-operate to activate mTORC1 signalling through enhancing glutaminolysis and 2-oxoglutarate (α-ketoglutarate) production [56].

In yeast auxotrophs, deprivation of leucine (and to a lesser degree lysine and histidine) reversibly inhibits TORC1-dependent phosphorylation of Sch9 (the yeast orthologue of S6K). Inhibition of protein synthesis with cyclohexamide results in accumulation of intracellular free amino acids, with leucine accumulating more than others, and increases Sch9 phosphorylation [57]. Taken together these observations are consistent with leucine primacy in TOR activation crossing evolutionary boundaries.

Bridging the GAP between leucine sensing and TOR activation

Unlike the GCN2 pathway, the most upstream mechanisms directly responsible for amino acid sensing permissive of TOR activation, and even the subcellular compartment in which this occurs, remain unresolved. TOR itself does not appear to bind amino acids or surrogates such as cognate tRNAs as GCN2 does. A number of candidates have been proposed as intermediates in TOR activation by amino acids. Some initial studies suggested a role for TSC1/TSC2 in amino acid sensing. However, budding yeast do not have TSC proteins, and mammalian TSC1- or TSC2-null cells are still sensitive to amino acid withdrawal, suggesting that amino acids signal to mTORC1 independently of TSC1/TSC2 [40,58].

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hVps34 (human vacuolar protein sorting 34) has also been proposed to participate in amino acid sensing. This class III PI3K (phosphoinositide 3-kinase) participates in the regulation of mTORC1 by amino acids, possibly through its effects on vesicle trafficking and/or through generation of membrane compartments necessary for mTORC1 activation [59,60].

Two independent groups, using complementary biochemical and genetic approaches, showed that the Rag family of small GTPases is required for amino acid sensing by mTORC1 [61,62]. Rag GTPases are orthologues of the yeast Gtr1p and Gtr2p GTPases, which function as obligate heterodimers and regulate the Gap1 amino acid permease and microautophagy. The critical role of Rags in mTORC1 signalling and its regulation of cellular growth is further evidenced by *in vivo* experiments in *Drosophila*, where constitutively active RagA leads to flies with increased cell and wing size, whereas dominant-negative RagA leads to flies with decreased wing size [62]. Mammalian Rags also exist in heterodimers with one Gtr1p-like (RagA or RagB) and one Gtr2p-like (RagC or RagD) partner [63]. Unlike the Rheb GTPase, purified Rag GTPases are unable to stimulate mTORC1 kinase activity *in vitro* [62].

Instead, Rag-mediated mTORC1 activation involves recruitment of the complex to the outer lysosomal membrane, where mTORC1 can interact with its activator Rheb [61,64]. The Rag heterodimer is anchored to the lysosomal membrane via the protein complex called 'Ragulator' [64,65] and activated by the presence of amino acids. RagA/B in its GTP-bound form with RagC/D in its GDP-bound form constitutes the active heterodimer, which recruits mTORC1 to the lysosomal membrane through an interaction with Raptor (regulatoryassociated protein of mTOR). The Ragulator complex is functionally analogous to the yeast EGO complex due to its interaction with Gtr1p and Gtr2p, and its regulation of amino acid signalling to TOR at the vacuolar membrane [57,64]. Thus spatial regulation of the mTORC1 complex has emerged as an important aspect of amino-acid-mediated control [66].

Lysosomes/vacuoles are a major site of protein degradation and amino acid recycling with high concentrations of free amino acids. On the basis of mTORC1 localization and activation at the cytoplasmic face of the lysosomal membrane, the relevant amino-acid-sensing mechanism has been proposed to sense lysosomal rather than cytoplasmic free amino acid pools [64]. A *Drosophila* siRNA (small interfering RNA) screen targeting the lysosomal components that might be involved in amino acid signalling to TOR revealed the requirement of V-ATPase (vacuolar ATPase), and this was confirmed in mammalian cells [67]. Although the ATPase activity of V-ATPase is required for amino acid permissive signalling, V-ATPase does not function in transport of amino acids between the cytoplasm and the lysosome. V-ATPase does, however, appear to function upstream of the Rag–Ragulator interaction, in which amino acids stimulate GEF (guaninenucleotide-exchange factor) activity of the pentameric Ragulator complex towards RagA and RagB [68]. In further support of the importance of lysosomal free amino acid pools, stimulation of amino-acid-starved cells with radioactively labelled amino acids leads to their rapid appearance in the lysosome [67].

At face value, this lysosome-centred rather than cytoplasmiccentred view of amino acid sensing appears to be at odds with a previous study identifying the leucyl-tRNA synthetase as the intracellular leucine sensor responsible for mTORC1 activation [69]. siRNA directed at the leucyl-tRNA synthetase renders HEK (human embryonic kidney)-293T cells unable to phosphorylate S6K in response to leucine or isoleucine withdrawal and restimulation. Consistent with its requirement for leucine-permissive mTORC1 activation, the leucyl-tRNA synthetase also localizes to the cytoplasmic face of the lysosomal membrane, interacts with the Rag GTPase and has GAP activity specifically towards RagD, promoting the active

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GDP-bound form in the presence of leucine. Its ability to stimulate mTORC1 is dependent on leucine binding, but is independent of its ability to charge leucine tRNA. Nonetheless, the subcellular localization of the free leucine, and whether other TOR-permissive amino acids such as arginine function through similar mechanisms, remain to be seen.

MECHANISMS OF GCN2- COMPARED WITH TOR-BASED TRANSLATIONAL CONTROL

Translation of mRNA is one of the most energy requiring processes in the cell. It must therefore be coupled to the availability of cellular energy and coding amino acids. The immediate response to energy or amino acid insufficiency is to repress translation. At the same time, cells increase translation of specific messages involved in metabolic adaptation, for example transcription factors controlling the expression of amino acid biosynthetic and transport genes. This process is known as translational derepression. Just asGCN2 and TOR pathways sense amino acids by different mechanisms, so do they differ in the ways they accomplish translational repression and derepression (Figure 1).

Translational regulation by GCN2

Translation initiation occurs upon pairing of the AUG start codon with Met-tRNA$_i^{Met}$ (initiator methionyl-tRNA), which is part of the so-called ternary complex together with eIF2 and GTP. This is followed by hydrolysis of eIF2-bound GTP and release of eIF2-GDP from the 43S ribosome. Phosphorylation of the α subunit of eIF2 by GCN2 inhibits GDP/GTP exchange by eIF2B, reducing ternary complex availability and thus repressing initiation of translation of most mRNAs.

Some mRNAs, however, contain short upstream ORFs (open reading frames), or μORFs (microORFs), that influence translation of the downstream full-length ORF depending on the status of eIF2α phosphorylation. Reinitiation of translation following μORF translation requires reassociation of the ternary complex. This depends both on the concentration of ternary complex (and hence eIF2α phosphorylation status) and the scanning distance/ time to the next AUG codon. Increasing the distance between μORFs increases the chance of ternary complex association before reaching the next AUG start codon [70]. Under non-starvation conditions, reinitiation of translation after the first μORF is relatively efficient, decreasing the chance of translation of the downstream full-length ORF. Under amino-acid-starvation conditions, reinitiation after the first μORF is less efficient, increasing the chance that the ribosome will scan past subsequent μORFs and reinitiate translation further downstream at the full-length ORF. Thus the distance between μORFs and timing of ribosomal reinitiation are critical factors in translational derepression of mRNAs such as *GCN4* upon intracellular amino acid starvation. Stabilization of ATF4 occurs by a similar mechanism [71], indicating conservation of this simple, yet elegant, translational control mechanisms from yeast to mammalian cells.

Translational regulation by mTOR

The mTORC1 pathway controls protein synthesis most directly by phosphorylating and inhibiting a repressor of cap-dependent mRNA translation, 4E-BP1. Translation of most cellular mRNAs is initiated via this common mechanism involving assembly of eIF4F (comprising eIF4E, eIF4G and eIF4A) on the 5′ 7-methyl-GTP cap. eIF4F is required for the loading of the small ribosomal subunit on the mRNA. 4E-BPs repress translation by inhibiting the formation of the eIF4F complex [72]. Phosphorylation of 4E-BPs by mTORC1 results in their dissociation from eIF4E, facilitating cap-dependent translation [40]. Acute inhibition of mTORC1 with rapamycin, or the more potent inhibitor Torin 1, reduces protein synthesis by approximately 30 % or 60 % respectively, without affecting the

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status of eIF2α phosphorylation [25]. Torin 1 inhibits translation of nearly all (99.8 %) cellular mRNAs, but the magnitude of repression is greatest for mRNAs containing 5′ TOP (terminal oligopyrimidine) motifs in their 5′-UTRs. Importantly, this class of mRNAs encodes all ribosomal proteins and major translation factors, therefore influencing the protein synthetic capacity of the cell. Torin-1-mediated translational repression requires 4E-BPs and is lost in cells lacking these proteins [25].

mTORC1 also regulates translation indirectly through phosphorylation of S6K. Phosphorylation on Thr^{389} activates S6K towards ribosomal protein S6, a component of the 40S ribosome important for translation in rapidly proliferating cells. It also phosphorylates a number of other downstream targets including eIF4B, eEF2K (eukaryotic translation elongation factor 2 kinase) and SKAR [72]. Phosphorylation of S6K targets can promote translation by a variety of direct and indirect mechanisms, including regulation of eIF4B recruitment, inhibition of PDCD4 (programmed cell death protein 4)-mediated inhibition of eIF4A, splicing-dependent translation of some mRNA messages via SKAR, and regulation of ribosome biogenesis [73,74]. The ability of amino acids to regulate phosphorylation of both S6K and 4E-BPs was shown in previous studies [47].

Although mTORC1 inhibition represses cap-dependent translation, it also selectively derepresses translation of select mRNAs that do not depend on cap-binding for ribosome entry. Conceptually, this parallels translational derepression upon GCN2 activation. However, instead of a μORF, it depends on an IRES (internal ribosome entry site) in the 5′-UTR for cap-independent ribosome recruitment. IRES-containing mRNAs are not only resistant to mTORC1 inhibition by Torin 1, but are in fact translated with greater efficiency under such conditions [25]. IRES-containing mRNAs are often involved in the response to stress, for example NRF2 (NF-E2-related factor 2), the master transcriptional regulator of genes in the Phase II response to oxidative/electrophilic stress. NRF2 is translationally up-regulated during oxidative stress despite the attenuation of global protein synthesis due to an IRES-dependent mechanism [75,76]. However, whether NRF2 is translationally derepressed upon amino acid starvation or mTORC1 inhibition remains to be elucidated.

Protein deficiency is expected to simultaneously repress mTOR and activate GCN2. How can efficient translational derepression occur when mRNA cap-binding and translation initiation are both inhibited by mTORC1 repression and GCN2 activation respectively? One possibility is that mRNAs subject to control by GCN2/eIF2α lack TOP motifs, limiting the effect of mTORC1/4E-BP-based repression on cap-binding. Another possibility is that IRES-containing mRNAs also contain μORFs; CAT1 (cationic amino acid transporter 1) is an example of such an mRNA [77]. IRES-mediated translational derepression can also occur despite phosphorylation of eIF2α. For example, translation of XIAP (X-linked inhibitor of apoptosis) occurs via an eIF2α-independent mechanism of translation initiation dependent on eIF5B [78]. Thus translational derepression probably depends on features within a particular 5′-UTR, as well as the status of both GCN2 and TOR activity.

CO-ORDINATION OF AMINO ACID STARVATION RESPONSES THROUGH GCN2 AND TOR

Why did separate amino-acid-sensing pathways evolve together in eukaryotes? Are they redundant or do they play separate roles? What is the connection between the two? Amino acid sensing through the TOR pathway may have evolved primarily to coordinate information on the presence of adequate nutrients (including amino acids), energy and other environmental conditions favourable for growth. Leucine and the other BCAAs are abundant in a variety of proteins. In mammals, they are also the only free amino acids to increase in peripheral blood after a meal in proportion to their levels in the diet (the

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remaining 17 are retained in the gut and liver and released in a controlled fashion) [79]. These qualities of BCAAs may be advantageous for a surrogate marker of availability of all amino acids.

GCN2-based sensing of individual amino acid deficiencies may have evolved for different reasons. Dietary amino acid deficiencies are common and must be detected rapidly in order to avoid negative effects ranging from reduced growth to fatty liver [80]. Protein sources with amino acid deficiencies include rice (low amounts of the EAA lysine), casein (limiting for both tryptophan and methionine) and gelatin (lacks tryptophan altogether). Xenobiotic anti-metabolites targeting individual amino acid metabolic pathways (for example, tRNA synthetase inhibitors) may also be widespread in Nature. Interestingly, yeast can make all 20 coding amino acids, and thus do not activate the GAAC response when amino acids are absent (for example in minimal growth media) [81]. However, they do activate the response when individual amino acids are deficient or in the presence of tRNA synthetase inhibitors. Thus the GCN2-based GAAC response may have evolved under different selective pressures than the TOR-based response, although both function to optimize growth potential and prioritize metabolic demands to fit environmental conditions.

Are responses to amino acid deprivation through GCN2 and TOR co-ordinated? In yeast, GCN4 translation is derepressed upon inhibition of TOR with rapamycin in a GCN2- and TOR1-dependent fashion [82]. Mechanistically, TOR inhibition may reduce inhibitory phosphorylation of GCN2, thus promoting eIF2α phosphorylation and GCN4 translational derepression. GCN4 is also a major effector of the transcriptional response to TOR inhibition by rapamycin on a scale equivalent to the canonical transcriptional activator and TOR substrate GLN3 [83,84]. Genes co-regulated by both GCN4 and GLN3 include those involved in nitrogen assimilation, amino acid transport, amino acid biosynthesis and other transcriptional activators.

Cross-talk between GCN2 and mTOR also exists in mammalian cell culture and animal models, although the direction of interaction appears to be different than in yeast. In human lymphocytic leukaemic cell lines, treatment with L-asparaginase, an enzyme that degrades asparagine in culture medium and activates GCN2, inhibits mTORC1 phosphorylation of its targets S6K and 4E-BP1 in a dose-dependent fashion [85]. Likewise, S6K activity is reduced in human T-lymphoblastoid cells exposed to various amino acid alcohols that selectively inhibit specific tRNA loading, including L-histidinol, L-leucinol, L-phenylalaninol and L-methioninol [86]. Both studies suggest cross-talk between GCN2 and mTORC1 signalling, with uncharged tRNAs initiating the changes in signal transduction.

Genetic evidence in mice is also consistent with GCN2 activation occurring upstream of mTORC1 repression. In response to dietary leucine deprivation or asparaginase treatment, phosphorylation of mTORC1 targets S6K and 4E-BP1 is reduced in the liver and pancreas, and this depends on the GCN2 kinase [24,27]. Leucine-deficient diets also improve insulin signalling in the liver as measured by increased phosphorylation of the insulin receptor, and whole-body insulin sensitivity as measured by an insulin tolerance test [87]. However, in GCN2-knockout mice on a leucine-deficient diet, mTOR signalling in the liver is increased and the improvement in insulin sensitivity is lost. Interestingly, GCN2-knockout mice on a leucine-deficient diet also develop hepatic steatosis due to increased expression of SREBP (sterol-regulatory-element-binding protein) 1c-dependent lipogenic genes including *Fasn* (fatty acid synthase), *ApoC4* (apolipoprotein C-IV) and *G6pd* (glucose-6-phosphate dehydrogenase), which are under the control of mTORC1 in the liver. This suggests a model in which failure to repress mTORC1 allows inappropriate activation of SREPB1c targets [88,89]. Potentially complicating this model is the fact that leucine deprivation on its own would be predicted to reduce mTORC1 activity by GCN2-independent mechanisms.

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Nonetheless, taken together these findings are consistent with a model in which GCN2 activation by pharmacological or dietary means can suppress mTORC1 activity.

By what mechanisms could GCN2 inhibit mTORC1 in mammals? Three ATF4 transcriptional targets may contribute to reduced mTORC1 signalling downstream of GCN2 activation. GADD34 (growth-arrest and DNA-damage-inducible protein 34) is a phosphatase that removes an inhibitory phosphate on TSC2, thus repressing mTORC1, whileatthe same time turning down the GCN2 responsebyremoving the phosphate on Ser^{51} of eIF2α [90]. 4E-BP1[91] and the TSC activator/mTORC1 repressor REDD1 (regulated in development and DNA damage responses 1) [92], can also be up-regulated at the transcriptional level by ATF4 upon endoplasmic reticulum stress, although their ability to effect mTORC1 activity following eIF2α phosphorylation by GCN2 has not been demonstrated [27].

AMINO ACID SENSING IN DR BENEFITS

Dietary protein/amino acid modulation and longevity

DR has been intricately associated with aging research since the discovery in the 1930s that reduced food intake extends longevity of experimental rodents [1]. Today, we know that the benefits of DR occur in a variety of organisms and are pleiotropic in nature. These benefits include enhanced metabolic fitness and improved resistance to multiple forms of acute stress, ranging from heat shock to surgical ischaemia/reperfusion injury. Still, lifespan extension in a variety of model organisms is the most studied and perhaps best understood of its pleiotropic benefits.

Classically, DR has been described as reduced food intake without malnutrition. DR is not a single intervention, but it rather loosely describes a variety of interventions ranging widely in both dietary composition and timing of food intake [93]. Despite a number of studies aimed at dissecting the nutritional basis of DR, no consensus yet exists onthe relative contributions of overall reduced calorie intake compared with the restriction of particular macronutrients such as protein [4].

In yeast, chronological lifespan can be extended by restriction of asparagine, glutamate or methionine in the medium, in addition to deletion of TOR1 or Sch9, inhibition of glutamine synthetase and rapamycin treatment [94]. Unfortunately in worms, another well-characterized model organism in which the genetics of longevity extension and DR are particularly well characterized, the lack of purified diets and the ability of starvation to increase the lifespan of adults makes these questions difficult to address.

In fruitflies, reduction of the calories via titration of the sole source of protein, yeast extract, provides greater longevity extension than isocaloric reduction of sucrose [4]. Adding back purified EAAs to a restricted sucrose/yeast-based diet optimized for longevity abrogates lifespan extension, whereas adding back EAA minus methionine (or to a lesser degree tryptophan) does not [5]. Perhaps most unexpectedly, adding back methionine alone (but not other individual EAAs) on top of the DR regimen abrogated a well-known negative consequence of DR, reduced fecundity. Although the involvement of reduced insulin-like peptide signalling is implicated in longevity benefits [5], the status of GCN2 or TOR under conditions of DR and EAA add-back remains to be reported.

In rodents, a number of studies have reported moderate lifespan extension upon dietary protein restriction as with DR [95]. However, interpretation of DR and protein restriction studies are both complicated by the fact that protein and carbohydrates are readily interconverted *in vivo*. A different approach was to reduce individual dietary EAAs, namely

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tryptophan and methionine. It is not clear why only these two have been tested in rodents for longevity benefits. In the case of tryptophan, there is some evidenceofincreased maximal lifespan and a delay in aging-related phenotypes in at least a subset of tryptophan-restricted rats [7,96]. The underlying mechanistic hypothesis in these studies involved reduction of serotonin, a downstream metabolite of tryptophan. Likewise, methionine restriction has been shown to extend lifespan in male rats and mice [6,8].

Interestingly, tryptophan and methionine are the two least abundant EAAs by weight in casein, a protein commonly used in purified diets for experimental rodents. Thus DR regimens using casein as the sole source of protein would be expected, at some level of restriction, to become limited for these two EAAs. Indeed, there are many overlapping phenotypes shared both by DR and isolated methionine restriction, including reduced adiposity, extended maximal longevity, increased resistance to acetaminophen toxicity in the liver, reduced insulin and IGF1 (insulin growth factor 1) levels and reduced thyroid hormone [8]. Nonetheless, there are differences as well, and future studies will be necessary to address whether the benefits of each are derived from fundamentally distinct or overlapping mechanisms.

To the best of our knowledge, other amino-acid-restricted diets have not been tested for lifespan extension, but have been shown to induce a number of other benefits. For example, methionine restriction contributes to adiposity resistance by altering the lipogenic/lipolytic balance [97], leucine deprivation improves insulin sensitivity [87] and tryptophan deprivation protects against surgical ischaemia/reperfusion injury to both kidney and liver [29]. The latter two benefits are absent from mice lacking GCN2.

Role of GCN2 and TOR in protein/amino acid restriction benefits

What roles do the TOR and/or GCN2 signal transduction pathways play in regulating longevity, metabolic fitness and stress resistance upon restriction of protein or specific amino acids? There is ample evidence that reduction of TOR signalling through genetic or pharmacological manipulation can extend longevity in a variety of organisms. For example, deletion of TOR1 extends lifespan in yeast and rapamycin treatment extends lifespan in rodents [98]. Genetic ablation of the downstream target of TOR, Sch9 in yeast and S6K in rodents, can also extend longevity [99,100]. In flies, 4E-BP is up-regulated upon DR and may contribute to translational derepression of nuclear-encoded mitochondrial electron transport chain components [101]. Because DR can reduce TOR signalling to downstream targets including S6K and 4E-BP, this could be a primary mechanism underlying its longevity effects (Figure 2).

Aside from the essential role of GCN2 in surgical stress resistance by short-term tryptophan deficiency [29], there is little in the DR or longevity literature on the potential role of this amino-acid-deprivation sensing kinase. This is perhaps surprising in light of established models of longevity extension involving amino acid restriction in rodents or amino acid imbalance in flies, combined with data suggesting the ability of GCN2 activation to inhibit mTOR activity upon amino acid deprivation. It may be that redundancy in mammalian eIF2α kinases, as well as multiple additional routes of mTOR inhibition, may abrogate the specific genetic requirement for GCN2 in a number of settings. Nonetheless, activation of GCN2 via dietary or anti-metabolite means would seem a productive area of future research, including the role of translational control in downstream benefits (Figure 2).

In yeast, GCN4 stabilization has been implicated in both chronological and replicative longevity extension [102,103]. In the replicative longevity model, lifespan is extended by knocking out any of a number of 60S ribosomal subunits or inhibiting 60S subunit biogenesis with small molecules [102]. By reducing the 60S subunit, translational initiation

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becomes inefficient, mimicking the effects of GCN2 activation and eIF2α phosphorylation on downstream GCN4 stabilization. Thus transcriptional reprogramming by GCN4 may play a role in longevity extension, whether or not it is stabilized upon GCN2 activation or by some other process.

In addition to translational control, GCN2 and TOR control a number of other processes that could also contribute to the reported benefits of DR, including autophagy, energy metabolism, immune function and food intake. Autophagy is an adaptive process that provides biological material (amino acids and lipids) to sustain anabolic processes [104] under conditions of nutrient depletion, including DR. mTORC1 negatively regulates autophagy by suppressing the ULK1 complex via phosphorylation [43]. In yeast, GCN2 and GCN4 are required for the induction of autophagy upon amino acid depletion [105,106]. DR also results in changes in metabolic pathways regulated, at least in part, by mTORC1, including fatty acid synthesis, glycolysis and the pentose phosphate pathway via the transcription factors HIF1α (hypoxia-inducible factor 1α) and SREBP [89,107]. GCN2 can also have an effect on fat metabolism. In addition to activation of amino acid biosynthesis and transport, GCN2 is required for suppression of fatty acid synthesis in the liver upon leucine deprivation [88]. Although this particular function of GCN2 is independent of ATF4, the broader role of this transcription factor in GCN2-dependent effects remains to be explored.

Immune function, including autoimmunity, can have a major impact on lifespan and aging-related disease in multicellular organisms. Activation of GCN2 upon local tryptophan depletion by the tryptophan-catabolizing enzyme IDO is one strategy to reduce T-cell proliferation and induce tolerance towards such unintended targets as apoptotic cells in the spleen [26] or the allogeneic fetus. Activation of the amino acid starvation response by the small molecule halofuginone, a prolyl-tRNA synthase inhibitor [28], prevents differentiation of inflammatory Th17 cells [30]. mTOR inhibition by rapamycin can also induce tolerance by suppressing T-cell proliferation, but is paradoxically pro-inflammatory in the context of innate immune activation [108,109].

Finally, GCN2 and mTOR can both participate through different mechanisms in behavioural control of food intake. Central administration of leucine activates hypothalamic mTOR and reduces food intake [110]. Diets lacking one or more EAAs cause an aversion to food intake centred in a different brain region, the APC (anterior piriform cortex) [111]. GCN2 is activated rapidly in the APC upon dietary amino acid deprivation or stereotactic injection of amino acid alcohol derivatives that compete for tRNA synthetases, resulting in uncharged tRNA accumulation [3,23]. Nonetheless, mice lacking GCN2 still display aversion to incomplete diets over the period of days to weeks [24,29,88], suggesting a redundant mechanism of amino acid deprivation sensing. Future studies will be required to elucidate the role of food aversion, if any, to the benefits of amino acid restriction.

Pharmacological activators of the amino acid starvation response as DR mimetics?

DR mimetics can be loosely defined as interventions that mimic some beneficial aspect of DR, for example lifespan extension, maintenance of metabolic fitness upon challenge with a high-fat diet or increased stress resistance. Metformin and rapamycin both extend lifespan in rodents, and are thus considered DR mimetics [98,112] (Figure 2). Halofuginone is a prolyl-tRNA synthetase inhibitor that activates the amino acid starvation response by mimicking proline deprivation. Like short-term EAA deprivation, halofuginone can increase resistance to renal ischaemia/reperfusion injury in a GCN2-dependent manner [29]. This serves as proof-of-principle that compounds that activate GCN2 and stimulate the amino acid starvation response can have benefits similar to dietary protein/amino acid restriction. Nonetheless, this anti-metabolite and others in its class are toxic due to on-target effects of

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tRNA synthetase inhibition. More desirable would be a DR mimetic that would activate the GCN2 kinase directly without inhibiting tRNA charging, or selectively inhibit amino acid sensing through the mTOR pathway. Our understanding of the mechanisms underlying amino acid sensing will probably improve the chances of identifying such a compound with potential beneficial uses in humans.

Acknowledgments

We thank Brendan Manning, Chris Hine and members of the Mitchell laboratory for critical feedback on the text before submission.

FUNDING

Work of the authors is funded, in part, by the National Institutes of Health National Institute on Aging [grant number AG036712], National Institute of Diabetes and Digestive and Kidney Diseases [grant number DK090629], Interdisciplinary Training in Genes and the Environment [grant number T32 ES016645], the American Federation for Aging Research, the Ellison Medical Foundation and the Glenn Foundation for Medical Research.

Abbreviations used

APC	anterior piriform cortex
ATF4	activating transcription factor 4
BCAA	branched-chain amino acid
DR	dietary restriction
EAA	essential amino acid
4E-BP	eIF4E-Binding protein
eIF	eukaryotic initiation factor
GAAC	general amino acid control
GAP	GTPase-activating protein
GCN	general amino acid control non-derepressible
HisRS	histidyl-tRNA synthetase
IDO	indoleamine 2,3-dioxygenase
IRES	internal ribosome entry site
mTOR	mammalian target of rapamycin
mTORC	mTOR complex
NRF2	NF-E2-related factor 2
ORF	open reading frame
μORF	microORF
PKR	double-stranded-RNA-dependent protein kinase
Rheb	Ras homologue enriched in brain
siRNA	small interfering RNA
S6K	S6 kinase
SREBP	sterol-regulatory-element-binding protein
TOP	terminal oligopyrimidine

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TOR	target of rapamycin
TSC	tuberous sclerosis complex
UTR	untranslated region
V-ATPase	vacuolar ATPase

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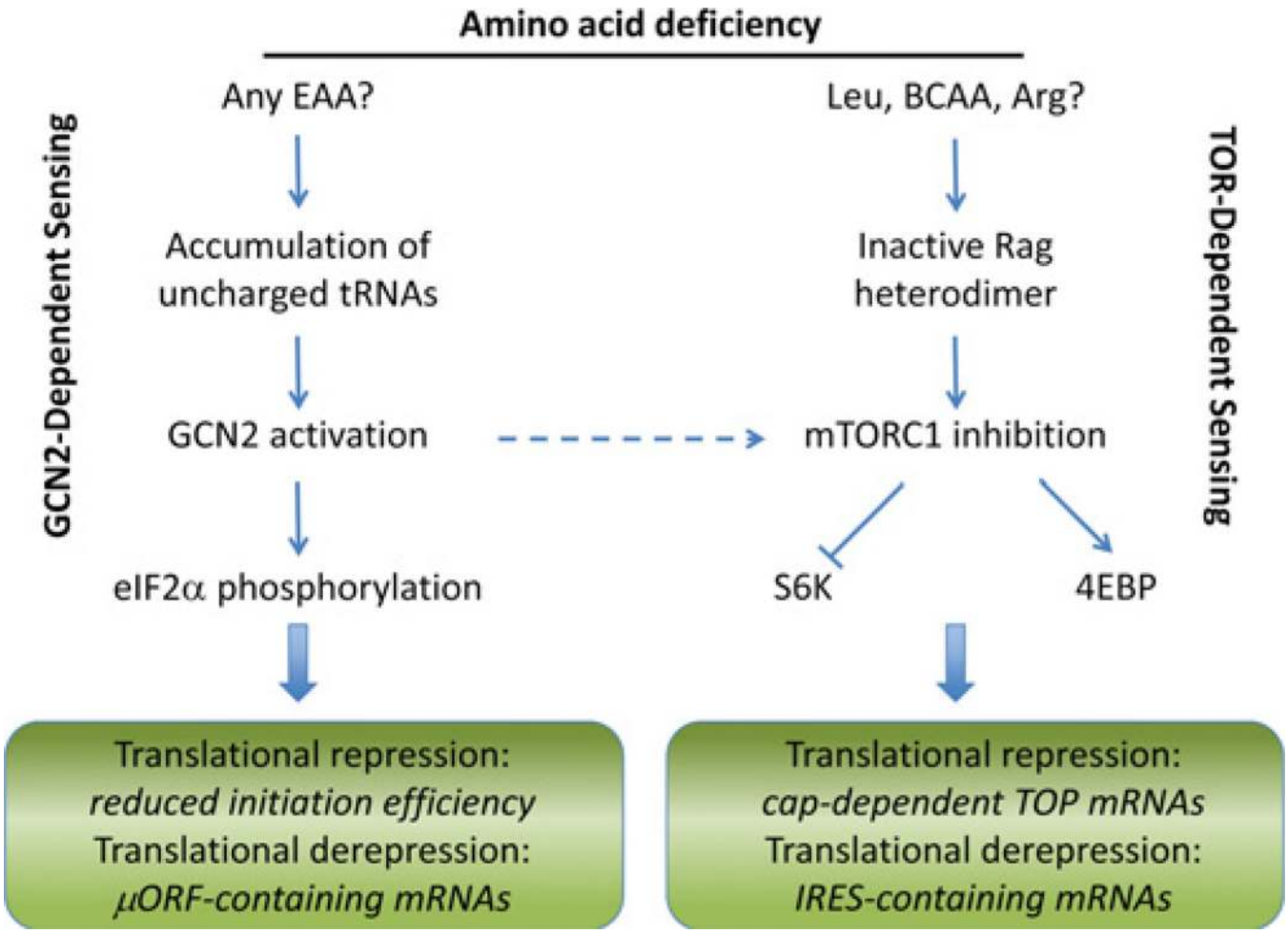

Figure 1. Integration of amino acid sensing with translational control

Two signal transduction mechanisms are involved in sensing intracellular amino acids: GCN2 senses the absence of many amino acids and the TOR pathway senses the presence of particular amino acids. Both repress general translation and derepress translation of specific messages through distinct mechanisms.

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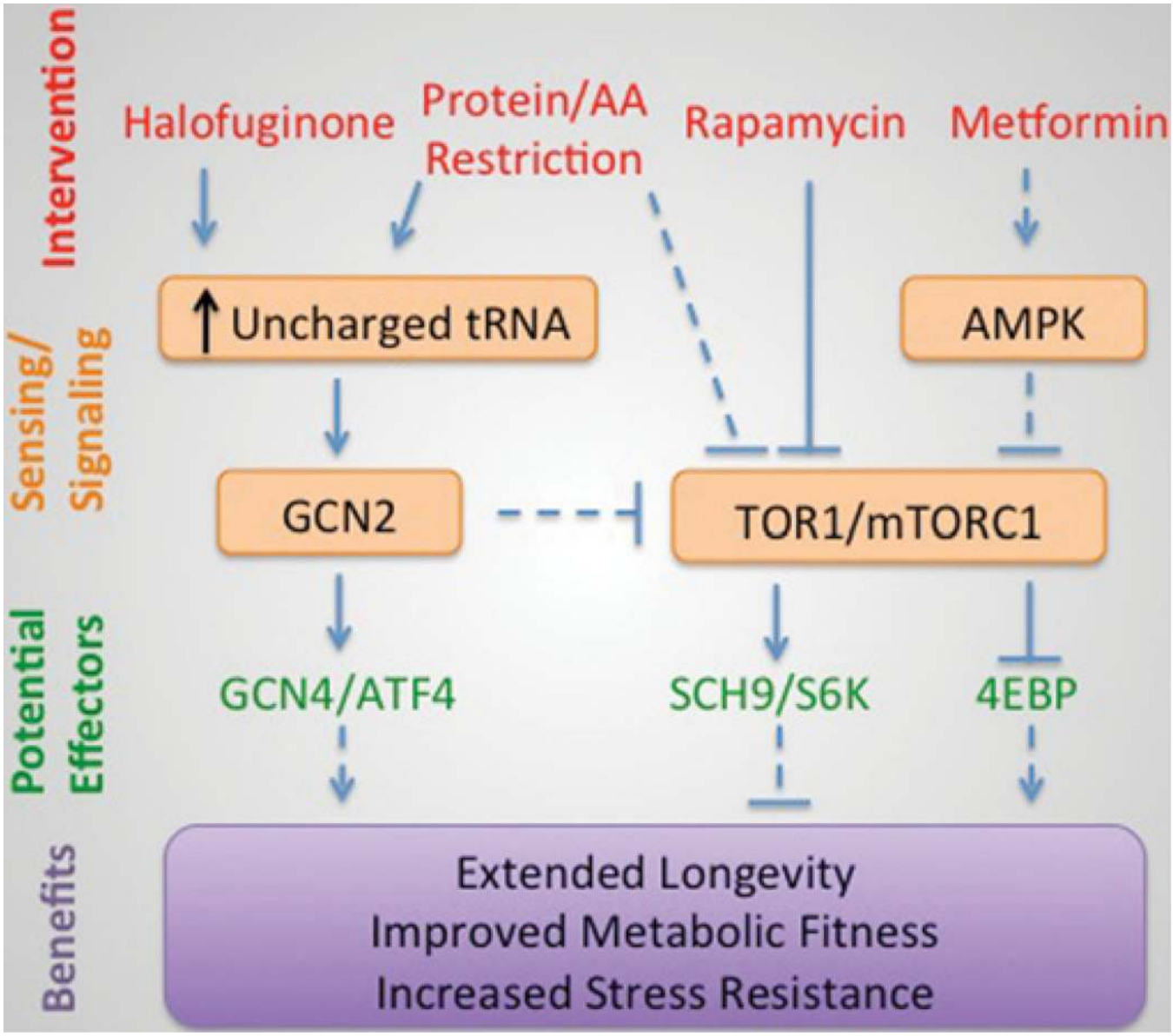

Figure 2. Model for amino acid sensing pathways in DR benefits

Modulation of activity of GCN2 or TOR pathways by dietary or pharmacological interventions can lead to benefits. Not all interventions necessarily lead to all benefits. Broken lines indicate indirect or hypothesized effects; solid lines indicate known or direct effects. In some cases, yeast/mammalian gene names are both indicated respectively. AMPK, AMP-activated protein kinase.