Time-resolved analysis of amino acid stress identifies eIF2 phosphorylation as necessary to inhibit mTORC1 activity in liver

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Running title: eIF2 phosphorylation is necessary to inhibit mTORC1 in liver

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ABSTRACT

Amino acid availability is sensed by general control nondepressible 2 (GCN2) and mechanistic target of rapamycin complex 1 (mTORC1), but how these two sensors coordinate their respective signal transduction events remains mysterious. In this study we utilized mouse genetic models to investigate the role of GCN2 in hepatic mTORC1 regulation upon amino acid stress induced by a single injection of asparaginase. We found that deletion of Gcn2 prevented hepatic phosphorylation of eukaryotic initiation factor 2 alpha (eIF2) to asparaginase and instead unleashed mTORC1 activity. This change in intracellular signaling occurred within minutes and resulted in increased 5′ terminal oligopyrimidine (TOP) mRNA translation instead of activating transcription factor 4 (ATF4) synthesis. Asparaginase also promoted hepatic mRNA levels of several mTORC1 inhibitors between 3 and 18 h and these were blunted or blocked in the absence of Gcn2, but their timing could not explain the early discordant effects in mTORC1 signaling. Pre-conditioning mice with a chemical endoplasmic reticulum (ER) stress agent before amino acid stress rescued normal mTORC1 repression in the liver of Gcn2−/− mice but not in livers with both Gcn2 and the ER stress kinase, Perk, deleted. Furthermore, treating wild type and Gcn2−/− mice with ISRIB, an inhibitor of PERK signaling, also failed to alter hepatic mTORC1 responses to asparaginase, although administration of ISRIB alone had an inhibitory GCN2-independent effect on mTORC1 activity. Taken together, the data show that ATF4 is not required but eIF2 phosphorylation is necessary to prevent mTORC1 activation during amino acid stress.

A variety of cytoplasmic and proteotoxic stresses activates the integrated stress response (ISR), a conserved survival strategy in eukaryotes. In mammals, the ISR is triggered by a family of kinases with a shared substrate; namely, the alpha subunit of eukaryotic initiation factor 2 (eIF2). Phosphorylation of eIF2 at serine 51 leads to global and gene-specific changes in translation which, depending on the timing and intensity of the stress, direct the cell/tissue/organism toward adaptation or cell death (1).

The translation factor eIF2 delivers initiator Met-tRNA and GTP to the 40S ribosomal subunit during the initiation step and is then released from the 80S monosome in the GDP-bound form. Participation of eIF2 in a new round of translation initiation requires GDP-GTP exchange by eIF2B, a guanine nucleotide exchange factor for eIF2. Phosphorylation of eIF2 at serine 51 leads to global and gene-specific changes in translation which, depending on the timing and intensity of the stress, direct the cell/tissue/organism toward adaptation or cell death (1).
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Amino acids steadily increased over 24 h (Figure S2) (9). Activation states of GCN2 and mTORC1 were tracked by assessing phosphorylation of their respective substrates, eIF2 and S6K1, respectively. Phosphorylation of eIF2 was increased within 15 min after injection and reached a maximum by 30 min (Figure 1B), which corresponded with the nadir in circulating glutamine (Figure S1). During this time frame, no significant change in mTORC1 activity was evident in the livers of wild type mice (Figure 1C and S3). In contrast, strong activation of mTORC1 was observed in the livers of Gcn2-/- mice as evidenced by the rapid increase in S6K1 phosphorylation at threonine 389 (Thr-389) that lasted for at least 6 h (Figure 1C and S3). Thus, Gcn2 deletion results in rapid and sustained activation of hepatic mTORC1 in response to amino acid stress.

**Hepatic Gcn2 status dictates the response of translational machinery to amino acid stress**

To assess the effect of amino acid depletion on gene-specific translation in liver, we conducted polysome profile analysis at multiple time points (Figure 2A). Half an hour following asparaginase injection we observed accumulation of 80S monosomes in wild type livers, but not in Gcn2-/- livers, (Figure 2B), showing that GCN2 is required for repression of mRNA translation initiation in response to amino acid depletion. This effect was sustained for at least 3 h in wild type mice, whereas in the livers of Gcn2-/- mice, little to no change in polysome profiles occurred in the period of 30 min to 3 h following asparaginase injection. Assessment of Atf4 mRNA distribution in polysome profile fractions revealed accumulation of Atf4 mRNA in the fractions heavily loaded with ribosomes as early as 1 h after asparaginase exposure (Figure 2C, left panel). Importantly, Gcn2-/- livers did not exhibit this shift in Atf4 mRNA distribution (Figure 2C, left panel). No apparent shift was found in the control mRNA encoding for actin (Figure 2C, right panel). Figure S4 shows the analysis of additional polysome profiles. These data indicate that (1) GCN2 kinase is required for translational induction of Atf4 mRNA following amino acid depletion by asparaginase, (2) gene-specific translation of Atf4 mRNA occurs very early following asparaginase injection, and (3) ATF4 synthesis following a single injection of asparaginase is a transient event, complete by 3 h post-injection.

We proceeded to test whether hyperactivation of mTORC1 in Gcn2-/- mice corresponded with maladaptive changes in gene-specific translation; namely the enhanced translation of TOP mRNAs (5). Quantitative analysis of the most responsive TOP mRNAs (encoding ribosomal proteins, Rps18 and Rps20, and poly(A) binding protein cytoplasmic 1, Pabpc1) in polysomal fractions revealed their accumulation in heavy polysomes 1-3 h following asparaginase injection (Figure 2D) in samples with the highest mTORC1 activity. Thus, altered TOP mRNA translation is consequential to Gcn2 deletion during amino acid stress.

We also assessed polysomal distribution of mRNAs that are reportedly translationally enhanced in mouse livers upon perfusion with a methionine deficient solution, namely chaperone Hspa5, amino acid transporter Slc3a2, and transferrin receptor Trf2 (22). None of these mRNAs exhibited any changes in translational efficiency upon asparaginase exposure (Figure S5). This observation suggests that hepatic response to amino acid stress by asparaginase is different from the one caused by methionine deficiency. In fact, recent studies demonstrate that hepatic responses to methionine restriction are mediated in a eIF2 phosphorylation-independent manner (23) and perhaps via its own unique pathway (24).

**Hyperactivation of mTORC1 in Gcn2-/- livers precedes transcriptional control of mTORC1 repressors**

To assess when ATF4 target genes serve as mTORC1 regulators during amino acid stress, we examined mRNA levels of Sessn, 4ebp1, Trib3, and Reddl, as well as a few classic targets of ATF4 – Fgf21, Asns, Atf5 (Figure 3A-H). No acute changes were observed in hepatic mRNA levels of any of the genes within the first hour following injection. A robust increase in Trib3 mRNA was observed 3-18 h following asparaginase exposure in wild type (up to 80 times over untreated control), but not Gcn2-/- livers. In the same time frame, moderate elevation was seen in Reddl mRNA levels (up to 30 times) and only
mild elevations in Sesn2 and 4ebp1 mRNAs (4-times maximum) were observed in wild type, but not in Gcn2— mice. These data clearly show that control of mTORC1 activity by known ATF4 targets occurs 3 or more hours following asparaginase exposure and thus cannot explain the immediate loss of control over mTORC1 activity in Gcn2— livers.

**Exposure to chemical ER stress does not activate mTORC1 in livers lacking Perk**

To check whether asparaginase may acutely induce ER stress we analyzed PERK activation (Figure 4A), which was assessed via its phosphorylation at Thr-980. No activation of PERK was observed in response to asparaginase exposure in either wild type or Gcn2— livers (Figure 4B), confirming that amino acid depletion by asparaginase increases eIF2 phosphorylation via GCN2 (25). Moreover, mice with genetic ablation of hepatic Perk via Cre-mediated recombination (liver-specific Perk—, ls-Perk—) displayed increased eIF2 phosphorylation and no hyperactivation of mTORC1 in response to asparaginase exposure, similar to wild type animals (Figure 4B).

To better understand if communication between the ISR and mTORC1 occurs more broadly in response to proteotoxic stress, we tested whether acute ER stress would cause similar activation of mTORC1 in livers bearing genetically disrupted Perk (Figure 4A). To test this idea, we injected tunicamycin, a potent inhibitor of glycosylation and an ER stress causing agent, to wild type mice, Gcn2— mice and ls-Perk— mice. As expected, tunicamycin activated PERK, evidenced by its autophosphorylation at Thr-980 (Figure 4C). The response was very similar in wild type and Gcn2— livers, whereas in ls-Perk— mice no phosphorylation of eIF2 was evident (26). Remarkably, no acute (over 90 min) activation of mTORC1 was observed in the livers of any mouse strains following tunicamycin injection. These data indicate that the type of cellular stress determines whether or not a functional ISR - mTORC1 communication axis exists.

**Pretreatment of Gcn2— mice with tunicamycin rescues hepatic mTORC1 repression following asparaginase exposure**

We then tested whether induction of eIF2 phosphorylation by alternative means in Gcn2— mice would help prevent activation of mTORC1 (i.e., ISR preconditioning). To do this, wild type and Gcn2— mice were injected with tunicamycin 30 min prior to asparaginase injection to induce phosphorylation of eIF2 (Figure 5A). Analysis of mTORC1 activity showed no hyperphosphorylation of S6K1 in the livers of Gcn2— mice preconditioned with tunicamycin (Figure 5B-D). However, genetic ablation of both Perk and Gcn2— in liver blocked phosphorylation of eIF2 to both tunicamycin and asparaginase and allowed for hyperactivation of mTORC1 once again (Figure 5B-D). These data suggest that ISR preconditioning by another kinase is sufficient to block mTORC1 activation in response to acute amino acid stress.

**ISRIB reduces mTORC1 activity by itself yet fails to prevent mTORC1 hyperactivation following asparaginase exposure in Gcn2— livers**

Finally, to explore the relationship between eIF2B and mTORC1 in response to asparaginase we utilized the small molecule inhibitor, ISRIB (Figure 5E), which locks eIF2B in its active dimer form and renders cells insensitive to eIF2 phosphorylation by PERK. To our surprise, administration of ISRIB significantly reduced hepatic mTORC1 signaling independent of GCN2 status (Figure 5F-I, compare bar graphs of control vs. ISRIB treated animals). This noted decrease in mTORC1 activity upon ISRIB exposure is consistent with the published observation of ISRIB causing decreased translation of TOP mRNAs (27). Nevertheless, administration of ISRIB 30 min prior to asparaginase did not affect mTORC1 activity in wild type mice, nor did it modify any measured outcome in wild type and Gcn2— mice exposed to asparaginase (Figure 5F-I).

Considering ISRIB functions as an inhibitor of PERK signaling, the results are consistent with asparaginase treatment of lsPerk— mice and lsPerk—-Gcn2— mice. Collectively, these data show that it is the phosphorylation of eIF2 and not ATF4 synthesis that directs mTORC1 regulation upon acute amino acid stress.
Discussion
This study utilized chemical activators of the amino acid response and the ER stress response to reveal new information regarding a vital role for the GCN2-eIF2 axis in acutely regulating hepatic mTORC1 in mice. The data show that hyperactivation of hepatic mTORC1 in Gcn2\(^{-/-}\) mice exposed to asparaginase occurs within minutes, preceding the timing of enhanced translation of Atf4 mRNA and increased transcription of mTORC1 inhibitors in wild type mice. The inappropriately high mTORC1 signaling in liver of Gcn2\(^{-/-}\) mice corresponds with movement of TOP mRNAs into polysomes, identifying a physiologically maladaptive outcome resulting from enhanced mTORC1 signaling. Preconditioning the ISR in Gcn2\(^{-/-}\) liver by activating PERK blocks mTORC1 activation following amino acid depletion which is reversed in the liver of mice lacking both Gcn2 and Perk. Importantly, hepatic mTORC1 activity is not unleashed by ER stress in Is-Perk mice, signifying that communication between the ISR and mTORC1 is specific to sensing amino acid levels. Together these results suggest that eIF2 phosphorylation is necessary to impart negative control of the ISR over mTORC1 during amino acid stress.

Rapid and robust activation of hepatic mTORC1 in Gcn2\(^{-/-}\) mice given asparaginase supports a model wherein the GCN2-eIF2 axis plays a suppressive, ATF4-independent role in regulating mTORC1 activity upon sensing amino acid stress. This suppressive role of GCN2 was first observed by us in mice fed a leucine devoid diet to Gcn2\(^{-/-}\) mice (10), then confirmed and further detailed using mice treated with asparaginase (8,9,19,25). This work also strongly supports the findings of Averous et al. who used a model of leucine deprivation in mouse embryonic fibroblasts (MEFs) (20) to conclude that GCN2 and eIF2 phosphorylation, but not ATF4, are required for continuous suppression of mTORC1 during leucine or arginine deprivation. Deficiency in either of these two amino acids in Gcn2\(^{-/-}\) MEFs strongly activated mTORC1 within 15 min. In our current and past in vivo models, we see hyperactivation of mTORC1 within the first 30-60 minutes of amino acid depletion which is sustained for at least 6 h. Based on time course results by ourselves and others, we conclude that ATF4 is not necessary to inhibit mTORC1 in response to amino acid deprivation. Our work also expands on the findings of Ye and colleagues, reporting that GCN2 induces the expression of Sestrin2 to inhibit mTORC1 (16). No considerable induction of Sestrin2 mRNA levels was observed upon asparaginase exposure in our model of amino acid deprivation. Considering that mTORC1 repression can be rescued by ISR preconditioning via tunicamycin-induced PERK, we conclude that eIF2 phosphorylation is required for acute inhibition of mTORC1 upon amino acid stress, in agreement with (20).

In our previous study of chronic exposure to asparaginase (8 days) obese Gcn2\(^{-/-}\) mice demonstrate PERK activation and eIF2 phosphorylation alongside highly activated mTORC1 (8). Thus, phosphorylation of eIF2 by PERK failed to prevent mTORC1 activation in obese mice. We reconcile these results by pointing out that the timing of PERK activation is very different in these two experimental models. In the current study, PERK is activated before exposure to amino acid stress, allowing it to function as a preconditioning agent. In the previous work, PERK is not activated in the excipient treated mice, only in asparaginase treated mice, indicating that PERK becomes engaged after or in concert with GCN2. Overall, these data suggest that phosphorylation of eIF2 is necessary but not sufficient to coordinate mTORC1 signaling upon amino acid stress and an intact GCN2-eIF2 axis is a vital necessity in this regard.

As part of the ISR to amino acid stress, ATF4-mediated induction of several mTORC1 inhibitors, namely Redd1 (28), Trib3 (29), Sesn2 (16), and 4ebp1 (30) are reported as playing a regulatory role. Our data show that out of these four candidates, the most robust induction was observed in the expression of Trib3 gene, coding for a negative regulator of insulin signaling (31), whereas hepatic levels of Redd1, Sesn2 and 4ebp1 mRNAs showed only modest increases if at all. In all the cases, these increases in mRNA levels were observed no earlier than 3 h following the injection of asparaginase, again confirming that acute inhibition of mTORC1 does not involve an ATF4-driven transcriptional program. Interestingly, our previous studies show that...
induction of Trib3 mRNA by asparaginase requires GCN2, but not ATF4, as Atf4−/− animals treated with asparaginase demonstrate 10-fold greater increases in Trib3 mRNA levels to asparaginase than wild type animals, whereas no induction whatsoever is observed in Gcn2−/− animals (8,32). This confirms that Trib3 gene is controlled by other transcriptional factors in addition to ATF4 (18), but whether TRIB3 represents the major effector of GCN2 pathway that contributes to sustained inhibition of hepatic mTORC1 in WT, as well as in Atf4−/− animals requires further investigation.

ATF4 is the central transcription factor of the ISR, but it is also utilized by the mTORC1 pathway to support anabolic processes (14,15). For instance, stimulation of mTORC1 with insulin in MEFs leads to the enhanced synthesis of ATF4 without eIF2 phosphorylation. This increase in ATF4 can be blocked by either cycloheximide, an inhibitor of protein synthesis, or rapamycin (inhibitor of mTORC1), suggesting that both protein synthesis and mTORC1 signaling are required for the increased ATF4 protein levels (14). The proposed function of such signaling cascade is to increase cellular uptake of essential amino acids increase the synthesis of the non-essential ones (15), thereby supporting anabolic reactions during insulin stimulation of mTORC1. Our data demonstrate that hyperactive mTORC1 per se is not a sufficient signal to induce enhanced synthesis of ATF4 as we observed no translational induction of ATF4 in Gcn2−/− animals with high mTORC1 activity. The fact that the fundamentally distinct stimuli - insulin vs. acute amino acid stress in the background of systemic Gcn2 disruption, produce one outcome in the form of hyperactive mTORC1, but distinct downstream response in the form of ATF4 production, implies the existence of yet-to-be-identified factors, other than mTORC1, that influence cellular decision whether to upregulate synthesis of ATF4.

Deregulation of mTORC1 is associated with many pathological conditions in the liver (11,33,34). Our study identifies GCN2 and its downstream effector eIF2 as essential components that coordinate hepatic mTORC1 during amino acid stress. These data complement and provide mechanistic insight as to why maladaptive activation of hepatic mTORC1 in Gcn2−/− mice associates with greater morbidity and metabolic toxicity (8) and have important implications for the clinical use of rapidly growing class of GCN2-activating drugs (35–44). Gcn2 is not an essential gene and so far is found to have 594 missense, 28 nonsense, and 33 frame-shifting mutations in the human population (45). Our study found that GCN2 governs important nutritional responses and, if dysfunctional, leads to altered whole body metabolism. It is tempting to speculate that as a result of this altered metabolism subjects bearing deleterious mutations in Gcn2 (EI2F2AK4) gene have higher chances of developing metabolic conditions later in life such as pulmonary arterial hypertension (46). This work provides a foundation for personalized approaches in usage of other GCN2-activating drugs as well as dietary approaches.

**Experimental Procedures**

### Animals

All animals received humane care according to the criteria outlined in the “Guide for the Care and use of Laboratory Animals” prepared by the National Academy of Sciences (NIH publication 86-23 revised 1985) and ARRIVE (articulated at www.nc3rs.org.uk/ARRIVE). Animal protocols were reviewed and approved by The Rutgers Institutional Animal Care and Use Committee. Adult (10–20 wk old) male and female wild type (WT) and Gcn2−/− mice on the C57Bl/6J genetic background (47) were used. Mice with albumin Cre-mediated deletion of Perk in liver (lsPerk−/−) were also used (48) and these mice were bred to Gcn2−/− mice to create a double knock down of Perk and Gcn2 in liver. All mice were bred at the Bartlett animal facility on the Rutgers University Cook campus. All mice had free access to food (5001 Laboratory Rodent Diet, LabDiet) and water and maintained on a 12h light-dark cycle (7am/7pm) with same sex littermates until experimental group assignment wherein mice were housed in individual plastic cages with soft bedding.

Animals were administered a single injection of either bacterial asparaginase (3 IU/g body weight, Elspar, Merck), tunicamycin (1 mg/kg body weight, Sigma #T7765) or ISRIB (0.25 mg/kg, Sigma #SML0843) and euthanized by decapitation at indicated time points. The
control groups were treated with PBS (vehicle for asparaginase) or 0.3% DMSO in PBS (vehicle for Tunicamycin and ISRIB). All mice were killed between 12 – 4 pm (ZT5-9).

**Serum amino acid profiling**

Trunk blood was collected into microcentrifuge tubes. Serum was collected by centrifugation after blood clotting on ice. Proteins of the serum were precipitated by the addition of 180 μL of a solvent (0.1% formic acid in methanol) to 60 μL of serum. The resulting cloudy mixture was filtered using Captiva Non-Drip Lipids Filtration Tubes (Agilent, Cat number A5400635). The recovered clear eluate in the amount of 20 μL was spiked with 5 μL of internal standard (equimolar mix of sarcosine and norvaline, 0.1 mM each) and analyzed using reversed-phase high pressure liquid chromatography (Agilent 1200 HPLC Instrument) according to the manufacturer’s protocol (Agilent publication #5990-4547EN). Briefly, amino acid separation was carried out using a gradient mix of mobile phase A (10 mM Na₂HPO₄, 10 mM Na₂B₄O₇, 5 mM NaN₃; pH 8.2) and mobile phase B (acetonitrile/methanol/water = 45/45/10) on ZORBAX eclipse Plus C18 column at the speed of 0.42 mL/min. To allow for amino acid detection by fluorescence, derivatization was done automatically using autosampler according to the manufacturer’s protocol mentioned above and consisted of two consequent steps: (i) conjugation to o-phthalaldehyde (OPA) and (ii) conjugation to fluorenylmethyloxycarbonyl chloride (FMOC). Standard curves for each amino acid were retrieved from analyzing a set of equimolar standard mixtures. Total 10 standard mixtures were used ranging from 2.25 μM to 900 μM of each amino acid. Analysis of chromatograms was performed using Agilent OpenLAB software.

**Immunoblotting**

Liver tissue was crushed using the Cellcrusher tissue pulverizer under liquid nitrogen conditions. The resulting tissue powder was lysed with RIPA buffer (25 mM HEPES pH 7.5, 10 mM DTT, 0.1% SDS, 1x protease inhibitor cocktail (Sigma P8340), 1mM sodium orthovanadate, 0.5% deoxycholate, 50 mM beta-glycerophosphate, 2mM EDTA, 1 mM microcystin, 50 mM NaF, 3 mM benzamidine) using the Polytom bench top homogenizer followed by heating the lysates for 5 min in Laemmli buffer. Primary antibodies used were as follows: anti-total p70 S6K (Cell Signaling Technology, CST 9202), anti-phospho(T389)-S6K (Cell Signaling Technology, CST 9205), and anti-4E-BP1 (Bethyl Laboratories A300-501A), anti-phospho(S51)-eIF2 (Cell Signaling Technology, CST 3597), anti-eIF2 alpha (Santa Cruz, sc-11386), anti-phospho(T980)-PERK (Santa Cruz, sc-3179S), anti-PERK (Santa Cruz, sc-3192S). Secondary antibody was Peroxidase-AffiniPure Goat Anti-Rabbit TgC (H+L) from Jackson ImmunoResearch (111-035-003). Images were taken with Fluorchem M imager (ProteinSimple), band densities were quantified using AlphaView software.

**Reverse Transcription Quantitative PCR (RT-qPCR)**

RNA was isolated from 10 mg of frozen tissue powder using TRI Reagent according to the manufacturer instructions. Quality of the isolated RNA was assessed by measuring A260/280 and A260/230 ratios; integrity was assessed by visualization of the rRNA on agarose gels. One microgram of the isolated RNA from each sample was used to generate cDNA using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher, 4368814). The resulting cDNA was used for quantitative analysis of gene expression using SYBR green system. Validated primers sequences are listed in Supporting Information.

**Polysomal profiling and RT-qPCT from sucrose gradients**

Frozen mouse livers were crushed at liquid nitrogen temperature with tissue pulverizer. About 50 mg of the frozen tissue powder were homogenized using blue plastic pestles in standard 1.5 mL microcentrifuge tubes using with lysis buffer (25 mM HEPES-KOH pH 7.5, 100 mM KCl, 5 mM MgCl₂, 0.5% deoxycholate, 1% NP-40, 1 mM DTT, 1x Protease inhibitor (Sigma P8340), SUPERase-In RNase Inhibitor (Thermo Fisher AM2696); tissue-to-lysis buffer ratio was 1:10. The lysates were incubated on ice for 5 minutes and centrifuged at 10,000 x g for 10 min to get rid of nuclei and cell debris. The cleared supernatants were loaded on 10-50% sucrose
eIF2 phosphorylation is necessary to inhibit mTORC1 in liver gradients and centrifuged at 100,000 x g for 3 h to resolve mRNA species loaded with different amounts of ribosomes. The resolved polysomes were collected in either 10 or 30 fractions from which RNA was isolated using TRI-Reagent for liquid samples according to the manufacturer’s protocol. The RNA precipitates were washed twice with 75% ethanol and resuspended in 20 μl of nuclease-free water. Quality of the isolated RNA was assessed by measuring A260/280 and A260/230 ratios; integrity was assessed by visualization of the rRNA on agarose gels. RT-qPCR was performed as described above with the exception that the relative values of polysomal Atf4 mRNA were normalized to exogenous spike-in Luciferase instead of Gapdh.

**Statistics**

In most graphical displays, liver samples from animal subjects are plotted individually alongside the mean value ± standard deviation (SD) or plotted as box-whisker plots where boxes extend from 25th to 75th percentiles and whiskers extend to the maximum and minimum values of the data set. Two group comparisons were analyzed by t-test assuming two-tailed distribution with unequal variance and with an alpha level of P < 0.05 (GraphPad Prism; La Jolla, CA). A table listing the number of replicate mice in each strain and treatment group is included in Table S1.

Additional details on experimental materials and procedures are available in Supporting Information.
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**FOOTNOTES**

The abbreviations used are: ATF4, activating transcription factor 4; eIF2, eukaryotic initiation factor 2; GCN2, general control nonderepressible 2; mTORC1, mammalian target of rapamycin complex 1; S6K1, ribosomal protein S6 kinase 1; 4E-BP1, eIF4E binding protein 1; ASNase, asparaginase; ER, endoplasmic reticulum; PERK, Protein kinase R-like ER resident kinase; ISR, integrated stress response; TRB3, tribbles 3; Rps, ribosomal protein; Pabpc1, poly(A) binding protein cytoplasmic 1; REDD1, regulated in development and DNA damage responses 1; Hspa5, heat shock protein family A5; Asns, asparagine synthetase; Fgf21, fibroblast growth factor 21; Sesn2, sestrin 2; Trf2, transferrin receptor 2; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Slc3a2, solute carrier family 3 member 2; CARE, CAAT enhancer binding protein ATF response element; TOP, 5′ terminal oligopyrimidine;
Figure 1. Gcn2 status determines the hepatic eIF2 and mTORC1 response to asparaginase (ASNase). (A) Schematic diagram of experimental setup. Mice were injected intraperitoneally with ASNase and killed at regular intervals over 24 h. Liver samples were analyzed for phosphorylation of eIF2 (p-eIF2) and p-S6K1. (B) Upper panels show representative immunoblots displaying p-eIF2 at serine 51 and eIF2α total protein. Quantitative analysis of individual values for p-eIF2 levels normalized to total eIF2α at each time point is summarized in the below bar graph. (C) Upper panels show representative immunoblots displaying p-S6K1 at threonine 389 and total levels of S6K1. Quantitative analysis of p-T389 band intensities at each time point is summarized in the below bar graph. Figure S3 shows images of additional biological replicates. In B and C, circles represent individual liver samples, and bars represent average values across the treatment groups ± SD. *, significantly higher than values at 0 which are PBS-treated, P<0.05 by t-test; †, significantly lower than values at 0 which are PBS-treated, p<0.05 by t-test.
**Figure 2.** Loss of Gcn2 prevents increased Atf4 mRNA translation efficiency in liver following asparaginase (ASNase) exposure and instead permits enhanced 5′ oligopyrimidine tract (TOP) mRNA translation. (A) Schematic diagram of polysomal profiling method. Polysomes from liver lysates were separated based on their different migration rates through sucrose gradients. Polysomal RNA was isolated from fractions across the gradient and translational efficiency was assessed by RT-qPCR analysis of RNA isolated from polysomal fractions. (B) Representative images of hepatic polysomal profiles from wild type (WT, left panel) and Gcn2−/− (right panel) mice treated with ASNase. An increase in the 80S peak height in WT reflects inhibition of mRNA translation initiation as early as 30 min after a single injection of asparaginase. This increase is not observed in Gcn2−/− following ASNase exposure. (C) Atf4 mRNA levels are present in heavier polysomes at 1 h after ASNase in WT but not in Gcn2−/− mice. Black dotted outlines of the polysomal profiles indicate the position of each fraction along the profile. Symbols represent values of relative Atf4 mRNA amount, expressed as percent of total mRNA value in all the fractions. (D) Assessment of select TOP mRNA (Rps18, Rps20, Pabpc1) levels across sucrose gradient fractions. Polysomal profile is shown below to indicate relative position of each fraction. Symbols represent values of relative amounts of mRNAs in the collected fractions, expressed as percent of total mRNA value in all the fractions.
Figure 3. Time-resolved changes in hepatic expression of select ATF4 target genes after asparaginase (ASNase) exposure. (A) Total RNA was isolated from livers of mice after a single injection of ASNase and select ATF4 target genes were quantitatively assessed using RT-qPCR. (B) Sesn2; (C) Redd1; (D) Asns; (E) Trib3; (F) 4ebp1; (G) Fgf21; (H) Atf5. Boxes extend from 25th to 75th percentiles as computed in GraphPad software using the following equation: $R = P \times (n + 1)/100$, where $P$ is the desired percentile and $n$ is the number of values in a data set. Whiskers extend to the maximum and minimum values of the data set. *, significantly different as compared to values at 0 which represents PBS injection, $p<0.05$ by t-test.
Figure 4. Loss of Perk does not unleash mTORC1 signaling upon asparaginase (ASNase) exposure or upon ER stress by tunicamycin (Tun). (A) Schematic diagram of molecular cascades tested – namely the ISR and mTORC1 pathway after a single injection of either ASNase or Tun. (B) Phosphorylation of S6K1 (p-S6K1) at threonine 389 is increased in livers of Gcn2−/− but not Is-Perk−/− mice. Two representative images are displayed next to mean values of band intensities for p-eIF2 and p-S6K1. “v” stands for PBS vehicle control. Mean values combine results at 15, 30 and 60 min after ASNase. (C) No increase in p-S6K1 is noted in the livers of Is-Perk−/− mice injected with Tun. Two representative images are displayed next to mean values of band intensities for p-eIF2 and p-S6K1. “v” stands for 0.3% DMSO vehicle control. Mean values combine results at 30, 60 and 90 min after a single injection of Tun. Circles represent individual data points, and bars represent average values across the treatment groups ± SD. *, significantly higher than values at the corresponding “v” groups, p<0.05 by t-test.
Pretreatment with tunicamycin (Tun) rescues normal control of mTORC1 by asparaginase (ASNase) in Gcn2−/− livers, whereas ISRIB does not. (A) Schematic diagram of the experiment. Wild type (WT), Gcn2−/− and Is-Perk−/− Gcn2−/− mice were injected with Tun 30 min prior to ASNase, after which livers were collected at the indicated time points. (B) Representative immunoblots display phosphorylation of eIF2 (p-eIF2), p-S6K1 and p-PERK. (C) Mean values of band intensities for p-eIF2 levels normalized to total eIF2α at indicated time points. (D) Mean values of band intensities for p-S6K1 at threonine 389 at indicated time points. “+” indicates positive control, which is Gcn2−/− liver collected 30 min after ASNase; “v” represents vehicle control. Individual data points are shown in circles, and bars represent average values across treatment groups ± SD. *, p<0.05 by t-test. (E) Schematic diagram of the experiment. WT and Gcn2−/− mice were administered ISRIB 30 min prior to ASNase, after which livers
eIF2 phosphorylation is necessary to inhibit mTORC1 in liver

were collected at the indicated time points. (F) Representative immunoblots display p-eIF2 and p-S6K1 in WT and Gcn2−/− livers. (G) Mean values of band intensities for p-eIF2 levels normalized to total eIF2α protein. (H) Mean values of band intensities for p-S6K1 at threonine 389. (I) Mean values of p-S6K1 at threonine 389 in both genetic strains combined (WT and Gcn2−/−) following a single injection of ISRIB alone. Individual data points are shown in circles, and bars represent average values across treatment groups ± SD. * p<0.05 by t-test as compared to vehicle treated control.
Time-resolved analysis of amino acid stress identifies eIF2 phosphorylation as necessary to inhibit mTORC1 activity in liver
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