GCN2 regulates the CCAAT enhancer binding protein beta and hepatic gluconeogenesis

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Xu X, Hu J, McGrath BC, Cavener DR. GCN2 regulates the CCAAT enhancer binding protein beta and hepatic gluconeogenesis. Am J Physiol Endocrinol Metab 305: E1007–E1017, 2013. First published July 30, 2013; doi:10.1152/ajpendo.00063.2013.—Mice deficient for general control nondepressible-2 (Gcn2) either globally or specifically in the liver display reduced capacity to maintain glucose homeostasis during fasting, suggesting the hypothesis that Gcn2 may regulate gluconeogenesis (GNG), which normally plays a key role maintaining peripheral glucose homeostasis. Gcn2-deficient mice exhibit normal insulin sensitivity and plasma insulin but show reduced GNG when administered pyruvate, a gluconeogenic substrate. The basal expression of phosphoenolpyruvate carboxykinase, a rate-limiting enzyme in GNG, is abnormally elevated in Gen2 knockout (KO) mice in the fed state but fails to be further induced during fasting. The level of tricarboxylic acid cycle intermediates, including malate and oxaloacetate, and the NADH-to-NAD ratio are perturbed in the liver of Gcn2 KO mice either in the fed or fasted state, which may directly impinge upon GNG. Additionally, the expression of the CCAAT enhancer-binding protein-β (C/EBPβ) in the liver fails to be induced in Gcn2 KO mice after 24 h fasting, and the liver-specific Cebp KO mice show reduced fasting GNG similar to that seen in Gcn2-deficient mice. Our study demonstrates that Gcn2 is important in maintaining GNG in the liver, which is likely to be mediated through regulation of C/EBPβ.

general control nondepressible-2; gluconeogenesis; glucose homeostasis

MATERIALS AND METHODS

Animals, diets, and experiments. Wild-type C57BL/6j mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Gen2 KO mice with C57BL/6j background were generated as previously described (28). Liver-specific Gcn2 knockout (Ltgcn2 KO) mice were generated by crossing Albumin-Cre transgenic mice to a floxed allele of Gcn2 (41). Liver-specific Cebp knockout mice (Ltcebp KO) were generated by crossing Albumin-Cre transgenic mice to a floxed allele of Cebp (34) provided by Dr. Esta Sternecke (National Cancer Institute). All mice were maintained on a 12:12-h light-dark cycle and were provided free access to 22% (kcal/100 kcal) fat rodent chow (5020; LabDiet, St. Louis, MO) and tap water before the experiments. Male mice around 6 mo old (body weight: 40 ± 5 g) were used exclusively unless otherwise indicated. Metabolic and biochemical measurements for fed (ad libitum) mice were performed on blood or organs sampled at 3 h after light cycle (ca. 10:00 A.M.). For the glucose tolerance test (GTT), mice were fasted overnight (14 h) and injected intraperitoneally with glucose solution (2 g/kg). For the insulin tolerance test (ITT), mice were fasted for 4 h and injected intraperitoneally with human insulin (0.75 U/kg; Eli Lilly, Indianapolis, IN). For pyruvate or glycerol loading to determine GNG, mice were fasted for 24 h and injected intraperitoneally with 1.5 g/kg sodium pyruvate or 1.5 g/kg glycerol, respectively. The GTT, ITT, pyruvate, and glycerol GNG experiments were conducted after methods previously described for measurement of these metabolic parameters in mice (25, 42). Blood glucose was measured from tail blood using a OneTouch Ultra glucometer (LifeScan, Milpitas, CA). All animal experiments were approved by the Pennsylvania State University Institutional Animal Care and Use Committee.

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Serum and liver metabolites measurement. Mice were killed by CO₂ euthanization following the recommended procedure of the American Veterinary Medical Association (AVMA Euthanasia Panel Report) and the Office of Laboratory Animal Welfare of the National Institutes of Health. Although these procedures minimize stress associated with CO₂ euthanization, some of the metabolic parameters measured may be impacted. Plasma was obtained from blood samples collected with Microvette CB 300LH (SARSTEDT, Nümbrecht, Germany). Posteuthanization, tissues were resected, snap-frozen in liquid nitrogen, and stored at −80°C. Plasma insulin and leptin were measured using the mouse insulin/leptin assay kit (MSD, Rockville, MD). Liver malate, oxaloacetate (OAA), lactate, and pyruvate contents were measured with assay kits from BioVision (Milpitas, CA). cAMP was measured with a CAMP Direct Immunoassay Kit (Abcam, Cambridge, MA). All assays were performed according to the manufacturer’s instructions. Liver glycogen was extracted and hydrolyzed with amyloglucosidase (Sigma, St. Louis, MO) as previously described (28), and glucose release was evaluated by glucose assay with the Glucose Assay Reagent (Sigma). Glucose production was expressed as the amount of glucose (μg) produced in 1 ml of medium.

RNA isolation and relative quantitative RT-PCR. RNA was extracted with the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) from tissues or cell cultures. RNA was quantitated by the Quant-It Ribogreen RNA Assay Kit (Invitrogen, Eugene, OR). RNA (1 μg) was used for reverse transcription with qScriptTM cDNA supermix (Biosciences, San Jose, CA), 50 nM bovine insulin (Sigma), and antibiotics. Cells were seeded in BioCoat Cellware, collagen I-coated 12-well plates (BD Biosciences, San Jose, CA) at a density of 5 × 10⁴ cells/well in 1 ml M199 medium for 1–2 days before the experiments.

Glucose production assay. Glucose production assay was performed on primary hepatocytes as previously described (18). The M199 medium was replaced with 500 μl glucose production buffer consisting of glucose-free DMEM (pH 7.4), without phenol red (Invitrogen), supplemented with 20 mM sodium lactate, 2 mM sodium pyruvate, and with a combination of 500 nM dexamethasone and 0.1 mM 8-(4-chloro-phenylthio)-cAMP (Sigma) for stimulation of glucose production. After 4 h of incubation, an aliquot of supernatant was sampled, and glucose concentration was measured with the Glucose Assay Reagent (Sigma). Glucose production was expressed as the amount of glucose (μg) produced in 1 ml of medium.

Primary hepatocyte isolation. Primary hepatocytes were isolated from livers of neonatal mice as previously described (11, 21). Briefly, the whole liver was taken and cut into 1- to 2-mm small pieces in Hanks’ balanced salt solution buffer and shaken at 37°C for 5 min in HBSS buffer with 5 mM EDTA and then for 20 min in HBSS buffer containing 2.5 mg/ml collagenase type I (Sigma), 0.1 mg/ml deoxyribonuclease I, type IV (Sigma), and 5 mM CaCl₂. The resulting cell suspension was poured through the nylon mesh and centrifuged at 250 g for 2 min. The cell pellet was washed two times with DMEM with 10% FBS and then resuspended in M199 medium with Earle salts (Invitrogen, Grand Island, NY), supplemented with 10% FBS, 2.0 mM l-glutamine, 20 ng/ml human epidermal growth factor (BD Biosciences, San Jose, CA), 50 nM bovine insulin (Sigma), and antibiotics. Cells were seeded in BioCoat Cellware, collagen I-coated 12-well plates (BD Biosciences, San Jose, CA) at a density of 5 × 10⁴ cells/well in 1 ml M199 medium for 1–2 days before the experiments.

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E1009
detection system (Applied Biosystems, Foster City, CA). Gapdh was
complified and used as an internal normalization of all other gene
mRNAs. No significant differences were seen in Gapdh mRNA levels
between Gcn2 or Cebpβ genotypes. Primers used for real-time PCR
are listed in Table 1.

Western blot analysis. Whole liver lysates were extracted with RIPA
buffer containing protease inhibitor cocktail (Sigma) and phosphatase
inhibitor cocktail 1, 2 (Sigma). Primary antibodies for phosphoerol-
pyruvate carboxykinase (PEPCK), C/EBPβ, CREB-1, phospho-
CREB-1 (Ser139) (Santa Cruz Biotech, Santa Cruz, CA), eIF2α and
eIF2α(pS52) (Invitrogen, Camarillo, CA), and α-tubulin (Sigma)
were used.

Data analysis. All data are expressed as means ± SE, including
numbers of mice as per strain-time. The two-tailed Student’s test was
used to evaluate statistical differences between paired samples, and
two-way ANOVA with replication was performed to analyze mea-
surements obtained by time course.

RESULTS

Phosphorylation of eIF2α by GCN2 in the liver is induced
by fasting. Mice were subjected to short-term or long-term
fasting, and liver samples were assessed by Western blot for
phosphorylation status of Ser51 of eIF2α, the regulatory phos-
phorylation site and target of the eIF2α kinases. A linear
increase in phosphorylated eIF2α was observed across a 24-h
fasting period (Fig. 1A). The induction of eIF2α phosphoryla-
tion in the liver is seen in both Gcn2 KO and wild-type mice
over a 72-h period but is substantially lower at each time point
in Gcn2 KO mice, suggesting that fasting-induced eIF2α phos-
phorylation is largely, but not exclusively, dependent upon
GCN2, particularly at the 72-h time point (Fig. 1B).

GNG is impaired in Gcn2 KO mice during starvation,
without impacting glucose tolerance. Gcn2 KO mice show
relatively low blood glucose at 24 and 48 h after fasting
(Fig. 2A), suggesting deficient glucose production or an alter-
ation in glucose tolerance or insulin sensitivity. Liver glycogen
content was not significantly altered in either the fed or fasted
state in Gcn2 KO mice (Fig. 2B), indicating the glycogen
storage in the fed state and glycogenolysis in the fasted state is
normal. In contrast, Gcn2 KO mice exhibit reduced glucose
production from exogenous pyruvate, a glucogenic substrate
(Fig. 2C), implicating a deficiency in GNG. However, no
genotypic differences were seen in GNG when glycerol was
administered to fasted mice (Fig. 2D), suggesting that the GNG
deficiency in Gcn2 KO mice lies within the early part of the
GNG pathway before the generation of glycerol 3-phospho-
late. Insulin levels, insulin sensitivity, and glucose tolerance
were all normal in Gcn2 KO, suggesting that reduced fasting
glucose levels and deficient GNG were not caused by changes
in serum insulin levels or insulin sensitivity of peripheral
tissues (Fig. 3, A–C). Although Gcn2 KO mice exhibited
normal insulin sensitivity in the initial glucose clearance seen
in the ITT test (Fig. 3B), the glucose recovery phase, which is
dependent upon GNG, was retarded.

Fig. 2. Fasting serum glucose levels and
glucogenesis are impaired in Gcn2 KO
mice. A: blood glucose of fed (0) WT and
Gcn2 KO mice and after 8, 12, and 24 h of
fasting (mean ± SE, n ≥ 24/strain-time; P =
0.004, KO vs. WT by 2-way ANOVA). B: liver glycogen content of fed and 24-h-
fasted mice of indicated genotypes (mean ±
SE, n = 8). C: blood glucose levels as a
function of time after ip injection of sodium
pyruvate in mice of indicated genotypes
(mean ± SE, n ≥ 12; P < 0.001, KO vs. WT
by 2-way ANOVA). D: blood glucose levels
as a function of time after ip injection
of glycerol in mice of indicated genotypes
(mean ± SE, n = 6; no significant differ-
ence, KO vs. WT by 2-way ANOVA).
GCN2 deficiency leads to imbalanced energy homeostasis. We measured the levels of key tricarboxylic acid (TCA) cycle intermediates and other metabolites that are known to influence hepatic GNG. We found a 69% increase in malate in the liver of Gcn2 KO mice in the fed state (Fig. 4A) but no difference in the fasted mice, whereas OAA levels were normal in fed Gcn2 KO mice but 74% higher than wild type in the fasted state (Fig. 4B). The pyruvate and lactate content also exhibited significant differences between genotypes in either the fasted or fed state (Fig. 4, C and D). The ratio of lactate to pyruvate, a surrogate measurement of the NADH-to-NAD⁺ ratio, was less than half of normal (Fig. 4E), indicating that Gcn2 KO mice may have altered energy homeostasis, although ATP levels were not different (data not shown). The mRNA encoding cytosolic malic enzyme-1, which is important for both production of NADPH and for regulating pyruvate cycling (5), was also found to be expressed at a lower level in the fed state in Gcn2 KO mice and failed to be induced after 24 h fasting (Fig. 4G). PEPCK protein levels were significantly reduced in fasted Gcn2 KO mice (Fig. 5, B and C), and this may partially explain the lack of PEPCK induction.

Fig. 3. GCN2-deficient mice exhibit normal insulin, glucose clearance, and insulin sensitivity. A: plasma insulin levels in the fed and 24-h-fasted mice of indicated genotypes (mean ± SE, n = 24). B: blood glucose levels as a function of time (expressed as a percentage of the starting level, KO starting blood glucose value: 168 ± 8 mg/dl; WT starting value: 150 ± 3 mg/dl) after ip injection of insulin in mice of indicated genotypes (mean ± SE, n = 6; P < 0.001, KO vs. WT by 2-way ANOVA). C: blood glucose levels as a function of time after ip injection of glucose in mice of indicated genotypes (mean ± SE, n = 12; no significant difference, KO vs. WT by 2-way ANOVA).

GCN2 and C/EBPβ expression in the liver regulates GNG. To determine if the metabolic differences seen in Gcn2 KO mice are due to functions of GCN2 in the liver, liver-specific Gcn2 KO mice (LiGcn2 KO) were generated by crossing the Albumin-Cre transgenic deleter strain to a floxed allele of Gcn2. We estimated the recombination deletion efficiency to be 69% in the liver of LiGcn2 KO mice. LiGcn2 KO exhibited normal fasting glucose, but similar to global Gcn2 KO mice GNG was impaired as demonstrated by significantly lower glucose synthesis from pyruvate administration (Fig. 6A). The expression of Pepck and G6pc mRNA was normal in LiGcn2 KO mice in the fed and fasted states, whereas basal expression of Cebpβ was abnormally high and failed to be further induced by fasting (Fig. 6B).

To directly assess the GCN2-dependent regulation of GNG in the liver, hepatocytes were isolated from neonatal Gcn2 KO and wild-type mice, and glucose production assays were performed. The Gcn2 KO primary hepatocytes exhibited a modest reduction in glucose production from pyruvate substrate stimulated by cAMP (Fig. 6C). The expression of gluconeogenic genes was examined in these primary hepatocytes, and the induction of Pepck, glucose-6-phosphatase (G6pase), and Cebpβ by cAMP and pyruvate was diminished in Gcn2 KO hepatocytes (Fig. 6D). Overall, these data support the hypothesis that reduced GNG in fasting Gcn2 KO mice is largely due to a GCN2 deficiency in the liver, and specifically in hepatocytes.
Fig. 4. Tricarboxylic acid cycle intermediates, reducing equivalents, and gene expression are perturbed in the liver of Gcn2 KO mice. 

A: liver malate content in fed and 24-h-fasted mice of indicated genotypes (mean ± SE, n = 8; *P < 0.05 as bracket indicated). B: liver oxaloacetate content in fed and 24-h-fasted mice of indicated genotypes (mean ± SE, n = 8; *P < 0.05 as indicated). C: liver pyruvate content in fed and 24-h-fasted mice of indicated genotypes (mean ± SE, n ≥ 8; *P < 0.05 as indicated). D: liver lactate content in fed and 24-h-fasted mice of indicated genotypes (mean ± SE, n ≥ 8, *P < 0.05 as indicated). E: NADH-to-NAD+ ratio (calculated from ratio of lactate/pyruvate level) in livers of fed and 24-h-fasted mice of indicated genotypes (mean ± SE, n ≥ 8, *P < 0.05 as indicated). F: liver malic enzyme-1 (Me1) mRNA in fed and 24-h-fasted mice of indicated genotypes (normalized to fed WT mice, mean ± SE, n = 8; *P < 0.05 as indicated). G: expression of Cebpβ, phosphoenolpyruvate carboxykinase (Pepck), G6pc, pyruvate carboxylase (Pcx), and Pdk4 mRNAs in livers of fed and 24-h- and 72-h-fasted mice of indicated genotypes (normalized to fed WT mice, mean ± SE, n = 8; *P < 0.05 as indicated).
C/EBPβ is a likely candidate for mediating GCN2-dependent regulation of GNG because it was previously shown that the eIF2α pathway regulates C/EBPβ expression (6), and mice deficient for C/EBPβ exhibit impaired GNG (3, 10, 19). We found that C/EBPβ mRNA and protein failed to be induced in the liver of Gcn2 KO mice (Fig. 6, E and F). To further probe the hepatic function of C/EBPβ, liver-specific Cebp KO (LiCebp KO) mice were generated using a floxed Cebp mouse (34) crossed to the Albumin-Cre deleter strain. The ablation of Cebpβ expression in LiCebp KO mice was evident in a 72-h fasting experiment where Cebpβ mRNA was virtually undetectable in contrast to robust expression in the wild type (Fig. 7A). The LiCebp KO mice showed normal random fed blood glucose levels, but blood glucose levels were significantly reduced after the 24-h fasting period (Fig. 7B). Glucose production from exogenous pyruvate was significantly reduced in LiCebpβ KO mice (Fig. 7C) similar to Gcn2-deficient mice. LiCebpβ KO mice exhibited altered gene expression profiles in the fed and/or fasted state for Pepek and G6pc (Fig. 7D). However, glucose clearance was normal in LiCebpβ KO mice, as assessed by GTT (Fig. 7E), suggesting that the perturbation in glucose homeostasis is unrelated to the action of insulin.

**DISCUSSION**

Hepatic glucose production in mice bearing a mutation in the regulatory phosphorylation site of the translation initiation factor eIF2α is insufficient to sustain perinatal/neonatal life, and pups die of hypoglycemia within a few hours after birth, leading to the speculation that one or more of the four known eIF2α kinases were likely to regulate hepatic glucose production (31). Although Gcn2 KO mice exhibit normal viability following birth, we found that Gcn2-deficient adult mice do indeed exhibit reduced glucose production when they are fasted or recovering from exogenous insulin administration. Phosphorylation of eIF2α at the regulatory Ser51 site exhibits a linear increase in the liver over a 24-h fasting period, and we showed that Gcn2 was primarily responsible for this induction although other eIF2α kinases likely contributed as well. Gcn2-deficient mice administered pyruvate, a glucogenic substrate, exhibited reduced conversion of pyruvate to glucose, strongly supporting the hypothesis GNG is reduced. Parallel experiments using glycerol as the glucogenic substrate suggested that reduced GNG in Gcn2-deficient mice is the result of an alteration in the GNG pathway between pyruvate and glycerol 3-phosphate.

Repressed GNG in Gcn2 KO mice is largely a deficiency of Gcn2 in the liver and hepatocytes, since we discovered that liver-specific Gcn2 KO mice and isolated hepatocytes exhibit impairment in glucose production and GNG. However, we observed a smaller genotype difference in pyruvate-derived GNG and no difference in fasting glucose in LiGcn2 KO, suggesting that either incomplete deletion of Gcn2 in the liver of these mice or extra hepatic sources of GNG contribute to the larger genotype difference in GNG seen in global Gcn2 KO mice. In particular, the kidney and intestine have been shown to conduct GNG (23), and these
organisms may exhibit different glucogenic substrate preferences compared with the liver (35).

Burgess and coworkers have shown that a deficiency of PEPCK in the liver results in an imbalance in the TCA cycle (4). Similarly, we found elevated malate and OAA in Gcn2-deficient mice, consistent with diminished cataplerosis. However, the amounts of PEPCK mRNA and protein were not reduced in Gcn2 KO mice; instead the basal level of PEPCK was elevated to a level equivalent to fasted mice and did not show a further induction in the fasted state. In addition, we found that Gcn2-deficient mice also exhibited an apparent misregulation of gluconeogenesis. A: blood glucose levels as a function of time after ip injection of sodium pyruvate in Gcn2 KO and WT mice after 24 h fasting (mean ± SE, n = 6; P = 0.003, KO vs. WT by 2-way ANOVA). B: expression of Cebpβ, G6pc, and Pepck mRNAs in livers of Gcn2 KO and WT mice after 24 h fasting (normalized to fed WT mice, mean ± SE, n = 4; *P < 0.05 as indicated). C: glucose production of primary Gcn2 KO and WT hepatocytes in glucose production medium supplemented with sodium pyruvate and cAMP or control medium (mean ± SE, n = 4; *P < 0.05 as indicated). D: expression of Pepck, G6pc, Pgc1a, and Cebpβ mRNAs in primary Gcn2 KO and WT hepatocytes in glucose production assay (normalized to control group, mean ± SE, n = 4; *P < 0.05 as indicated). E: expression of CEBPβ mRNA in livers of fed and 24-h-fasted WT and Gcn2 KO mice (normalized to fed WT mice, mean ± SE, n = 8; *P < 0.05 as indicated). F: CEBPβ (LAP) protein from liver lysates of fed and 24-h-fasted mice of indicated genotypes. Top, Western blot; bottom, CEBPβ protein relative to tubulin (mean ± SE, n = 3; *P < 0.05 as indicated).

Fig. 6. Liver-specific Gcn2 KO (LiGcn2 KO) mice and Gcn2-deficient primary hepatocytes display misregulation of gluconeogenesis. A: blood glucose levels as a function of time after ip injection of sodium pyruvate in LiGcn2 KO and WT mice after 24 h fasting (mean ± SE, n ≥ 6; P = 0.003, KO vs. WT by 2-way ANOVA). B: expression of Cebpβ, G6pc, and Pepck mRNAs in livers of LiGcn2 KO and WT mice after 24 h fasting (normalized to fed WT mice, mean ± SE, n = 4; *P < 0.05 as indicated). C: glucose production of primary Gcn2 KO and WT hepatocytes in glucose production medium supplemented with sodium pyruvate and cAMP or control medium (mean ± SE, n ≥ 4; *P < 0.05 as indicated). D: expression of Pepck, G6pc, Pgc1a, and Cebpβ mRNAs in primary Gcn2 KO and WT hepatocytes in glucose production assay (normalized to control group, mean ± SE, n = 4; *P < 0.05 as indicated). E: expression of CEBPβ mRNA in livers of fed and 24-h-fasted WT and Gcn2 KO mice (normalized to fed WT mice, mean ± SE, n = 8; *P < 0.05 as indicated). F: CEBPβ (LAP) protein from liver lysates of fed and 24-h-fasted mice of indicated genotypes. Top, Western blot; bottom, CEBPβ protein relative to tubulin (mean ± SE, n = 3; *P < 0.05 as indicated).
of preexisting metabolic imbalances before fasting. To further examine the role of metabolite differences in Gcn2 KO mice, it will be necessary to assess the level of these metabolites in the cytosol and mitochondria separately.

GNG in mice globally deficient for the C/EBPβ transcription factor was previously shown to be impaired (3, 9, 10, 19). C/EBPβ is normally induced upon fasting and is required for the induction of Pepck mRNA (8, 13, 27). We found that Gcn2-deficient mice have elevated basal expression of Cebpβ mRNA, but the C/EBPβ protein and mRNA failed to be induced in response to fasting. This pattern is remarkably similar to that seen in PEPCK expression in Gcn2 KO mice, suggesting that the well-known regulation of PEPCK by C/EBPβ during fasting is GCN2-dependent. We show herein that ablating C/EBPβ or Gcn2 specifically in the liver leads to a reduction in GNG, supporting the hypothesis that the expression and action of GCN2 and C/EBPβ in the liver modulates GNG. However, GCN2 in skeletal muscle may also play a role, since this tissue is a major source of the gluconeogenic substrates pyruvate and lactate, which are imbalanced in Gcn2 KO mice. Leucine deprivation in Gcn2 KO mice results in a loss of skeletal muscle (2), suggesting that GCN2 may also act in skeletal muscle to regulate metabolic homeostasis during nutrient deprivation.

Our studies suggest that the function of GCN2 and C/EBPβ in regulating GNG lies within the initial steps of the GNG pathway between pyruvate and glycerol 3-phosphate (Fig. 8), since several intermediates and the expression of key enzymes that regulate these steps are perturbed in GCN2- and C/EBPβ-deficient hepatocytes. The conversion of OAA to phosphoenolpyruvate (PEP) by PEPCK is often cited as the key regulatory step in GNG, and we discovered alterations in PEPCK mRNA and/or protein expression and OAA levels in mice deficient for GCN2 and/or C/EBPβ. Specifically, we found that PEPCK mRNA and protein in GCN2-deficient mice were derepressed in the fed state and failed to be further induced upon fasting. The simplest prediction from these results would be elevated hepatic glucose output in the fed state with no
further increase upon fasting. However, we observed euglycemia in the fed state but hypoglycemia and poor response to glucogenic substrates during fasting. We speculate that, although the metabolic perturbation seen in the fed state did not significantly impact glucose homeostasis, they set the stage for impaired hepatic glucose output during fasting. In addition, we think it is likely that GCN2 and C/EBPβ regulate more than just a single step in the GNG pathway, since we observed multiple alterations in both the fed and fasted state, some of which cannot be readily explained by altered GCN2 expression. We also discovered reduced levels of cAMP and phospho-CREB in fasted Gcn2 KO mice, which, in addition to negatively impacting the induction of PEPCK and G6Pase, may disrupt the normal energy and metabolite balance of hepatocytes necessary for GNG. However, this interpretation is confounded by discordance in the metabolic state at which we see the reduced levels of cAMP and phospho-CREB (fasted state) vs. the elevation of PEPCK at the fed state and lack of further induction during fasting.

We speculate that the reduction in cAMP and phospho-CREB is due to an alteration of glucagon signaling. Pancreatic and serum glucagon levels are not significantly different in Gcn2 KO mice (unpublished data), suggesting that reduced glucagon signaling is autonomous to hepatocytes and downstream of the glucagon receptor. We have not detected diminished gene expression of the other key players in glucagon signaling, including adenylate cyclase, TORC1, and PKA, but have not excluded possible differences in their activities.

The expression of C/EBPβ, particularly at the translation initiation level, has been shown to be regulated by the eIF2α pathway (6, 40). The two major isoforms of C/EBPβ, C/EBPβ liver activator protein (LAP) and C/EBPβ liver inhibitor protein (LIP), are encoded by a single mRNA species but initiated by two different translation start codons (24). The LIP and LAP C/EBPβ isoforms were previously shown to be differentially regulated by eIF2α phosphorylation (6). However, in our study, we found that both the mRNA and protein levels of C/EBPβ were differentially regulated as a function of Gcn2 with equal impact on the relative expression of LAP and LIP, suggesting that GCN2-dependent regulation is mediated by transcription of C/EBPβ rather than by translational control.

GCN2 is activated by uncharged tRNAs that increase inversely proportional to amino acid levels, and hence GCN2 is the sensor of amino acid deprivation. How activation by amino acid deprivation is physiologically connected to regulation of GNG is unknown. We speculate that GCN2 and C/EBPβ may regulate glucogenic amino acids that are converted to TCA intermediates followed by cataplerosis to PEP (Fig. 8). During the first 24 h of fasting mice lose up to 40% of their liver mass, which is in part due to proteasome degradation that releases amino acids that can be redeployed for synthesis of essential proteins or used for GNG (32, 38). GCN2 and C/EBPβ are indeed known to regulate the response to amino acid deprivation (7, 15, 26, 37). GCN2 in the piriform cortex of the brain also regulates aversive feeding behavior to diets lacking essential amino acids (16, 20). Therefore, a growing body of evidence suggests that GCN2 plays multiple roles of regulating metabolic responses to nutritional changes that entail the action of GCN2 in the liver and/or central nervous system.

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DISCLOSURES

The authors declare no conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS

Author contributions: X.X. and D.R.C. conception and design of research; X.X., J.H., and D.R.C. performed experiments; X.X., J.H., B.C.M., and D.R.C.
REFERENCES


