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Article

# The GCN2 eIF2α Kinase Regulates Fatty-Acid Homeostasis in the Liver during Deprivation of an Essential Amino Acid

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## SUMMARY

**Metabolic adaptation is required to cope with episodes of protein deprivation and malnutrition. GCN2 eIF2α kinase, a sensor of amino acid deficiency, plays a key role in yeast and mammals in modulating amino acid metabolism as part of adaptation to nutrient deprivation. The role of GCN2 in adaptation to long-term amino acid deprivation in mammals, however, is poorly understood. We found that expression of lipogenic genes and the activity of fatty acid synthase (FAS) in the liver are repressed and lipid stores in adipose tissue are mobilized in wild-type mice upon leucine deprivation. In contrast, GCN2-deficient mice developed liver steatosis and exhibited reduced lipid mobilization. Liver steatosis in *Gcn2*$^{-/-}$ mice was found to be caused by unrepressed expression of lipogenic genes, including *Srebp-1c* and *Fas*. Thus, our study identifies a novel function of GCN2 in regulating lipid metabolism during leucine deprivation in addition to regulating amino acid metabolism.**

## INTRODUCTION

Organisms have evolved a repertoire of adaptations to cope with episodes of malnutrition and starvation. To maintain vital functions during prolonged malnutrition, proteins, carbohydrates, lipids, and other biomolecules are mobilized from skeletal muscle, adipose tissues, and specific internal organs including the liver (Anthony et al., 2004; Heard et al., 1977; Munro, 1975). Amino acid deprivation in mammals is particularly problematic as nine amino acids cannot be synthesized de novo; consequently, some proteins must be degraded to redeploy essential amino acids for synthesis of proteins vital for survival. Recycling of nonessential proteins in skeletal muscle and some internal organs such as liver results in overall reduction in body weight (Adibi, 1976; Kerr et al., 1978).

Adaptive changes to amino acid deprivation have been extensively studied in yeast, where they have been shown to be regulated by the general control nonderepressible 2 (GCN2) kinase, which is activated by uncharged tRNA-dependent mechanisms (Hinnebusch, 1994; Wek et al., 1995). Activated GCN2 kinase phosphorylates eukaryotic initiation factor 2α (eIF2α) and results in derepression of the translation of the transcription factor GCN4. Elevated levels of GCN4 stimulate the expression of hundreds of genes, including enzymes required to synthesize all 20 major amino acids (Hinnebusch, 1997; Mueller and Hinnebusch, 1986).

The response to amino acid deprivation in mammals is characterized by repression of protein synthesis and upregulation of amino acid biosynthesis and transporters (Anthony et al., 2004; Averous et al., 2003; Kilberg et al., 2005). GCN2 is thought to play a dual role in mammals by upregulating the synthesis of nonessential amino acids and regulating a complex array of adaptation to cope with the loss of essential amino acids (Anthony et al., 2004; Berlanga et al., 1999; Maurin et al., 2005; Sood et al., 2000; Zhang et al., 2002b). Rodents exhibit an aversive behavior toward food deficient in essential amino acids, which is lost in *Gcn2* mutant mice (Hao et al., 2005; Maurin et al., 2005). Fetal development is also impaired in *Gcn2*$^{-/-}$ mice if dietary essential amino acids are lacking during gestation (Zhang et al., 2002b).

As essential amino acid limitation in nature is almost always correlated with nutrient deprivation or starvation, we speculated that GCN2 may act as a master regulator of metabolic adaptation to nutrient deprivation. The potential coordinate regulation of diverse metabolic pathways during essential amino acid deprivation was suggested by a recent study in cultured hepatocyte-derived HepG2 cells showing that, when any of the essential amino acids is absent, expression of fatty acid synthase (FAS), a key component of lipid synthesis, is downregulated (Dudek and Semenkovich, 1995). Inasmuch as lipid synthesis and adaptation to essential amino acid deprivation are biochemically distinct, we speculated that this response reflects an underlying mechanism modulating multiple metabolic pathways in response to nutrient deprivation. As deficiency of amino acid is sensed by GCN2, we hypothesized that GCN2 might be involved in repressing lipid synthesis during amino acid deprivation. To investigate this hypothesis, we examined the response of *Gcn2*$^{+/+}$ and *Gcn2*$^{-/-}$ mice to deprivation of the essential amino acid leucine. We found that lipid synthesis was repressed in the livers of *Gcn2*$^{+/+}$ mice during prolonged leucine

**Table 1. Phenotypic Differences between Wild-Type (+/+) and *Gcn2* Knockout (−/−) Mice on Control and Leucine-Deficient Diet**

| | +/+ | | −/− | |
|---|---|---|---|---|
| Parameter | Control Diet | (−)leu Diet | Control Diet | (−)leu Diet |
| 17 Days | | | | |
| Food intake reduction (%) | −1.7 ± 3.1 | −32.0 ± 3.8* | −1.8 ± 6.0 | −34.4 ± 4.2* |
| Body weight reduction (%) | 6.7 ± 1.6 | −28.6 ± 1.0* | 5.4 ± 2.0 | −27.4 ± 2.2* |
| Liver weight (mg) | 1092.8 ± 37.8 | 569.4 ± 31.6* | 1135.0 ± 73.1 | 966.0 ± 54.6 |
| Liver weight/body weight (%) | 4.5 ± 0.1 | 3.7 ± 0.2* | 4.1 ± 0.3 | 5.6 ± 0.1* |
| Adipose tissue weight (mg) | 854.4 ± 138.1 | 25.4 ± 10.4* | 745.4 ± 125.1 | 231.8 ± 25.3* |
| Adipose tissue weight/body weight (%) | 3.5 ± 0.6 | 0.2 ± 0.1* | 2.8 ± 0.5 | 1.3 ± 0.1* |
| 7 Days | | | | |
| Food intake reduction (%) | 2.6 ± 3.0 | −21.8 ± 4.7* | 2.2 ± 4.2 | −21.2 ± 5.1* |
| Body weight reduction (%) | 2.5 ± 1.5 | −11.8 ± 1.0* | 2.1 ± 2.2 | −10.0 ± 1.6* |
| Liver weight (mg) | 948.0 ± 65.9 | 911.0 ± 74.8 | 1059.0 ± 64.0 | 1247.0 ± 49.9* |
| Liver weight/body weight (%) | 4.4 ± 0.1 | 4.3 ± 0.3 | 4.4 ± 0.1 | 5.2 ± 0.3* |
| Adipose tissue weight (mg) | 461.0 ± 104.8 | 234.0 ± 47.7 | 562.0 ± 66.3 | 468.0 ± 50.4 |
| Adipose tissue weight/body weight (%) | 2.1 ± 0.3 | 1.1 ± 0.2* | 2.2 ± 0.2 | 1.9 ± 0.2 |
| Liver cholesterol content (mg/g) | 59.5 ± 3.6 | 59.0 ± 1.5 | 58.2 ± 1.0 | 56.7 ± 1.9 |
| Liver triglyceride content (mg/g) | 54.8 ± 1.4 | 56.5 ± 2.2 | 54.3 ± 1.8 | 100.0 ± 1.8* |
| Liver glycogen content (mmol/mg) | 148.0 ± 7.6 | 146.0 ± 4.0 | 151.0 ± 4.6 | 150.0 ± 9.8 |
| Serum cholesterol (mg/dl) | 138.9 ± 3.0 | 147.0 ± 6.8 | 138.0 ± 4.7 | 149.0 ± 8.3 |
| Serum triglycerides (mg/dl) | 54.3 ± 1.6 | 35.0 ± 1.5* | 55.9 ± 1.4 | 25.6 ± 1.6* |
| Serum free fatty acid (mEq/l) | 0.7 ± 0.0 | 0.4 ± 0.1* | 0.7 ± 0.1 | 1.1 ± 0.1* |
| Serum insulin (ng/ml) | 0.6 ± 0.1 | 0.2 ± 0.0* | 0.7 ± 0.1 | 0.2 ± 0.0* |
| Blood glucose (mg/dl) | 178.7 ± 10.0 | 168.4 ± 7.1 | 182.2 ± 7.0 | 169.5 ± 7.0 |
| Fatty liver | No | No | No | Yes |

Two- to three-month-old mice were maintained on either nutritionally complete amino acid diet (control diet) or diet devoid of leucine ((−)leu diet) for 17 or 7 days. Numbers and sex of mice used: for 17 days, n = 5 (3 male, 2 female) per treatment per genotype; for 7 days, control diet: n = 6 (3 male, 3 female) per genotype, (−)leu diet: n = 8 (5 male, 3 female) per genotype. Values represent mean ± SEM. Total food intake refers to the total amount of control or leucine-deficient diet consumed during 17 or 7 days. *$p < 0.01$ by two-tailed Student's t test for effect of (−)leu diet versus control diet within the same strains of mice. For all parameters examined, no significant difference was observed between the two *Gcn2* strains fed control diet or between genders in response to (−)leu diet.

deprivation, whereas lipid synthesis continued unabated in $Gcn2^{-/-}$ mice, resulting in severe liver steatosis. Failure to downregulate lipid synthesis was found to be due to persistent expression of sterol regulatory element-binding protein 1c (SREBP-1c) and its downstream transcriptional targets underlying fatty-acid and triglyceride synthesis.

## RESULTS

### Leucine Deprivation Results in Radical Changes in Liver and Adipose Tissue Mass

A pilot experiment was conducted to assess the effect of leucine deprivation in mice as a function of *Gcn2* genotype. $Gcn2^{+/+}$ and $Gcn2^{-/-}$ mice were maintained on either a control diet or a diet lacking leucine for 17 days. Leucine deprivation resulted in a reduction in food intake and body weight in both $Gcn2^{+/+}$ and $Gcn2^{-/-}$ mice (Table 1). $Gcn2^{+/+}$ mice fed the leucine-deprived diet for 17 days experienced a 48% loss of liver mass and a dramatic 97% loss of abdominal adipose mass, whereas $Gcn2^{-/-}$ mice showed no apparent loss of liver mass and much less severe loss of adipose tissue (69% reduced) compared to mice fed the control diet. A second leucine-deprivation feeding experiment was conducted for a period of 7 days to better assess the more immediate effects of leucine-deprivation. Liver mass was similar in $Gcn2^{+/+}$ mice fed the leucine-deprived diet compared to the control diet during this shorter period, whereas adipose tissue was reduced by almost 50% (Table 1). In contrast, liver mass was significantly increased in GCN2-deficient mice fed

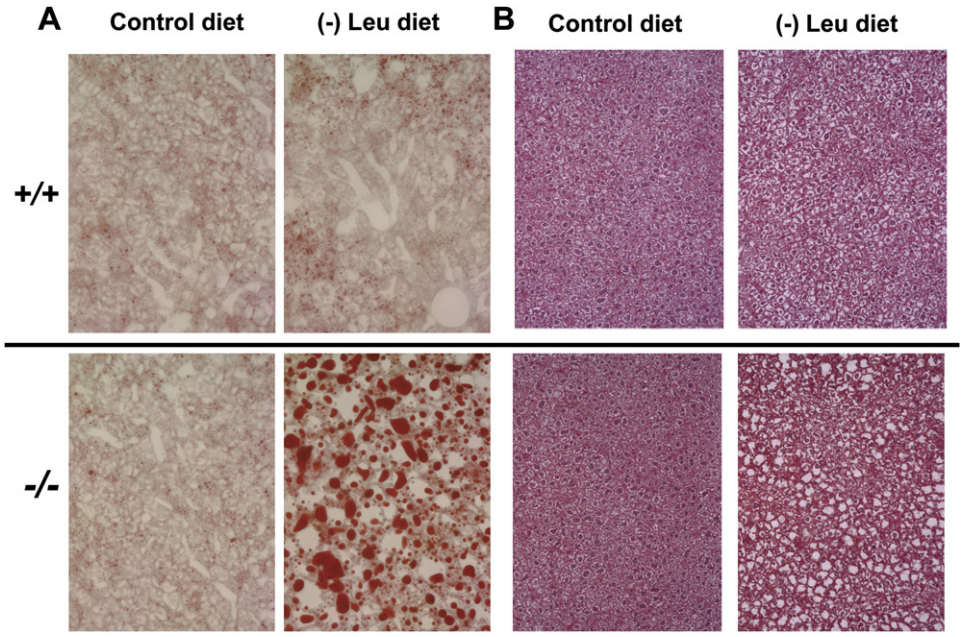


**Figure 1. Leucine-Deprived *Gcn2*$^{-/-}$ Mice Exhibit Severe Liver Steatosis**

Liver histology for wild-type (+/+) and *Gcn2* KO (−/−) mice. Mice were fed either control diet or leucine-deficient diet for 7 days. Liver tissue sections from control and (−)leu diet group animals were stained with oil red O (A, 40× magnification) or hematoxylin and eosin (B, 20× magnification). Lipid deposits are detected as red-stained areas in (A) and white empty areas in (B). Images shown are representative of several animals for each group.

the leucine-deprived diet compared to the control diet, but no significant difference in adipose mass was seen.

### *Gcn2*$^{-/-}$ Mice Display Severe Liver Steatosis When Fed a Leucine-Deprived Diet

The livers of *Gcn2*$^{-/-}$ mice fed a leucine-deficient diet for 7 days appeared very pale, suggesting severe liver steatosis. This observation was confirmed by histological examination of the liver, which revealed extensive lipid deposition manifested as macro- and microvesicular steatosis (Figures 1A and 1B). Leucine deprivation had no effect on total cholesterol in the liver in either strain of mice. By contrast, leucine deprivation dramatically increased liver triglycerides in *Gcn2*$^{-/-}$ mice compared to *Gcn2*$^{+/+}$ mice (Table 1). As observed for liver, cholesterol in serum was not affected by leucine deprivation in either *Gcn2*$^{+/+}$ or *Gcn2*$^{-/-}$ mice. Leucine deprivation reduced triglyceride levels in serum significantly in *Gcn2*$^{+/+}$ mice and significantly more in *Gcn2*$^{-/-}$ mice. Leucine deprivation resulted in significantly decreased free fatty acids in serum in *Gcn2*$^{+/+}$ mice; in contrast, fatty-acid levels increased in serum in *Gcn2*$^{-/-}$ mice. Taken together, these results suggest that leucine deprivation rapidly results in liver steatosis caused by deposition of triglycerides in the *Gcn2*$^{-/-}$ mice. Leucine deprivation resulted in decreased insulin levels in both genotypes perhaps in part due to decreased food intake, but serum glucose was unaffected by this diet (Table 1). Liver glycogen content was equivalent across both diets and genotypes (Table 1). Both strains of mice showed a normal glucose clearance when challenged with glucose injection (see Figure S1 in the Supplemental Data available with this article online).

### Genes Related to Triglyceride Synthesis Are Not Repressed in *Gcn2*$^{-/-}$ Mice Fed a Leucine-Deprived Diet

The accumulation of hepatic triglycerides in *Gcn2*$^{-/-}$ mice fed a leucine-deprived diet is likely to reflect an imbalance in hepatic triglyceride synthesis, β-oxidation, and/or uptake and/or secretion of fatty acids. For this reason, we examined changes in levels of mRNA expression for proteins related to each of these processes. We first investigated whether genes underlying the synthesis of triglycerides are differentially regulated in *Gcn2*$^{+/+}$ and *Gcn2*$^{-/-}$ mice during leucine deprivation. These proteins included acetyl CoA carboxylase 1 (ACC1) and FAS, which together catalyze the rate-limiting step in production of palmitate 16:0; stearoyl CoA desaturase (SCD), which catalyses the synthesis of oleic acid (18:1, n-9); ATP-citrate lyase (ACL), which catalyzes the conversion of citrate to CoA and oxaloacetate; and glucose-6-phosphate dehydrogenase (G6PD) and malic enzyme (ME), which generate NADPH for synthesis of fatty acids (Horton and Shimomura, 1999). Levels of *Fas* mRNA and protein were greatly reduced in the livers of *Gcn2*$^{+/+}$ mice fed a leucine-deficient diet but were not reduced in *Gcn2*$^{-/-}$ mice (Figures 2A and 2B). Similarly, FAS enzyme activity was reduced by 74% in the livers of *Gcn2*$^{+/+}$mice but was increased

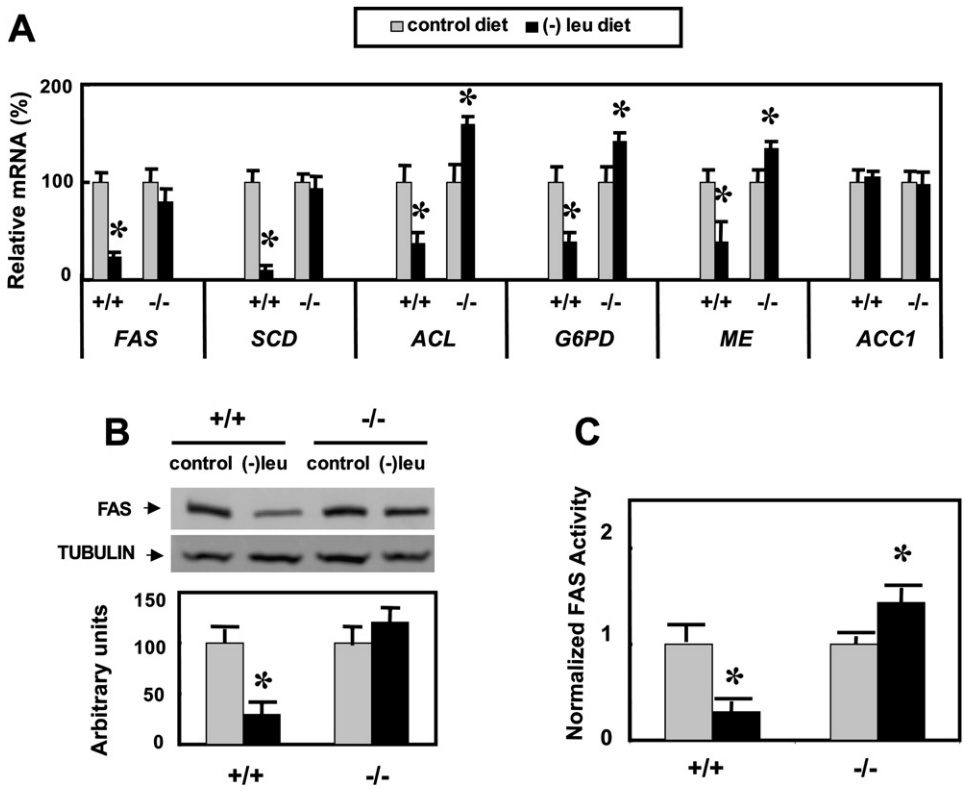


**Figure 2. Lipogenic Genes Are Not Repressed in Livers of Leucine-Deprived *Gcn2*$^{-/-}$ Mice**

Expression of lipogenic genes in the livers of *Gcn2* KO (−/−) and wild-type (+/+) mice fed either control diet or leucine-deficient diet for 7 days. Data are mean ± SEM of at least two independent real-time PCR experiments per animal (A) or western blot (B) with mice of each diet for each experiment (WT: control diet [n = 6], (−)leu diet [n = 8]; KO: control diet [n = 6], (−)leu diet [n = 8]). *p < 0.0001 by two-tailed Student's t test for effect of (−)leu diet versus control diet compared within the same strains of mice.

(A) Expression of *Fas*, *Scd*, *Acl*, *G6pd*, *Me*, and *Acc1* mRNAs.

(B) FAS protein (top, western blot; bottom, FAS protein relative to tubulin and normalized to control diet group within same strain of mice).

(C) FAS enzyme activity.

1.5-fold in *Gcn2*$^{-/-}$ mice under leucine deprivation (Figure 2C). Leucine deprivation also resulted in a large decrease in the mRNA levels of *Acl*, *Scd*, *G6pd*, and *Me* in the livers of *Gcn2*$^{+/+}$ mice. In contrast, the expression of these lipogenic genes was not repressed in the livers of leucine-deprived *Gcn2*$^{-/-}$ mice and, in the case of ACL, G6PD, and ME, their mRNA expression was increased (Figure 2A). The increase in G6PD and ME expression may enhance the production of NADPH, which is required as the reducing power to drive fatty-acid synthesis. Only *Acc1* mRNA, among the fatty-acid synthesis genes, was expressed at equivalent levels in mice fed control or leucine-deficient diets in the livers of both *Gcn2*$^{+/+}$ and *Gcn2*$^{-/-}$ mice (Figure 2A).

The expression of cholesterol synthesis genes including 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase, HMG-CoA reductase, and farnesyl diphosphate synthase was unchanged in the livers of either *Gcn2*$^{+/+}$ or *Gcn2*$^{-/-}$ mice maintained on a leucine-deficient diet (data not shown). These results are consistent with the observation that leucine deprivation did not result in a significant change in liver cholesterol levels in *Gcn2*$^{+/+}$ or *Gcn2*$^{-/-}$ mice (Table 1).

## Expression of SREBP-1c and PPARγ Is Repressed in Wild-Type, but Not *Gcn2*$^{-/-}$, Mice Fed a Leucine-Deprived Diet

Lipogenic genes in the liver are regulated primarily by SREBP-1c (Brown and Goldstein, 1997) and secondarily by peroxisome proliferator-activated receptor γ (PPARγ) (Herzig et al., 2003) and carbohydrate response element-binding protein (ChREBP) (Dentin et al., 2006; Iizuka et al., 2006). Expression of *Srebp-1c* mRNA in liver was reduced 43% by leucine deprivation in *Gcn2*$^{+/+}$ mice, whereas it was increased by 176% in *Gcn2*$^{-/-}$ mice (Figure 3A). Reduced expression of *Srebp-1c* mRNA in *Gcn2*$^{+/+}$ mice was accompanied by an 80% reduction in the precursor and mature, nuclear-localized SREBP-1c protein, whereas it was unchanged in the livers of *Gcn2*$^{-/-}$ mice (Figures 3B and 3C). In contrast, the expression of SREBP-1a precursor protein was not altered in either diet or genotype (Figure 3B). Regulators of SREBP processing including SCAP, S1P, and Insig1 and 2a (Goldstein et al., 2006) were examined. *Scap* mRNA was reduced in the livers of *Gcn2*$^{+/+}$ mice and *S1p* mRNA was increased in the livers of *Gcn2*$^{-/-}$ mice under leucine deprivation, while the other genes remained unchanged in both strains of mice.

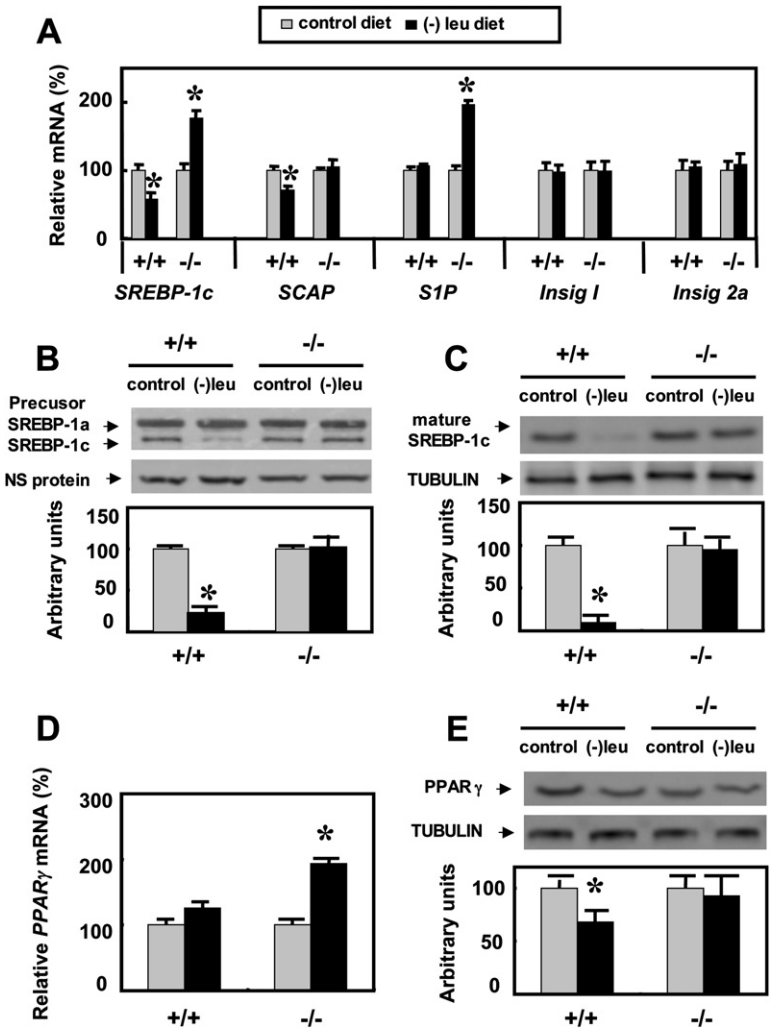


**Figure 3. GCN2-Dependent Regulation of SREBP-1c and PPARγ**

Expression of lipogenic regulatory factors in the livers of *Gcn2* KO (−/−) and wild-type (+/+) mice fed either control diet or leucine-deficient diet for 7 days. Data are mean ± SEM of at least two independent real-time PCR experiments (A and D) or western blot (B, C, and E) with mice of each diet for each experiment (WT: control diet [n = 6]; (−)leu diet [n = 8]; KO: control diet [n = 6]; (−)leu diet [n = 8]). *p < 0.0001 by two-tailed Student's t test for effect of (−)leu diet versus control diet within the same strains of mice.

(A) Expression of *Srebp-1c*, *Scap*, *S1p*, *Insig1*, and *Insig2a* mRNA.

(B) SREBP-1c precursor protein (top, western blot; bottom, SREBP-1c protein relative to NS protein and normalized to control diet group within same strains of mice).

(C) SREBP-1c nuclear mature form of SREBP-1c (top, western blot; bottom, SREBP-1c protein relative to tubulin and normalized to control diet group within same strain of mice).

(D) Expression of *Pparγ* mRNA.

(E) PPARγ protein from nuclear extraction (top, western blot; bottom, PPARγ protein relative to tubulin and normalized to control group within same strain of mice).

Leucine deprivation had no significant effect on levels of *Pparγ* mRNA in the livers of *Gcn2*$^{+/+}$ mice, but PPARγ protein was moderately decreased (Figures 3D and 3E). Curiously, *Pparγ* mRNA was increased in the livers of leucine-deprived *Gcn2*$^{-/-}$ mice, but PPARγ protein was unchanged (Figures 3D and 3E). Accumulation of fat could also be controlled by ChREBP-stimulated glycolysis and lipogenesis (Dentin et al., 2006; Iizuka et al., 2006). Glucose levels, however, were unchanged in leucine-deprived *Gcn2*$^{+/+}$ or *Gcn2*$^{-/-}$ mice (Table 1). The expression of *Chrebp* and its downstream target gene, liver pyruvate kinase (*L-pk*), was also unchanged in the livers of both *Gcn2*$^{+/+}$ and *Gcn2*$^{-/-}$ mice under leucine deprivation (Figure S2).

## β-Oxidation Genes Are Upregulated in *Gcn2*$^{-/-}$ Mice Fed a Leucine-Deprived Diet

Impaired β-oxidation of fatty acids is another potential cause of liver steatosis (Kersten et al., 1999; Leone et al., 1999). To examine whether genes related to this process are differentially regulated by leucine deprivation in *Gcn2*$^{+/+}$ and *Gcn2*$^{-/-}$ mice, the expression level of mRNAs encoding the transcription factor PPARα and its target genes fatty acyl-CoA oxidase (ACO) and long- and medium-chain acyl-CoA dehydrogenase (LCAD and MCAD) were examined. Leucine deprivation had no effect on levels of *Pparα*, *Aco*, *Lcad*, and *Mcad* mRNA in the livers of *Gcn2*$^{+/+}$ mice, whereas the expression of these genes was markedly increased in *Gcn2*$^{-/-}$ mice (Figure 4A). Thus, the expression of β-oxidation genes is not impaired in *Gcn2*$^{-/-}$ mice; rather, these genes are induced, perhaps in response to increased fat accumulation in the liver.

## Fatty-Acid Transport Genes Are Upregulated in *Gcn2*$^{-/-}$ Mice Fed a Leucine-Deprived Diet

In addition to triglyceride synthesis and β-oxidation, misregulation of triglyceride uptake and secretion could also contribute to fatty liver (Bradbury, 2006). We therefore examined genes related to the uptake of fatty acids, including fatty acid binding protein (FABP), fatty acid translocase (CD36), fatty acid transport protein (FATP), and lipoprotein lipase (LPL) mRNAs. These genes were all significantly increased in the livers of leucine-deprived *Gcn2*$^{-/-}$ mice, whereas *Gcn2*$^{+/+}$ mice exhibited no

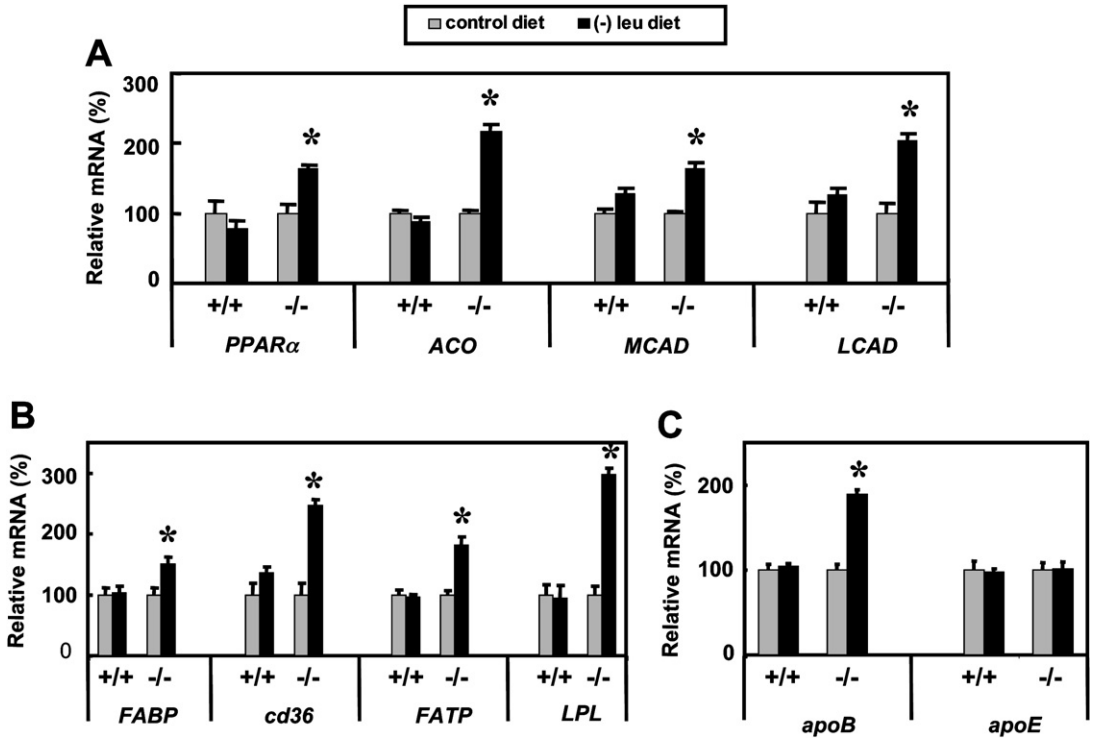


**Figure 4. β-Oxidation and Fatty-Acid Transport Genes Are Upregulated in Livers of *Gcn2*$^{-/-}$ Mice Fed a Leucine-Deficient Diet**

Expression of β-oxidation and fatty-acid transport genes in the livers of *Gcn2* KO (−/−) and wild-type (+/+) mice fed a leucine-deficient diet for 7 days compared with control diet. Data are mean ± SEM of at least two independent real-time PCR experiments with mice of each diet for each experiment (WT: control diet [n = 6]; (−)leu diet [n = 8]; KO: control diet [n = 6]; (−)leu diet [n = 8]). *p < 0.0001 by two-tailed Student's t test for effect of (−)leu diet versus control diet within the same strains of mice.

(A) *Pparα*, *Aco*, *Lcad*, and *Mcad* mRNA.

(B) *Fabp*, *cd36*, *Fatp*, and *Lpl* mRNA.

(C) *ApoB* and *ApoE* mRNA.

significant increase in the expression. Expression of *ApoB* and *ApoE* mRNAs, which encode key proteins in lipid secretion and transport, was also examined. *ApoB* mRNA was increased in the livers of leucine-deprived *Gcn2*$^{-/-}$ mice, but *ApoE* mRNA was unchanged in both strains of mice (Figure 4B).

## Leucine-Deprivation-Induced Liver Steatosis in *Gcn2*$^{-/-}$ Mice Is Prevented by Treatment with C75, a Potent FAS Inhibitor

We postulated that failure to repress liver FAS expression and induced FAS enzyme activity were major factors contributing to persistent lipid synthesis and liver steatosis in leucine-deprived *Gcn2*$^{-/-}$ mice. To test this hypothesis, we examined the ability of C75, a potent FAS inhibitor, to repress lipid synthesis and the development of fatty liver in leucine-deprived *Gcn2*$^{-/-}$ mice. C75 has been shown to inhibit FAS expression significantly in liver 24 hr after a single i.p. injection (Kim et al., 2002). We found that liver steatosis was prevented in leucine-deprived *Gcn2*$^{-/-}$ mice that had received three doses of C75 over the course of the 7 day period (Figure 5A). Moreover, *Fas* mRNA and protein levels were repressed in the livers of *Gcn2*$^{-/-}$ mice (Figures 5B and 5C) to levels similar to those seen in leucine-deprived wild-type mice (Figures 2A and 2B). We also speculated that the activation of *Pparα* mRNA and increased expression of β-oxidation genes in the livers of leucine-deprived *Gcn2*$^{-/-}$ mice were secondary responses to increased triglycerides and therefore should be ablated by C75 administration. Indeed, the levels of *Pparα* and *Aco* mRNA in the livers of leucine-deprived *Gcn2*$^{-/-}$ mice were reduced by C75 treatment (Figures 5D and 5E).

## Phosphorylation of eIF2α and Regulation of Amino Acid Biosynthetic Genes Are GCN2 Dependent in the Liver under Leucine Deprivation

Short-term leucine deprivation in cultured mouse embryonic fibroblasts was previously shown to result in potent activation of GCN2 and phosphorylation of eIF2α (Harding et al., 2003; Zhang et al., 2002b). In response, ATF4 is induced translationally in cultured cells and activates the expression of C/EBPβ (Chen et al., 2005) and amino acid metabolism and transport genes including asparagine synthetase (*Asns*) (Harding et al., 2003; Siu et al., 2002). However the GCN2 dependence of ASNS regulation had not been tested in a whole animal system. We found that the phosphorylated state of eIF2α in the livers was

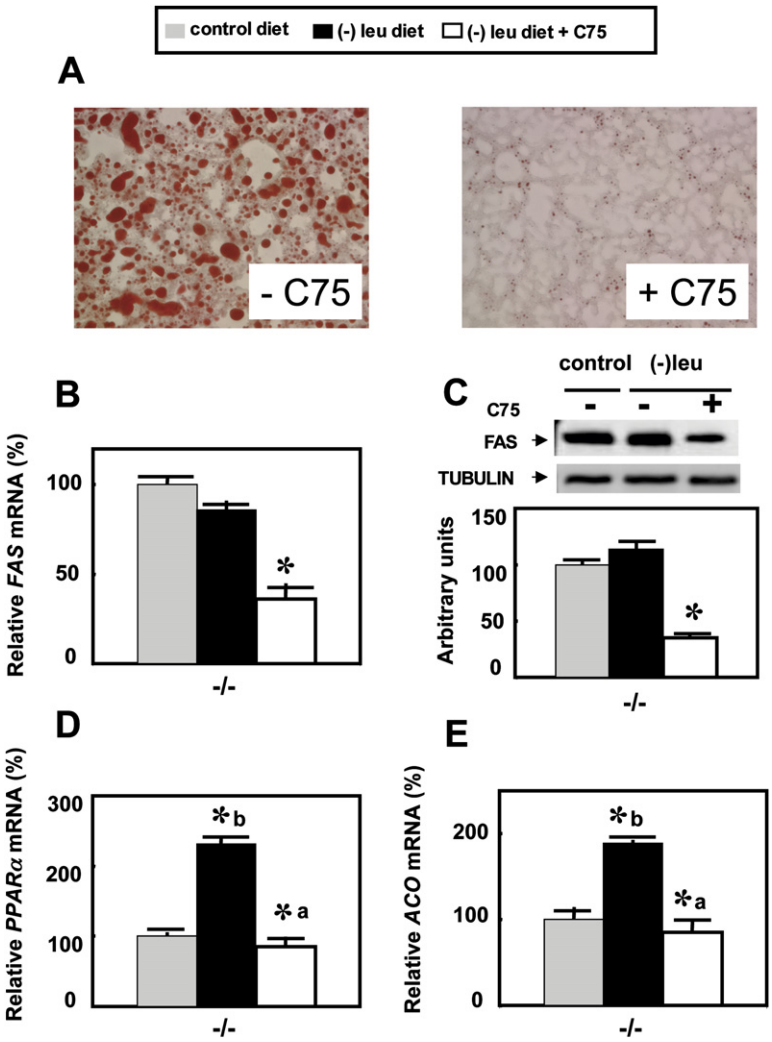


**Figure 5. Leucine-Deprivation-Induced Liver Steatosis in *Gcn2*$^{-/-}$ Mice Is Prevented by Inhibition of FAS Expression**

*Gcn2* KO (−/−) mice were fed a leucine-deficient diet for 7 days and were given C75 at a dose of 20 mg/kg body weight in 200 μl RPMI or only RPMI every other day by i.p. injection during this period. Error bars in (B)–(E) = SEM.

(A) Liver histology for *Gcn2* KO mice. Sections from animals were stained with oil red O (40× magnification). Lipid deposits are detected as red-stained areas.

(B and C) Expression of *Fas* mRNA (B) and FAS protein (C) (top, western blot; bottom, FAS protein relative to tubulin and normalized to control diet group within same strain of mice). *p < 0.0001 by two-tailed Student's t test for effect of C75 treatment on *Gcn2* KO mice fed (−)leu diet versus no C75 treatment on *Gcn2* KO mice fed (−)leu diet or versus *Gcn2* KO mice fed control diet. Control diet (n = 6); (−)leu diet (n = 5); (−)leu diet + C75 (n = 3).

(D and E) Expression of *Pparα* and *Aco* mRNA. *a: p < 0.001 by two-tailed Student's t test for effect of C75 treatment versus no C75 treatment on *Gcn2* KO mice fed (−)leu diet. *b: p < 0.001 by two-tailed Student's t test for effect of (−)leu diet versus control diet. Control diet (n = 6); (−)leu diet (n = 5); (−)leu diet + C75 (n = 3).

markedly increased in wild-type mice consuming a leucine-deficient diet for 7 days compared to mice fed a complete diet, whereas *Gcn2*$^{-/-}$ mice lacked this induction (Figure 6A). *Asns* and *C/ebp*β mRNAs were expressed at low levels in the livers of both strains of mice when fed a complete diet (Figure 6B and 6C). The expression of these mRNAs, as well as the C/EBPβ protein, was induced markedly in the livers of leucine-deprived *Gcn2*$^{+/+}$ mice (Figures 6B–6D). In contrast, *Asns* mRNA and *C/ebp*β mRNA and protein were not induced by the leucine-deprived diet in the livers of *Gcn2*$^{-/-}$ mice (Figures 6B–6D). To determine whether ATF4 was responsible for the GCN2-dependent regulation of lipogenesis and *Asns* mRNA expression, *Atf4*$^{-/-}$ mice were subjected to a leucine-deprived diet for 7 days. Unlike *Gcn2*$^{-/-}$ mice, *Atf4*$^{-/-}$ mice did not develop fatty liver under leucine deprivation, and liver *Fas* mRNA was repressed to an extent similar to in *Atf4*$^{+/+}$ mice (Figure S3). However, *Asns* mRNA was not induced in the livers of leucine-deprived *Atf4*$^{-/-}$ mice (Figure S3), indicating that leucine-deprivation-induced *Asns* mRNA expression is dependent upon both GCN2 and ATF4 as was previously shown for cultured cells (Harding et al., 2003; Siu et al., 2002).

## DISCUSSION

During episodes of extended fasting, lipid synthesis in the liver is downregulated and lipolysis in adipose tissue is upregulated in concert with an overall shift in the balance of anabolic and catabolic metabolism (Finn and Dice, 2006). Considering the known metabolic pathways of amino acid and lipid metabolism, it could not have been anticipated that deprivation of a single essential amino acid would impact lipid metabolism. Yet we found that lipid synthesis is downregulated in the liver and adipose tissue mass is reduced in wild-type mice fed a leucine-deficient diet for several days. Based upon the unexpected findings of this study, we propose that limitation of even a single essential amino acid triggers a global nutrient-deprivation response that includes the modulation of lipid synthesis. As essential amino acid limitation in nature occurs during general nutrient deprivation, we speculate that detection of essential amino acid limitation acts to mobilize a global

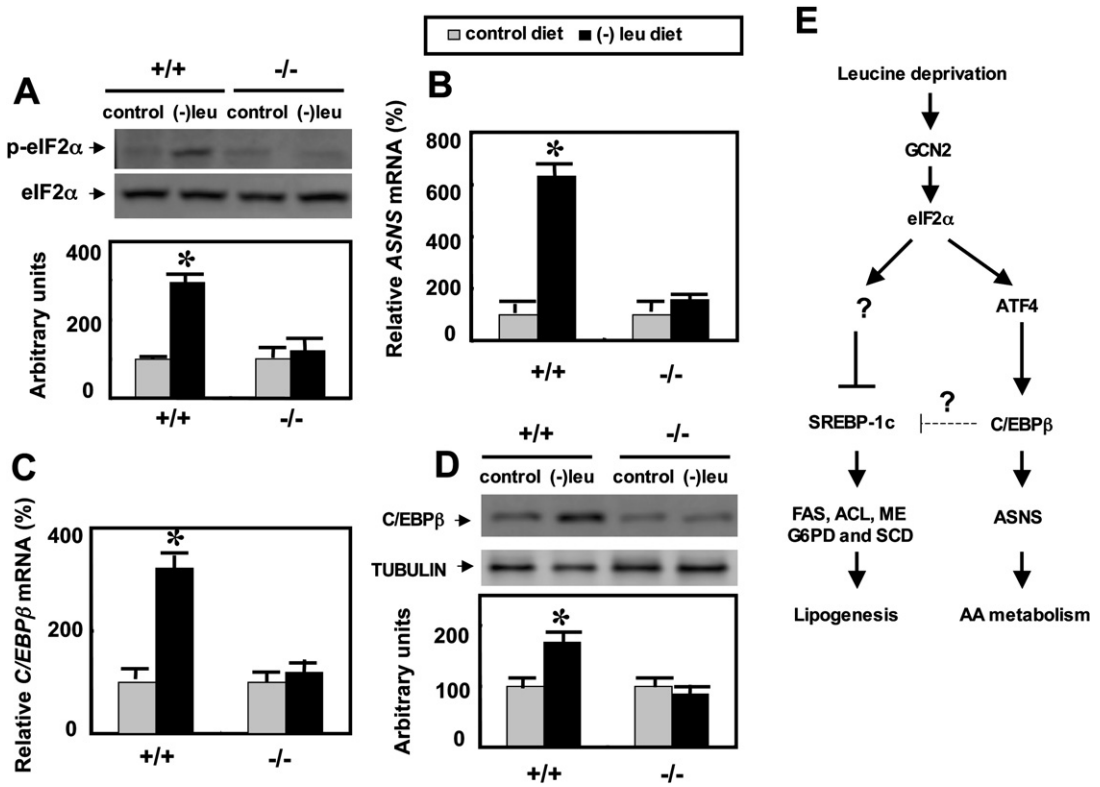


**Figure 6. Phosphorylation of eIF2α and Regulation of Amino Acid Biosynthetic Genes Are GCN2 Dependent in the Liver under Leucine Deprivation**

*Gcn2* KO (−/−) and wild-type mice (+/+) were fed control diet or leucine-deficient diet for 7 days. *$p < 0.0001$ by two-tailed Student's t test for effect of (−)leu diet versus control diet within the same strains of mice. (WT: control diet [n = 6]; (−)leu diet [n = 8]; KO: control diet [n = 6]; (−)leu diet [n = 8].) Error bars in (A)–(D) = SEM.

(A) Phosphorylation of eIF2α (top, western blot; bottom, phospho-eIF2α relative to eIF2α and normalized to control diet group within same strain of mice).

(B) *Asns* mRNA.

(C) *C/ebpβ* mRNA.

(D) C/EBPβ protein from nuclear extraction (top, western blot; bottom, C/EBPβ protein relative to tubulin and normalized to control diet group within same strain of mice).

(E) Model of GCN2-dependent regulation of lipid metabolism in liver. Leucine deprivation results in activation of GCN2 and phosphorylation of eIF2α. This is followed by repression of SREBP-1c and its downstream lipogenic genes and, in parallel, activation of ATF4 and C/EBPβ, as well as their downstream amino acid regulatory genes.

adaptive metabolic response. The critical role of GCN2 in regulating lipid metabolism was shown by the rapid development of liver steatosis in $Gcn2^{-/-}$ mice during leucine deprivation. Following a switch from a complete diet to a leucine-deficient diet, $Gcn2^{+/+}$ mice downregulate mRNAs encoding proteins linked to the synthesis of fatty acids and triglycerides including SREBP-1c, ACL, FAS, SCD, G6PD, and ME. These results are consistent with an in vitro study showing that *Fas* mRNA expression is repressed by leucine deprivation in HepG2 cells (Dudek and Semenkovich, 1995). In contrast, $Gcn2^{-/-}$ mice fail to downregulate these mRNAs, and some are even induced. Consistent with changes in its gene and protein expression, FAS enzyme activity is suppressed significantly by leucine deprivation in the livers of $Gcn2^{+/+}$ mice but is increased 1.5-fold in $Gcn2^{-/-}$ mice. The importance of GCN2-dependent repression of FAS was further demonstrated by the ability of a potent FAS inhibitor to prevent liver steatosis in leucine-deprived $Gcn2^{-/-}$ mice. Increased fat accumulation in liver could also arise from other sources, including mobilization of free fatty acids from adipose tissue and increased dietary intake (Fong et al., 2000). However, leucine-deprived $Gcn2^{-/-}$ mice have a higher percentage of adipose tissue relative to body weight and consume less food compared to mice fed a control diet. Thus, failure to repress lipogenesis is likely to be the major cause of liver steatosis in these mice. We speculate that our studies on leucine-deprived mice can be extended to a deficiency of any of the essential amino acids, inasmuch as Dudek and Semenkovich (1995) showed that omission of any of the nine essential amino acids from growth medium resulted in the repression of *Fas* mRNA and protein in HepG2 cells, whereas depriving these cells of nonessential amino acids had no effect.

The decreased expression of lipogenic genes including *Fas*, *Acl*, *Scd*, *G6pd*, and *Me* in response to leucine deprivation may reflect repression of transcription, inasmuch as these genes were previously shown to be regulated by the transcription factor SREBP-1c as a function of nutritional state (Horton and Shimomura, 1999). SREBP-1c belongs to a basic helix-loop-helix/leucine-zipper transcription factor family, which also includes SREBP-1a and SREBP-2 (Brown and Goldstein, 1997). Among the SREBP family of transcription factors, SREBP-1c preferentially regulates genes involved in triglyceride and fatty-acid synthesis, whereas SREBP-2 regulates genes related to cholesterol synthesis. We found that mRNA and protein levels of SREBP-1c are reduced by leucine deprivation in the liver and that this effect is GCN2 dependent. The observation that SREBP-1a protein levels do not change in response to leucine deprivation suggests that SREBP-1c is the isoform required to inhibit triglycerides in the liver during leucine deprivation. No change in the expression of SREBP-2 or its target genes was observed in the livers of either strain of mice under leucine deprivation (unpublished data). Triglyceride-enriched fatty liver, with no increase in cholesterol, has been previously described in transgenic mice that overexpress SREBP-1c in liver (Shimano et al., 1997). PPARγ, a secondary activator of lipogenic gene expression in the liver (Herzig et al., 2003), exhibits a modest reduction in the livers of leucine-deprived wild-type mice and may therefore also contribute to the reduction in triglyceride synthesis.

In addition to altered triglyceride synthesis, impairment of β-oxidation and/or imbalance between triglyceride uptake and secretion could also contribute to increased accumulation of triglycerides in the livers of *Gcn2*$^{-/-}$ mice. We showed that the transcription factor PPARα and its target genes related to β-oxidation are significantly upregulated in the livers of leucine-deprived *Gcn2*$^{-/-}$ mice. The increased expression of β-oxidation genes, however, is not sufficient to prevent the increased triglyceride accumulation in the livers of *Gcn2*$^{-/-}$ mice under these conditions. We surmise that the increase of PPARα and its downstream targets in β-oxidation is a secondary response to the high level of triglyceride synthesis occurring in leucine-deprived *Gcn2*$^{-/-}$ mice. In fact, it has been shown that PPARα can be activated by fatty acids and peroxisome proliferators (Chakravarthy et al., 2005; Forman et al., 1997). This interpretation is also supported by our finding that *Pparα* and *Aco* expression is repressed by treatment with a FAS inhibitor.

In addition to its key role in regulating β-oxidation, PPARα has been shown to positively regulate the expression of genes controlling fatty-acid uptake, including *Lpl*, *Fabp*, *cd36*, and *Fatp* (Motojima et al., 1998; Tordjman et al., 2001). We found that leucine deprivation results in upregulation of these PPARα target genes facilitating fatty-acid uptake into the liver. Thus, an increase in fatty-acid uptake in leucine-deprived *Gcn2*$^{-/-}$ mice is likely a contributing factor to liver steatosis in these animals. However, expression of *ApoB*, which functions in the secretion of triglycerides from liver, was also upregulated by leucine deprivation, which would tend to reduce fatty-acid accumulation. Moreover, we found that further activation of PPARα with the agonist WY compound in mice deprived of leucine was not able to prevent the development of fatty liver in *Gcn2*$^{-/-}$ mice (unpublished data). Taken together, these results suggest that increases in fatty-acid synthesis and uptake in the liver outweigh apparent increases in β-oxidation and secretion in *Gcn2*$^{-/-}$ mice, resulting in fatty liver.

GCN2-dependent regulation of lipogenesis is likely to entail regulation of SREBP-1c, as we found that the level of *Srebp-1c* mRNA was substantially reduced in the liver of wild-type mice, but not *Gcn2* KO mice, fed a leucine-deficient diet. The liver X nuclear transcription factor α (LXRα), together with the retinoid receptor (RXR), is a critical regulator of SREBP-1c transcriptional activation (Chen et al., 2004; Liang et al., 2002) and therefore may mediate GCN2-dependent regulation of SREBP-1c. Preliminary studies have not shown a difference in the expression of LXRα in the liver between *Gcn2* genotypes under leucine deprivation (unpublished data). However, LXRα/RXR transcriptional activity is regulated by oxysterols and potentially other metabolites (Schultz et al., 2000), which will require further study. The transcription factor C/EBPβ, which we found was GCN2-dependently induced in the livers of leucine-deprived mice, may potentially negatively regulate LXRα (Steffensen et al., 2002).

Previous studies have shown that *Asns* gene expression is potently activated in fibroblasts and hepatocytes upon culturing in medium deprived of one or more essential amino acids and that this induction is GCN2 and ATF4 dependent (Harding et al., 2003; Siu et al., 2002). C/EBPβ has also been found to be induced in cultured cells by essential amino acid deprivation (Marten et al., 1994; Siu et al., 2001), but its GCN2 dependence had not been tested. In cell culture, high levels of phospho-eIF2α are transient and limited by activation of phosphatase (Novoa et al., 2001), raising the question of how long-term leucine deprivation would impact GCN2 activation. Moreover, organisms are capable of reallocating essential amino acids stored in various organs and tissues (Anthony et al., 2004), and consequently, the effect of dietary leucine deprivation on these pathways could not be assumed to be the same in whole organisms subjected to prolonged leucine deprivation. Nonetheless, we found that the level of phospho-eIF2α was indeed elevated by long-term leucine deprivation and was GCN2 dependent. Moreover, *Asns* mRNA and *C/ebpβ* mRNA and protein were markedly induced in the livers of leucine-deprived wild-type mice but not GCN2-deficient mice. Both ATF4 and C/EBPβ are critical transcriptional regulators of the *Asns* gene, so one or both of these factors may act directly or indirectly to repress *Srebp-1c* transcription by leucine deprivation. Our results, however, exclude ATF4 as a potential mediator of GCN2-dependent regulation of lipogenesis, as leucine-deprived *Atf4*$^{-/-}$ mice did not develop fatty liver and *Fas* mRNA was repressed similarly to in *Atf4*$^{+/+}$ mice. However, we did find that the GCN2-dependent regulation of *Asns* is dependent upon ATF4.

Normally, mammals avoid eating food deficient in one or more essential amino acids, and this behavior requires the function of GCN2 in the central nervous system (Hao et al., 2005; Maurin et al., 2005). This effect, however, is a short-term response and is likely to be predicated upon the availability of food choice. However, if animals are not given a food choice over a longer period, aversive behavior toward essential amino acid-deficient foods would be maladaptive. Not surprisingly, then, we failed to detect a difference between *Gcn2*$^{+/+}$ and *Gcn2*$^{-/-}$ mice in food intake during our several-day leucine-deprivation experiments. Instead, both genotypes continued to consume food, but at a reduced rate. We speculate that reduced food intake is likely due to an indirect metabolic effect of leucine deficiency on appetite.

A previous study showed that liver mass is reduced in mice fed a leucine-deprived diet (Anthony et al., 2004) but did not investigate the impact on adipose tissue. We were then surprised to observe an almost complete obliteration of adipose tissue in leucine-deprived wild-type mice after 17 days and a 50% reduction after only a week. The adipose tissue of *Gcn2*$^{-/-}$ mice is somewhat spared from this drastic reduction. We have not extensively examined the cause of the reduction in adipose tissue, but we speculate that it is due to enhanced lipolysis, as other factors that influence adipose tissue mass (e.g., lipogenesis, fatty-acid import, and cell death) do not act so quickly to radically reduce adipose tissue. Moreover, rapid reduction of adipose tissue during fasting is known to be caused by increased lipolysis (Finn and Dice, 2006), and our observation of repressed lipid synthesis in the liver combined with reduction in adipose tissue is characteristic of a fasting response.

In summary, our data suggest a novel function for GCN2: downregulating genes related to triglyceride synthesis and enzyme activity during leucine deprivation. By regulating genes related to the synthesis of fatty acids, GCN2 has a profound effect on the synthesis and storage of triglycerides and overall energy homeostasis. As essential amino acid limitation in nature is almost always correlated with other deprivation or starvation, we speculate that GCN2 may act as a master regulator of metabolic adaptation to nutrient deprivation.

## EXPERIMENTAL PROCEDURES

### Animals, Diets, and C75 Treatment

Wild-type C57BL/6J (*Gcn2*$^{+/+}$) mice were obtained from The Jackson Laboratory. *Gcn2*$^{-/-}$ mice backcrossed onto the C57BL/6J background for eight generations were generated as previously described (Zhang et al., 2002b). All mice used were in the C57BL/6J genetic background. Two- to three-month-old male and female *Gcn2*$^{+/+}$ and *Gcn2*$^{-/-}$ mice were maintained on a 12 hr light/dark cycle and were provided free access to commercial rodent chow and tap water prior to the experiments. Control (nutritionally complete amino acid) and (−)leu (leucine-deficient) diets were obtained from Research Diets, Inc. All diets were isocaloric and compositionally the same in terms of carbohydrate and lipid component. At the start of the feeding experiment, mice were acclimated to control diet for 10 days and then randomly assigned to either control diet group, with continued free access to the nutritionally complete diet, or (−)leu diet group, with free access to the diet devoid of the essential amino acid leucine for either 17 or 7 days. Treatment experiments with C75 (Calbiochem), an inhibitor of *Fas* gene expression, were performed on *Gcn2*$^{-/-}$ mice fed the leucine-deficient diet. In the C75-treated group, mice were injected intraperitoneally (i.p.) with 20 mg/kg body weight C75 in 200 μl of RPMI on the 2nd, 4$^{th}$, and 6th day on the leucine-deficient diet; in the non-C75-treated control group, mice were injected i.p. with 200 μl of RPMI accordingly. The dose of C75 was determined based on a previous report showing that 24 hr after a single i.p. injection of C75 at a dose of 30 mg/kg body weight, significant reduction of liver FAS expression results (Kim et al., 2002). Food intake and body weight were recorded daily. Animals were killed by $CO_2$ inhalation, and trunk blood was collected for the assays described below. Body, liver, and adipose tissue weight were recorded at the time of sacrifice. Livers were isolated and either put into 10% paraformaldehyde buffer immediately for histological study (described later) or snap frozen and stored at −20°C for future analysis. These experiments were approved by the Pennsylvania State University Institutional Animal Care and Use Committee.

### Serum Measurements

Serum was obtained by centrifugation of clotted blood, snap frozen in liquid nitrogen, and stored at −20°C. Serum triglyceride, total cholesterol, and free fatty-acid levels were determined enzymatically using triglyceride reagent (Sigma), Infinity cholesterol reagent (Thermo), and NEFA C reagent (Wako), respectively. Serum insulin was measured using the Mercodia Ultrasensitive Rat Insulin ELISA kit (ALPCO Diagnostic). All assays were performed according to manufacturer's instructions.

### Liver Lipid and Glycogen Assays

Lipids were extracted using chloroform/methanol (2:1, v/v) and evaporated in a heat block, and the pellets were dissolved in water as previously described (Herzig et al., 2003). Liver triacylglycerol and total cholesterol content were determined using the commercial reagents or kits described above. Values were calculated as mg/g wet tissue. Liver glycogen content was assayed using glucose assay reagent (G3293, Sigma) and enzyme amyloglucosidase (Sigma) as previously described (Passonneau and Lauderdale, 1974). Values were calculated as μmol/mg wet tissue.

### Oil Red O and Hematoxylin and Eosin

Frozen sections of liver (5 μm) were stained with oil red O. Formalin-fixed, paraffin-embedded sections (5 μm) of liver were stained with hematoxylin and eosin for histology.

### FAS Enzyme Activity Assay

FAS activity was performed as described previously (Kim et al., 1981) with some modifications. The rate of NADPH oxidation was measured at 340 nm before and after addition of the substrate malonyl-CoA. The concentration of enzyme was adjusted to assure a linear reaction rate.

### RNA Isolation and Relative Quantitative RT-PCR

Total RNA was prepared from frozen liver using an RNase mini kit (QIAGEN). Quantitative RT-PCR was performed using coamplification of GAPDH as an internal control. One microgram of RNA was reverse transcribed with random primers (Promega), and quantitative PCR was performed with a qPCR core kit for SYBR green I (Eurogentec) by 7000 Sequence Detection System (Applied Biosystems). PCR products were subjected to a melting curve analysis. At a specific threshold in the linear amplification stage, the cycle differences between amplified GAPDH and cDNAs of interest were used to determine the relative quantity of genes of interest. Primers used for real-time PCR are listed in Table S1.

### Western Blot Analysis

Nuclear extraction from frozen liver was performed as previously described (Tai et al., 2000). Whole-cell lysates from frozen liver were

isolated using Tris-based lysis buffer. Protease inhibitor and phosphatase inhibitors were added to all buffers before experiments. Western blot was performed as previously described (Zhang et al., 2002a) using 30 μg of protein for each sample. Protein concentration was assayed using Bio-Rad reagent. Primary antibodies (anti-FAS antibody [BD Scientific], anti-PPARγ antibody [Upstate], and anti-SREBP-1c antibody [Santa Cruz Biotechnology]) were incubated overnight at 4°C followed by ECL Plus (Amersham/Pharmacia) visualization of specific proteins. Band intensities were measured using ImageQuant (Molecular Dynamics/Amersham/Pharmacia). Intensity values were normalized to α-tubulin or nonspecific protein on the membrane.

### Data Analysis

All data are expressed as mean ± SEM for experiments including numbers of mice as indicated. The two-tailed Student's t test was used to evaluate statistical differences between control diet group and (−)leu diet group with or without C75 treatment within the same strains of mice. There was no significant effect on parameters shown between mice of different strains when fed a control diet. Means for all parameters examined in current study were calculated independently for male and female mice. No statistical difference in the response of male and female mice to leucine deprivation was observed (two-tailed Student's t test, $p > 0.05$).

### Supplemental Data

Supplemental Data include three figures and one table and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/5/2/103/DC1/.

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