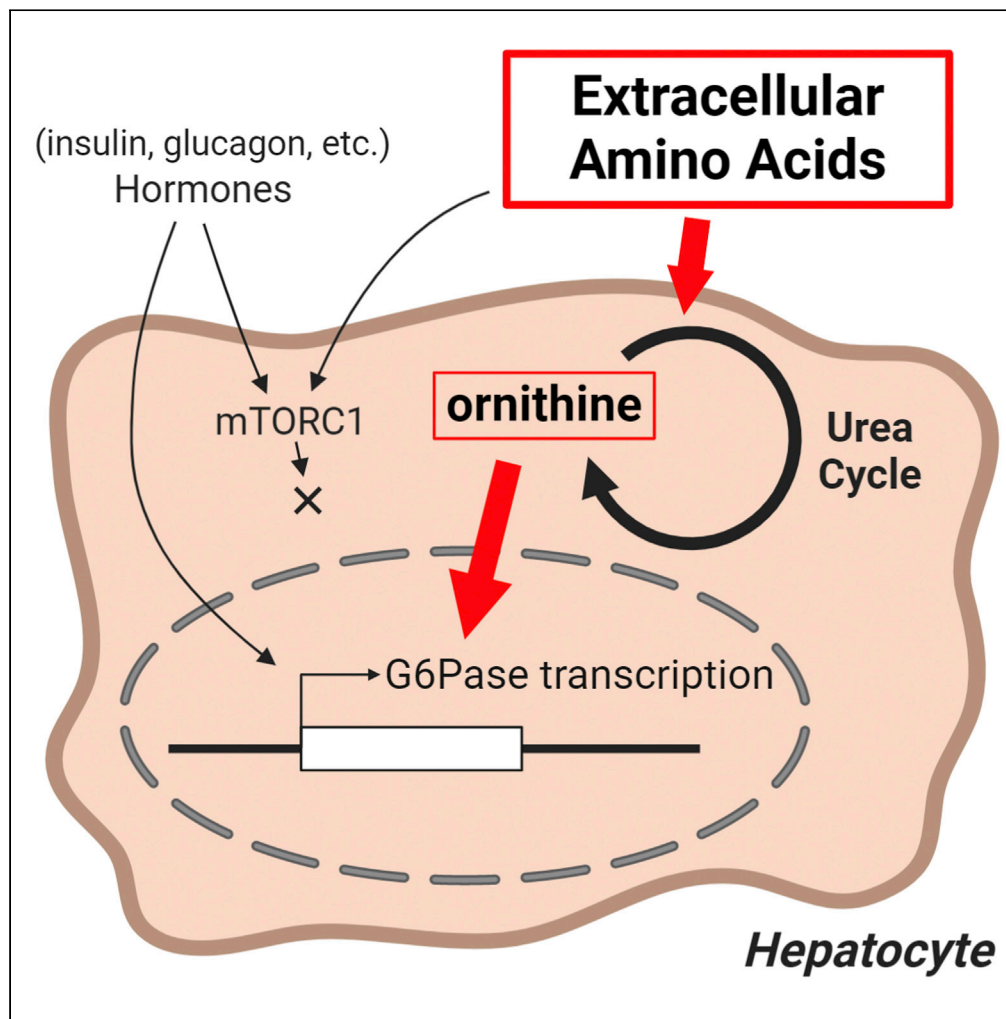**Article**

A novel amino acid signaling process governs glucose-6-phosphatase transcription

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Highlights

Amino acids regulate G6Pase transcription in hepatocytes independently of hormones

Urea cycle activity changes reflecting the extracellular amino acid concentration

Ornithine regulates G6Pase mRNA level in the same manner as proteinogenic amino acids

Amino acids/ornithine signals are independent of canonical mTORC1 pathway

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Article

A novel amino acid signaling process governs glucose-6-phosphatase transcription

Sara Fukushima,[1,3] Hiroki Nishi,[1,3] Mikako Kumano,[1] Daisuke Yamanaka,[2] Naoyuki Kataoka,[1] Fumihiko Hakuno,[1,4,*] and Shin-Ichiro Takahashi[1,*]

SUMMARY

Emerging evidence has shown that amino acids act as metabolic regulatory signals. Here, we showed that glucose-6-phosphatase (G6Pase) mRNA levels in cultured hepatocyte models were downregulated in an amino-acid-depleted medium. Inversely, stimulation with amino acids increased G6Pase mRNA levels, demonstrating that G6Pase mRNA level is directly controlled by amino acids in a reversible manner. Promoter assay revealed that these amino-acid-mediated changes in G6Pase mRNA levels were attributable to transcriptional regulation, independent of canonical hormone signaling pathways. Metabolomic analysis revealed that amino acid starvation induces a defect in the urea cycle, decreasing ornithine, a major intermediate, and supplementation of ornithine in an amino-acid-depleted medium fully rescued G6Pase mRNA transcription, similar to the effects of amino acid stimulation. This pathway was also independent of established mammalian target of rapamycin complex 1 pathway. Collectively, we present a hypothetical concept of "metabolic regulatory amino acid signal," possibly mediated by ornithine.

INTRODUCTION

Protein nutrition has a significant impact on systemic metabolism in animals. We previously reported that a low-protein diet causes animals to have reduced levels of insulin-like growth factor-I (IGF-I) in the serum, leading to moderate growth retardation (Takahashi *et al.*, 1994; Takenaka et al., 1996). Moreover, a low-protein diet or a protein-devoid diet decreases serum insulin levels, enhances systemic insulin sensitivity, and induces hepatic triacylglycerol accumulation (Toyoshima et al., 2010, 2014; Nishi et al., 2018). Regarding the mechanism of fatty liver development caused by a low-protein diet, we demonstrated that serum amino acids could serve as a metabolic regulatory signal directly in hepatocytes, promoting *de novo* lipogenesis (Nishi et al., 2018). It was surprising because we initially presumed that hepatic lipid accumulation could be attributable to enhanced insulin sensitivity. These results, however, suggested that in the context of lipid metabolic regulation, which is dependent on protein nutritional status, amino acids might act as signal transducers themselves and exhibit some insulin-like activities.

In contrast, in an earlier study, we found that when rats are fed a protein-deprived diet, mRNA levels of gluconeogenic enzymes (glucose-6-phosphatase, G6Pase; phosphoenolpyruvate carboxykinase) in the liver decreased, resulting in weak gluconeogenic activity (Toyoshima et al., 2010). Transcriptional regulation of these mRNAs in response to various hormones has been extensively studied. For example, glucagon and glucocorticoid increase mRNA transcription of gluconeogenic enzymes through cAMP-response elements and glucocorticoid-response elements, respectively, located at the 5′ upstream region of their promoters. In contrast, insulin represses their transcription through the insulin-response element by inhibiting the nuclear localization of forkhead box protein O, a transcription factor that enhances transcription of gluconeogenic enzyme genes (Vander Kooi et al., 2005; Gautier-Stein et al., 2005; Vander Kooi et al., 2003; Tang et al., 1999). Based on this information, here, we explored the mechanisms underlying the downregulation of gluconeogenic enzyme transcription in response to protein deficiency.

Mammalian target of rapamycin complex 1 (mTORC1) is a well-documented kinase and an important nutrient signal mediator. It is known to respond to amino acid stimulation and to modulate cellular metabolism (Sabatini, 2017; González and Hall, 2017). In the presence of amino acids, mTORC1 is recruited to

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Table 1. Experimental media compositions (mg/L)

|	Full	Zero	EAA	NEAA	BCAA	ΔGly	· · ·
Glycine	30.0	0	0	30.0	0	0	
L-Alanine	35.6	0	0	35.6	0	35.6	
L-Serine	42.0	0	0	42.0	0	42.0	
L-Threonine	95.0	0	95.0	0	0	95.0	
L-Cystine	48.0	0	0	48.0	0	48.0	
L-Methionine	30.0	0	30.0	0	0	30.0	
L-Glutamine	584.0	0	0	584.0	0	584.0	
L-Asparagine· H_2O	60.0	0	0	60.0	0	60.0	
L-Glutamic acid	58.8	0	0	58.8	0	58.8	
L-Aspartic acid	53.2	0	0	53.2	0	53.2	
L-Valine	94.0	0	94.0	0	94.0	94.0	
L-Leucine	105.0	0	105.0	0	105.0	105.0	
L-Isoleucine	105.0	0	105.0	0	105.0	105.0	
L-Phenylalanine	66.0	0	66.0	0	0	66.0	
L-Tyrosine	72.4	0	0	72.4	0	72.4	
L-Tryptophan	16.0	0	16.0	0	0	16.0	
L-Lysine· HCl	146.0	0	146.0	0	0	146.0	
L-Arginine· HCl	84.0	0	0	84.0	0	84.0	
L-Histidine	31.0	0	31.0	0	0	31.0	
L-Proline	46.0	0	0	46.0	0	46.0	
EBSS	10%	10%	10%	10%	10%	10%	
Vitamin solution	1%	1%	1%	1%	1%	1%	
NaHCO₃	2200	2200	2200	2200	2200	2200	
D-Glucose	4500	4500	4500	4500	4500	4500	

the lysosomal membrane where the small GTPase Ras homolog enriched in the brain (Rheb) activates mTORC1 kinase activity (Jewell et al., 2013). However, the full mechanism underlying the amino acid signaling remains unclear. Thus, the discovery of new mechanisms mediating the amino acid signaling, other than the mTORC1 system, is necessary.

Therefore, in this study, we aimed to elucidate potential mechanisms underlying amino acid signaling by examining G6Pase mRNA regulation in cell culture models using hepatocyte-derived cells. Here, we illustrate a hypothetical mechanism for amino acid signaling mediated by the urea cycle and present a novel concept for systemic metabolic regulation in animals to maintain energy homeostasis governed by amino acids.

RESULTS

Amino acids regulate G6Pase mRNA levels in hepatocyte-derived cells independently of hormone signals

To examine the effects of amino acids on G6Pase mRNA expression, we subjected hepatocyte models to serum-free, amino-acid-modified media. For these experiments, we prepared amino-acid-sufficient medium (Full) as the control medium and amino-acid-depleted medium (Zero), which was prepared by depriving the Full medium of total amino acids (see Table 1 for media compositions). Cells were cultured in Full or Zero medium for 24 h to analyze G6Pase mRNA levels. All three hepatocyte models (rat primary hepatocytes, H4IIE rat hepatoma cells, and HepG2 human hepatoma cells) cultured in Zero medium exhibited lower levels of G6Pase mRNA than did those cultured in the Full medium, similar to the results observed in the Full medium with insulin stimulation (Figures 1A–1D). When cells were cultured with dibutyryl-cAMP (Bt₂cAMP: a long-acting analog of cAMP, which is the second messenger of glucagon/epinephrine), or dexamethasone (Dex: a synthetic glucocorticoid), G6Pase mRNA levels increased; meanwhile, a decrease in G6Pase mRNA upon amino acid deprivation was still observed in the presence of Bt₂cAMP,
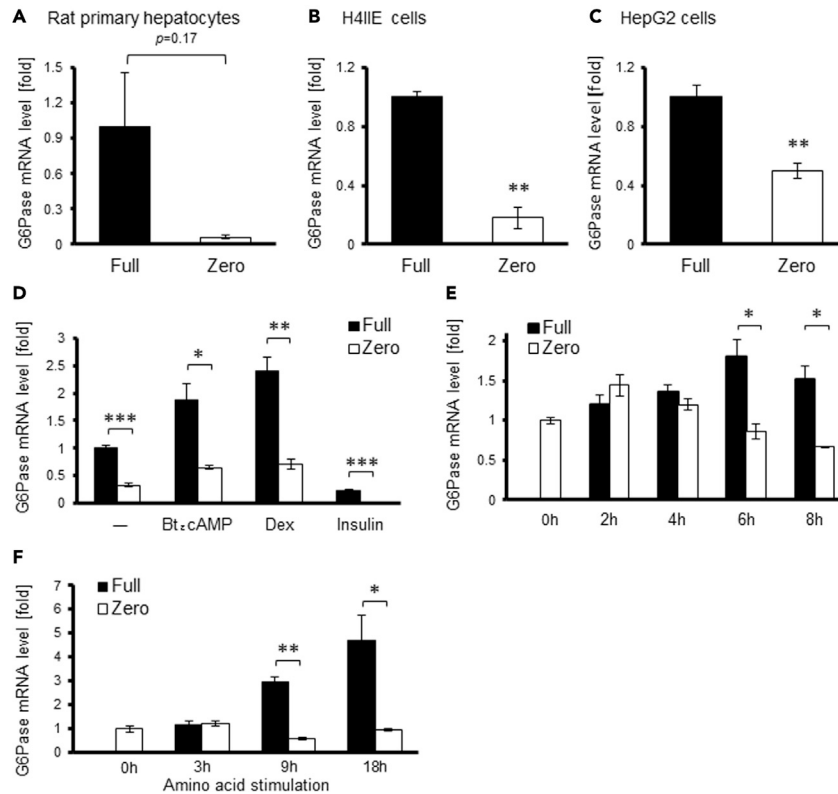
Figure 1. Amino acids control G6Pase mRNA levels in hepatocyte-derived cells

(A–C) G6Pase mRNA levels in rat primary hepatocytes (A), H4IIE rat hepatoma cells (B), and HepG2 human hepatoma cells (C) cultured for 24 h in a medium containing 20 amino acids (Full) or in a medium with no amino acids (Zero).

(D) G6Pase mRNA levels in H4IIE cells cultured for 24 h in Full or Zero medium containing 1 mM dibutyryl-cAMP (Bt_2cAMP), 1 μM dexamethasone (Dex), or 10 nM insulin.

(E) G6Pase mRNA levels in H4IIE cells cultured in Full or Zero medium for the indicated time.

(F) G6Pase mRNA levels in H4IIE cells amino-acid-starved by preculturing in Zero medium for 6 h and retreated with Full or Zero medium by continuous culturing at the indicated times.

Data information: In (A–F), G6Pase mRNA levels were quantified using real-time qPCR and normalized against 18S rRNA levels. Bar graphs are presented as fold change of columns on the far left. Bar: mean ± S.E.M., *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. A-F: n = 3, Student's t-test.

Dex, or insulin (Figure 1D). Upon exposure to the Zero medium, G6Pase mRNA levels in H4IIE cells gradually decreased but started increasing in the Full medium and the differences became significant in 6 h (Figure 1E). Additionally, when cells were cultured in the Zero medium for 6 h, followed by amino acid stimulation, G6Pase mRNA levels increased again (Figure 1F). In comparison with G6Pase, Actb (a housekeeping gene) mRNA levels did not change, and mRNA levels of Igfbp1, a gene that we reported previously to increase with amino acid deficiency, increased in the Zero medium (Figure S1). These results suggest that amino acids from the medium positively regulate the transcription of G6Pase in cells, rather than being permanently damaged by the harsh amino-acid-free condition. Besides, the reaction is independent of well-known gluconeogenic regulatory hormones.

Amino acids upregulate the transcription of G6Pase independently of hormone signals

We then assessed the degradation and transcription rates of G6Pase mRNA in H4IIE cells. When cells were treated with actinomycin D, a general transcription inhibitor, the G6Pase mRNA had a lower degradation rate in the Zero medium than in the Full medium (Figure 2A), suggesting that amino acid deprivation-induced decrease in G6Pase mRNA was not due to a difference in mRNA degradation rate.

To test G6Pase transcription activity, we constructed a rat G6Pase promoter (−1100 to +174 upstream region of the rat G6Pase gene: FL)-driven luciferase reporter (FL_{WT}) (Figure S2A). After transfection with FL_{WT},
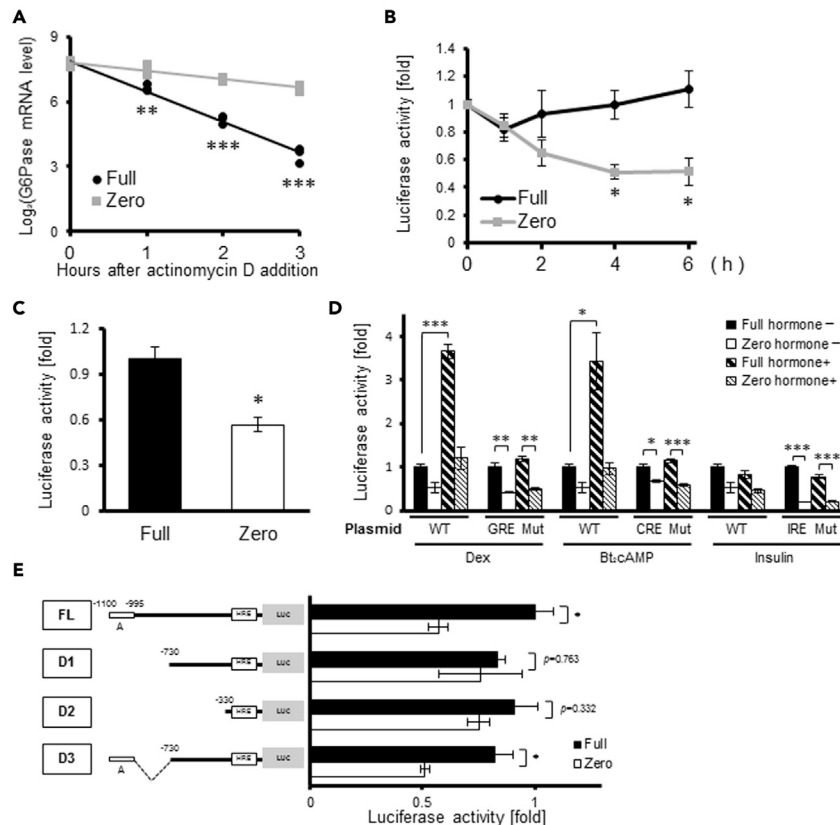
Figure 2. Amino acids increase G6Pase mRNA by enhancing transcriptional activity

(A) H4IIE cells were cultured in Full or Zero medium containing 10 μg/mL actinomycin D for the indicated time, and G6Pase mRNA level was quantified by real-time qPCR. Values are shown as the logarithm to the base 2.

(B–E) G6Pase promoter activities were analyzed using a luciferase reporter assay. Rat G6Pase promoter region ranging from −1100 to +174 bp (B and C) and indicated mutant constructs (D, E) were inserted into the pGL4.11 *firefly* luciferase reporter plasmid, which was transfected into the H4IIE cells. After 24 h, media were changed to Full or Zero medium and cultured for the indicated time (B). Or, 24 h after transfection, cells were subjected to 4-h amino acid starvation in Zero medium followed by another 6-h culture in Full or Zero medium (C–E). Luciferase activities in the cell lysates were measured.

Data information: Bar graphs are presented as fold change of columns on the far left. Bar: mean ± S.E.M., *p < 0.05, **p < 0.01, ***p < 0.001. (A) n = 3, Student's *t*-test; B, C, and E: n = 4, Student's *t*-test; D: n = 4, Tukey-Kramer test.

H4IIE cells were cultured in Full or Zero medium and then luciferase activity was measured. In the Zero medium, the activity gradually decreased, while it remained constant in the Full medium. Significant differences could be observed by culturing cells for more than 4 hr (Figure 2B). The H4IIE cells were next subjected to 4 h amino acid starvation in the Zero medium, followed by amino acid stimulation in the Full medium for another 6 h. As a result, cells cultured in the Full medium displayed higher levels of luciferase activity than those in the Zero medium, indicating that amino acids increase G6Pase mRNA levels by enhancing transcription (Figure 2C).

Because the FL promoter region contains established hormone response elements (cAMP-response element [CRE]; glucocorticoid response element [GRE]; insulin-response element [IRE]), inactive mutations were introduced into these elements to evaluate their roles in amino-acid-mediated regulation of G6Pase expression (Figure S2B) (Xu et al., 2007). When H4IIE cells were transfected with FL$_{WT}$ or mutant promoters, Dex and Bt$_2$cAMP treatment enhanced luciferase activity in FL$_{WT}$-transfected cells, while GRE and CRE mutations disrupted the response (Figure 2D). In contrast, luciferase activity of cells cultured in the Full medium was still higher than that in the Zero medium, regardless of mutations. Although insulin stimulation did not affect luciferase activity in Full and Zero medium, luciferase activity was still higher in the Full medium than in the Zero medium, even in the cells transfected with the IRE mutant reporter (Figure 2D).

Collectively, these results further indicate that amino acids increase G6Pase transcription in hepatocyte-derived cells independently of the established hormonal signals.

To determine the cis-elements involved in response to amino acids, we constructed plasmids where the 5′ region upstream of the FL promoter was deleted (D1, D2), resulting in the discovery of a −1100 to −730 region, distant from known hormone response elements (around −200 to +1), that was important for the response to amino acids (Figure 2E). Furthermore, when only the −1100 to −995 region (region A) was connected directly to the amino-acid-nonresponsive D1 promoter, the response to amino acids was rescued (Figure 2E), indicating that region A includes an amino acid response element associated with G6Pase transcription regulation.

Specific amino acids increase G6Pase mRNA levels in H4IIE cells

To determine the role of each amino acid in enhancing G6Pase transcription in H4IIE cells, we cultured the cells with either essential-amino-acid (EAA) or nonessential-amino-acid (NEAA)-containing medium. Cells cultured in the medium containing only NEAAs had the same levels of G6Pase mRNA as those in the Full medium; meanwhile, cells cultured in the medium with only EAAs had slightly elevated G6Pase mRNA levels compared to those cultured in the Zero medium (Figure 3A). Furthermore, when H4IIE cells were cultured in the media containing fixed concentrations of each amino acid for more accurate comparisons, the cells exhibited similar dose-responsive G6Pase induction in the Full and NEAA medium, whereas just a slight increase was observed in the EAA medium (Figure 3B).

We next determined which amino acid played a pivotal role in the acceleration of G6Pase transcription. H4IIE cells were cultured in the medium containing only a single amino acid for 24 h, and the result showed that isoleucine, leucine, valine, arginine, asparagine, aspartic acid, and glutamine increased or tended to increase G6Pase mRNA levels compared with levels in cells cultured in the Zero medium (Figure 3C). Most of these amino acids, except for leucine and asparagine, also displayed dose-dependent effects on G6Pase mRNA levels (Figure 3D). These results suggested that the slight upregulation of G6Pase mRNA in the EAA medium could be attributable to branched-chain amino acids (BCAAs), and the full increase in the NEAA medium might be due to arginine, aspartic acid, and glutamine.

BCAAs increase G6Pase mRNA levels to a small extent independently of the mTORC1 activity

To verify our hypothesis, we cultured H4IIE cells in a medium containing only three BCAAs for 24 h and analyzed G6Pase mRNA levels. The BCAA medium partially increased G6Pase mRNA transcription to levels similar to those observed in the EAA medium (Figure S3A). In addition, when BCAAs were depleted in the EAA medium, the enhancing effect of the EAA medium on G6Pase mRNA was terminated (Figure S3B), suggesting that BCAAs acted as the G6Pase mRNA regulatory factors. However, when we analyzed G6Pase mRNA degradation and transcription by actinomycin D and a series of luciferase reporters, neither was significantly affected by either BCAAs or EAAs (Figures S3C and S3D).

The integrated nutrient-sensing complex, mTORC1, is activated by amino acids, and it is involved in various metabolic reactions, such as protein synthesis and autophagy (Sabatini, 2017; Huang and Fingar, 2014). Leucine, one of the BCAAs, is the most familiar activator of mTORC1 among the amino acids (Wolfson et al., 2015). These reports imply that the mTORC1 signal is also involved in the regulation of G6Pase mRNA transcription. Thus, we treated H4IIE cells with torin 1, an mTOR-specific inhibitor, to investigate its role. However, when H4IIE cells were stimulated by amino acids, the G6Pase mRNA levels increased compared with those with no amino acid stimulation, even in the presence of torin 1 (Figure 3E). Besides, when cells were cultured in the BCAA medium, G6Pase mRNA levels also did not significantly change regardless of torin 1 treatment (Figure S3E), suggesting that the amino acid signal in association with G6Pase upregulation is independent of the well-known mTORC1 signaling pathway.

Ornithine upregulates G6Pase mRNA levels

We next evaluated the effects of arginine, aspartic acid, and glutamine on G6Pase mRNA. These amino acids are all directly involved in urea metabolism; therefore, we hypothesized that the urea cycle is involved in G6Pase regulation. Thus, we carried out a metabolome analysis using lysates from H4IIE cells cultured in the Full and Zero medium for 24 h and compared the profiles of metabolites from the urea cycle (Figure 4A). The results showed that levels of aspartate, argininosuccinate, arginine, and ornithine were significantly lower, while only the level of citrulline was much higher in cells cultured in the Zero medium. Urea concentration was also lower in the Zero medium and was increased by amino acid stimulation (Figures S4A and S4B).
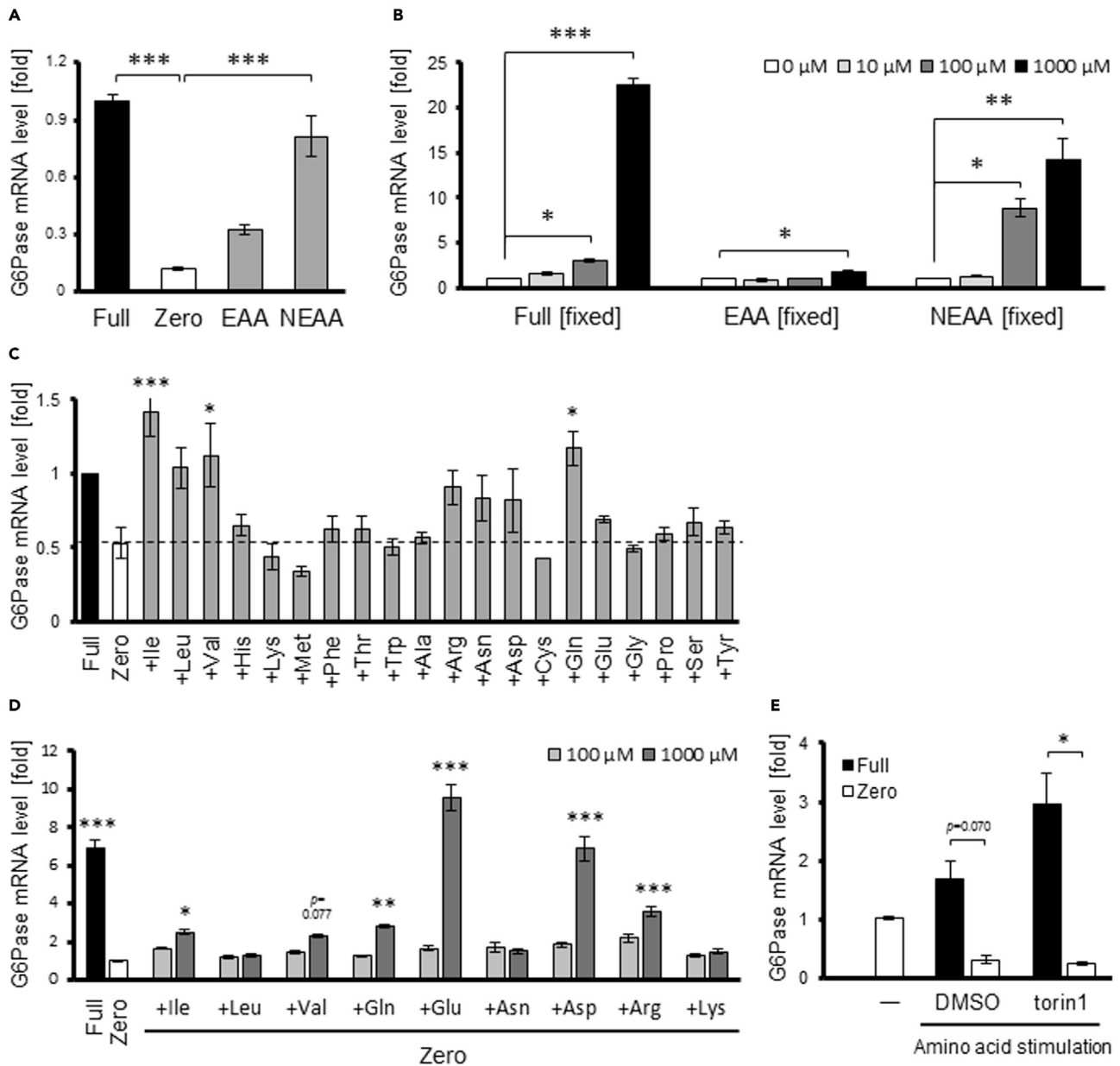
Figure 3. Varying effects of amino acids on G6Pase transcription

(A–C) H4IIE cells were cultured for 24 h in various culture media, including media containing: 20 amino acids (Full), no amino acids (Zero), Zero plus essential amino acids (EAAs), nonessential amino acids (NEAAs) (A and B), or Zero plus single amino acid (C). G6Pase mRNA levels were quantified using real-time qPCR. In A and C, each amino acid concentration was identical to the concentration of the corresponding amino acid in the Full medium. In B, indicated fixed concentrations were applied to all the amino acids.

(D) H4IIE cells were cultured for 24 h in Full, Zero, or Zero plus 100 or 1000 μM of a single amino acid. G6Pase mRNA levels were quantified using real-time qPCR.

(E) H4IIE cells were amino-acid-starved by 6 h preculturing in Zero medium. Then, the medium was replaced with Full or Zero medium for amino acid stimulation with or without 50 nM torin 1, and the cells were cultured for another 18 h. G6Pase mRNA levels were quantified using real-time qPCR. Samples collected just before amino acid stimulation are indicated with "-."

Data information: All G6Pase mRNA levels were normalized against 18S rRNA levels. Bar graphs are presented as fold change of columns on the far left. Bar: mean ± S.E.M., *p < 0.05, **p < 0.01, ***p < 0.001. A: n = 3, Tukey-Kramer test; B-D: n = 3, Dunnett's test vs Zero; E: n = 3, Student's *t*-test.

Furthermore, when we added ornithine, citrulline, or fumarate to Full and Zero medium and cultured H4IIE cells for 24 h, only ornithine treatment could fully rescue the G6Pase mRNA levels in the Zero medium in a dose-dependent manner (Figures 4B and S4C). Besides, ornithine-driven G6Pase transcription was observed
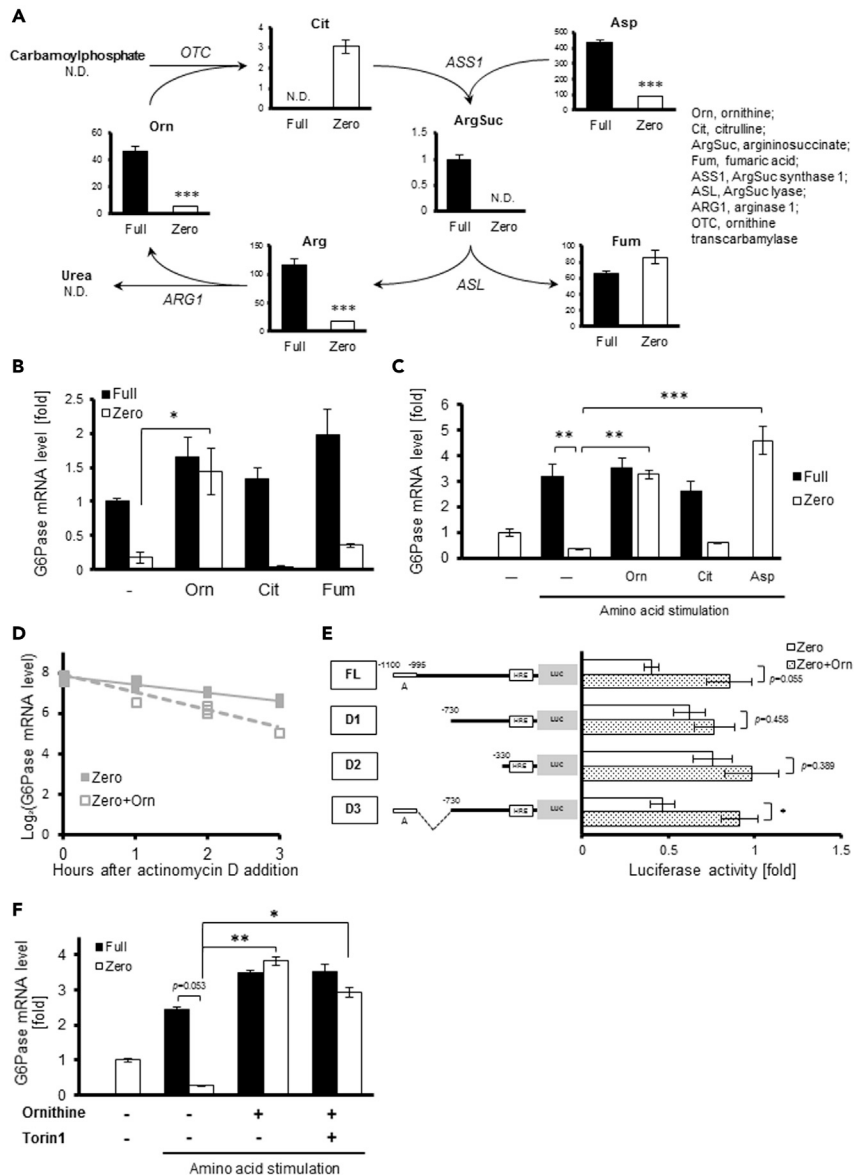
Figure 4. Ornithine takes part in the amino acid signaling that regulates G6Pase transcription

(A) H4IIE cells were cultured in a medium containing 20 amino acids (Full) or in a medium with no amino acids (Zero) for 24 h, and hydrophilic metabolites were extracted from the cells for metabolome analysis. Metabolites related to the urea cycle are shown. All values in the graphs except for ArgSuc represent molecular amounts [pmol/10^6 cells]. ArgSuc concentration is shown as a relative value.

(B) H4IIE cells were cultured for 24 h in Full or Zero medium supplemented with 4 mM ornithine (Orn), 4 mM citrulline (Cit), or 4 mM fumaric acid (Fum), and G6Pase mRNA level was quantified by real-time qPCR.

(C) H4IIE cells were amino-acid-starved by 6 h preculturing in Zero medium. Then, media were changed to Full or Zero medium containing 4 mM of ornithine or citrulline, or Zero medium containing 4 mM of aspartic acid for amino acid stimulation, and cells were cultured for another 18 h. G6Pase mRNA levels were quantified using real-time qPCR.

(D) H4IIE cells were cultured in Full, Zero, or Zero plus 4 mM ornithine medium containing 10 μg/mL actinomycin D for the indicated times, and G6Pase mRNA level was quantified using real-time qPCR. Values are shown as the logarithm to the base 2.

(E) G6Pase promoter activities were analyzed using the luciferase reporter assay. The pGL4.11 *firefly* luciferase reporter plasmids carrying rat G6Pase promoter regions (Figure 1I) were transfected into H4IIE cells. After 24 h, cells were subjected to a 4-h amino acid starvation in Zero medium. Media were changed to Full, Zero, or Zero plus 4 mM ornithine medium and cultured for another 6 h. Luciferase activities of the cell lysates were measured.

Figure 4. Continued
(F) H4IIE cells were amino-acid-starved by 6-h preculturing in Zero medium. Then, media were changed to Full or Zero medium for amino acid stimulation with or without 4mM ornithine and 50 nM torin1, and cells were cultured for another 18 hr G6Pase mRNA levels were quantified using real-time qPCR. Samples collected just before amino acid stimulation are indicated with "-."

Data information: All G6Pase mRNA levels were normalized against 18S rRNA levels. Bar graphs are presented as fold change of columns on the far left. Bar: mean ± S.E.M., *p < 0.05, **p < 0.01, ***p < 0.001. A: n = 4, Student's *t*-test; B, C, and F: n = 3, Tukey-Kramer test; E: n = 4, Student's *t*-test.

in not only H4IIE cells but also in HepG2 cells and rat primary hepatocytes (Figures S4D and S4E). These results suggested that under amino-acid-deprived conditions, the argininosuccinate-synthase-1 (ASS1)-catalyzed reaction in the urea cycle was interfered with, presumably due to aspartate shortage, resulting in the reduction of ornithine levels, which, in turn, led to G6Pase mRNA downregulation.

To test this hypothesis, we stimulated H4IIE cells with aspartate after amino acid starvation in the Zero medium. As expected, aspartate supplementation recapitulated the result of ornithine supplementation (Figure 4C). When we assessed degradation and transcription of G6Pase mRNA upon ornithine supplementation, ornithine supplementation did not slow the G6Pase mRNA degradation but accelerated transcription rate, similar to the total amino acid stimulation (Figures 4D and 4E). Interestingly, region A of the G6Pase promoter was again found to be responsible for the ornithine-mediated upregulation of G6Pase transcription (Figure 4E).

Similar to leucine, ornithine also activates mTORC1 (Kokubo et al., 2015). We, therefore, treated cells with torin 1 again and evaluated the involvement of mTORC1 in the ornithine-mediated G6Pase transcription regulation. Consequently, G6Pase mRNA levels were also not significantly affected by torin1 treatment (Figure 4F), indicating that the "ornithine signaling" pathway also functioned independently of mTORC1 signaling in H4IIE cells.

DISCUSSION

Glucose metabolism in the animal liver is strictly controlled by the endocrine and nervous systems (Yabaluri and Bashyam, 2010; Petersen et al., 2017). In this study, however, our findings showed that G6Pase mRNA transcription in hepatocyte-derived cells was affected by only the ambient amino acid concentrations, without any hormonal stimulations and neuronal access (Figure 1). Additionally, this amino-acid-induced increase in G6Pase mRNA transcription occurred regardless of the presence of representative sugar-metabolic hormones, and we found that the DNA promoter region responsible for amino acid signaling was distinct from the established hormone response elements (HREs) (Figures 1D and 2E). Remarkably, ornithine treatment phenocopied the amino acid treatment, and we found that the amino-acid-responsive region was the same as the ornithine-responsive region (Figure 4E). In addition, levels of ornithine, a urea cycle metabolite, increased under amino-acid-sufficient conditions, suggesting that ornithine directly mediates the upregulation of G6Pase transcription independently of the canonical hormonal signals. The findings illustrate that the amino acid signaling that we discovered could be the third regulatory mechanism for glucose metabolism, in addition to the endocrine and nervous systems. Several reports have shown that amino acids also enhance secretion of hormones (e.g. IGF-I) (Gheni et al., 2014; Alba-Roth et al., 1988; Takahashi et al., 1994), modulate the action of some hormones (e.g. insulin, glucagon) (Toyoshima et al., 2010; Holst et al., 2017), and regulate the reaction of the central nervous system mainly via the hypothalamus (e.g. satiety, feeding behavior, glucose homeostasis) (Morrison and Laeger, 2015; Petersen et al., 2017). Based on these results, the three systems appear to interact intimately with each other to maintain energy homeostasis.

Concentrations of each of the amino acids varied since the medium amino acid composition was based on that of Dulbecco's modified Eagle's medium (DMEM), and most of them were higher than physiological levels (≈ several hundred micromolar) (Nishi et al., 2018). However, the experiments performed using multiple amino acid concentrations showed that the Full amino acids, NEAAs, and ornithine exhibited a dose-dependent effect on G6Pase transcription and that they could significantly influence G6Pase transcription even at a concentration as low as 100 µM (Figures 3B and S4C). Among the analyzed amino acids, ornithine, when administered alone, was the most efficient positive regulator of G6Pase transcription at a physiological concentration (<100 µM) (Figures 3D and S4C). In addition, high-dose Asp, Arg, and Gln supplementation might increase cellular ornithine levels through urea cycle reactions, which presumably explains upregulation of G6Pase mRNA by those amino acids. Thus, we propose that amino acid/ornithine signals could also be relevant in a physiological setting.

Table 2. Primer list

Primer set	Sequence (5′ → 3′)	Purpose
#1 Fw	GCTCGCTAGCCTCGATCATCTATCTGTACACACATTTGTCCCCT	plasmid
#1 Rv	TCTTGATATCCTCGAGATCACAGACACCAAGACAAACCAGTCCT	plasmid
#2 Fw	GCTCGCTAGCCTCGATGGAACTGGGTCAGTTACTGACATTTATCAG	plasmid
#2 Rv	= #1 Rv	plasmid
#3 Fw	GCTCGCTAGCCTCGAAGCCACAGTTGAAACAGACTCTGCC	plasmid
#3 Rv	= #1 Rv	plasmid
#4 Fw	GTGTGCCTCTTTTGCTCTTTTACGTAAATCACCCTG	IRE mut.
#4 Rv	GCAAAAGAGGCACACAAAAAGAGCCTGATCGGCCATTGG	IRE mut.
#5 Fw	TGTTTTTGATGCTCTGTTTTGATATTTTACGTAAATCACCCTG	GRE mut.
#5 Rv	AGAGCATCAAAAACATAAGTCGATGCCATTGGCGGAGC	GRE mut.
#6 Fw	ACCGACTGCGTCCAGTGTTTGCATCAACCTACTG	GRE mut.
#6 Rv	CTGGACGCAGTCGGTACGTAAAATATCAAAACAGAGCATC	GRE mut.
#7 Fw	TCACCCTGAACATGTTTCTAGAAACCTACTGATGATGCACC	CRE mut.
#7 Rv	ACATGTTCAGGGTGAAAGCTTTAAAATAGCAAAACAGGC	CRE mut.
rG6Pase Fw	CTACCTTGCGGCTCACTTTC	qPCR
rG6Pase Rv	ATCCAAGTGCGAAACCAAAC	qPCR
rIgfbp1 Fw	TGCCGCTCAACAGAAAGCAG	qPCR
rIgfbp1 Rv	TCCAGGGATCTTCTTCCCACTC	qPCR
rActb Fw	CTAAGGCCAACCGTGAAAAGAT	qPCR
rActb Rv	AGGGACAACACAGCCTGGA	qPCR
r18S Fw	TCCCAGTAAGTGCGGGTCATA	qPCR
r18S Rv	CGAGGGCCTCACTAAACCATC	qPCR
hG6Pase Fw	GGCTCAACCTCGTCTTTAAGTG	qPCR
hG6Pase Rv	CTCCCTGGTCCAGTCTCACA	qPCR
h18S Fw	GATGGAAAATACAGCCAGGTCCTA	qPCR
h18S Rv	TTCTTCAGTCGCTCCAGGTCTT	qPCR

mut, mutant.

As shown in Figure 3, varying effects on G6Pase transcription were displayed by each amino acid. For example, NEAAs increased the mRNA levels to the same level as total amino acids, whereas EAAs or BCAAs increased them to a smaller extent. Moreover, while ornithine enhanced G6Pase transcription just like total amino acids, BCAAs affected neither transcription nor degradation of G6Pase mRNA (Figure S3). These results suggest that, although BCAAs have particular effects, ornithine-associated signals have a predominant role in G6Pase transcription regulation in response to extracellular amino acids.

mTORC1 is the primary representative of a signal-mediating molecule related to nutrient-sensing. Leucine, arginine, and methionine (or S-adenosylmethionine) have been demonstrated to stimulate

mTORC1 (Wolfson et al., 2015; Chantranupong et al., 2016; Gu et al., 2017), and another report has shown that ornithine also activates mTORC1 (Kokubo et al., 2015). Thus, we initially suspected that mTORC1 is also involved in the amino-acid-dependent regulation of G6Pase. However, our results revealed that this was not the case. For example, leucine or arginine treatment increased or tended to increase G6Pase mRNA levels in H4IIE cells, whereas methionine did not (Figure 3C). Remarkably, a potent mTOR inhibitor, torin 1, did not have apparent effects on G6Pase transcription induced by amino acids, BCAAs, or ornithine (Figures 3E, 4F, and S3E). These results strongly suggest that mechanisms independent of the well-known mTORC1 system must be involved, and here, we identified the ornithine (urea cycle)-mediated mechanism as a novel type of amino acid signaling pathway. Because there are no obvious conserved motifs in the amino acid/ornithine-responsive sequence (region A), how ornithine functions to accelerate G6Pase transcription remains to be elucidated, but investigation for potentially responsible molecules is in progress in our laboratory.

Several studies have shown that nutrient-sensing systems and several animal phenotypes in response to protein nutritional status are highly conserved across many species, from yeast and flies to rodents and humans (Chantranupong et al., 2015; González and Hall, 2017). For example, when rats and fruit flies (*Drosophila melanogaster*) are fed a low-protein diet, the expression of insulin-like peptides decreases, resulting in mild growth retardation (Takahashi et al., 1994; Takenaka et al., 2000; Post and Tatar, 2016; Agrawal et al., 2016). These findings indicate that the amino acid signaling system and its ability to regulate metabolism are important not only for mammals but also for most eukaryotic organisms. In recent years, new types of putative amino-acid-sensing mechanisms, distinct from mTORC1 signaling, have been reported. Aminoacyl-tRNA synthetases have been found to aminoacylate lysine residues of certain proteins as a post-translational modification to modulate their activity (He et al., 2017); specific amino acids (e.g. arginine) have been found to bind to certain transcription factors to enhance their affinity to DNA (Geiger et al., 2016). Although these mechanisms are not yet widely known and not broadly studied, these systems are probably parts of a complicated amino acid signaling system. In addition to these, our current study has identified a novel form of amino acid signaling, which is possibly mediated through ornithine.

In catabolism, the amino groups of amino acids are cleaved, followed by conversion to urea through the urea cycle in the mammalian liver. The remaining carbon skeletons derived from several amino acids (glucogenic amino acids) enter the gluconeogenic process to produce glucose. Therefore, in the context of gluconeogenesis, the hepatic urea cycle activity can be used to approximate substrate supply. As shown in Figure 4A, amino acid availability may primarily affect ASS1 catalytic performance and, consequently, decrease cytosolic ornithine levels, when cells are deprived of environmental amino acids. Therefore, it may be beneficial for cells to utilize intracellular ornithine concentrations as indicators of both the urea cycle activity and amino acid supply. Because amino acids have many molecular species and a variety of chemical properties, it is probably difficult for cells to monitor amino acids comprehensively, and it may need many kinds of specific sensing machinery. As such, it is reasonable to hypothesize that the expression of the gluconeogenic enzyme G6Pase is regulated by the urea cycle metabolite, ornithine, downstream of amino acid signaling. In fact, a decrease in G6Pase mRNA levels in response to amino acid starvation was also observed in cell lines derived from other G6Pase-expressing tissues, the kidney and intestine, whereas ornithine did not significantly affect them (Figure S5), implying that ornithine-mediated amino acid signaling functions specifically in the liver.

Dietary proteins and amino acids have a significant impact on systemic metabolism in animals. For example, dietary protein concentration greatly affects satiety and food intake, and a low-protein diet stimulates fibroblast growth factor 21 secretion from the liver, which results in an increased metabolic rate (Morrison and Laeger, 2015; Laeger et al., 2014). In addition, the low-protein diet increases insulin sensitivity, enhances hepatic lipid accumulation, and inhibits gluconeogenesis (Toyoshima et al., 2010; Nishi et al., 2018). Interestingly, we previously demonstrated that hepatic *de novo* lipid synthesis is enhanced directly by extracellular amino acids in a cell-autonomous manner (Nishi et al., 2018), and G6Pase transcription is also regulated directly by amino acids (Figure 1). These findings provide a novel insight into amino acids that can exhibit a part of insulin-like activities, substituting for insulin. In the evolutionary context, it is not very surprising because amino-acid-sensing systems and their metabolic regulation are highly conserved from yeasts to mammals. Insulin-like peptides or endocrine systems exist only in higher multicellular organisms, being absent in yeasts (Chantranupong et al., 2015). It indicates that amino acid signals

may be as important as endocrine and nervous systems in regulating metabolism, even in higher organisms.

This study demonstrated that G6Pase transcription enhancement in response to amino acids is mediated possibly by ornithine signaling, at least in part, independently of the established hormonal signals. The results we present here will contribute to the understanding of the amino-acid-dependent mechanism in animal metabolic regulation and shed light on a novel aspect of the physiological functions of amino acids.

Limitations of the study

In this study, we have illustrated that ornithine mediates amino acid signaling to regulate G6Pase transcription in hepatocytes. However, to confirm this more accurately, metabolomic dynamics in response to amino acid/ornithine treatment needs to be tested. Furthermore, here, we have examined the effects of single amino acids, but it is possible that specific combinations might also be important for amino acid signaling. Considering that several of the experimental settings used in this study were nonphysiologic, the possibility that amino acids other than ornithine are potentially involved, depending on the physiological context, cannot be excluded. In addition, molecular evidence showing the direct involvement of ornithine in the amino acid signaling system is still lacking, and therefore, identifying transcription factor(s) that can associate with the promoter sequence that we discovered is necessary.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102778.

AUTHOR CONTRIBUTIONS

S.F., H.N., M.K., D.Y., N.K., F.H., and S-I. T. designed the experiments and critically discussed with each other. S.F., H.N., and M.K. performed the experiments. S.F., H.N., M.K., D.Y., N.K., F.H., and S-I. T. contributed to materials and analysis tools. H.N, F.H., and S-I. T prepared the manuscript.

DECLARATION OF INTERESTS

Authors have no conflicts of interest to declare.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
insulin from bovine pancreas	Sigma-Aldrich	#16634; CAS: 11070-73-8
dexamethasone	Sigma-Aldrich	#D1756; CAS: 50-02-2
dibutyryl-cAMP	Sigma-Aldrich	#D0260; CAS: 16980-89-5
torin1	Selleck	#S2827; CAS: 1222998-36-8
actinomycin D	Sigma-Aldrich	#A1410; CAS: 50-76-0
Collagenase Type X	Wako	#035-17861; CAS: 9001-12-1
Critical commercial assays		
ReverTra Ace qPCR RT Master Mix	Toyobo	Cat #FSQ-301
THUNDERBIRD SYBR qPCR Mix	Toyobo	Cat #QPS-201
Dual-Luciferase Reporter Assay System	Promega	Cat #E1910
Experimental models: Cell lines		
H4IIE-C3	ATCC	CRL-1600
HepG2	ATCC	HB-8065
HEK293T	ATCC	CRL-3216
Caco2	ATCC	HTB-37
Experimental models: Organisms/Strains		
Wistar rats for hepatocyte preparation	Charles River Laboratories Japan, Inc.	Crlj:WI, male
Recombinant DNA		
pGL4.11[luc2]	Promega	Cat #E6661
FL_{WT} construct	This paper	N/A
CRE, GRE, and IRE mutant construct	This paper	N/A
D1-3 construct	This paper	N/A
Software and algorithms		
JMP® Pro	SAS Institute Inc.	https://www.jmp.com/ja_jp/software/predictive-analytics-software.html
Other		
Metabolome analysis	Human Metabolome Technologies	https://humanmetabolome.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Fumihiko Hakuno (hakuno@g.ecc.u-tokyo.ac.jp).

Materials availability

Plasmids generated in this study are available from the corresponding author upon reasonable request.

Data and code availability

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request..

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell culture and cell experiments

H4IIE-C3 cells (rat hepatoma cell line, ATCC CRL-1600), HepG2 cells (human hepatoma cell line, ATCC HB-8065), and HEK293T cells (human embryonic kidney, ATCC CRL-3216) were grown in DMEM supplemented with 10% FBS and antibiotics under 5% CO_2 at 37°C. Caco2 cells (human intestine, ATCC HTB-37) were grown in DMEM supplemented with MEM Non-essential amino acid solution (Sigma Aldrich), 20% FBS, and antibiotics under 5% CO_2 at 37°C. When cells reached sub-confluency, media were changed to the experimental media (Table 1), and then cells were cultured for the indicated durations. The amino acid composition of the Full medium was based on the formulation of DMEM and, in the other culture media used in the experiments, each amino acid concentration was the same as that of the corresponding amino acid in the Full medium, unless otherwise stated.

Rat primary hepatocytes were prepared as described in a previous report (Nishi et al., 2018). Parenchymal hepatocytes were isolated from 8-10 weeks old male Wistar rats by perfusion of the liver *in situ* with collagenase in perfusion buffer [8 g/l NaCl, 0.4 g/l KCl, 0.56 g/l $CaCl_2$, 0.078 g/l $NaH_2PO_4 \cdot 2H_2O$, 0.151 g/l $Na_2HPO_4 \cdot 12H_2O$, 2.38 g/l HEPES, 0.006 g/l Phenol red, 350 mg/l Collagenase Type X (Wako, Osaka, Japan), 150 mg/l, Trypsin inhibitor from soybean (#T9003, Sigma Aldrich), 130 mg/l Trypsin inhibitor from chicken egg white (#T9253, Sigma Aldrich), 0.35 g/l $NaHCO_3$, pH7.4]. Rats were purchased from Charles River Laboratories Japan, INC. Cells were suspended in Williams' E medium (Sigma Aldrich) supplemented with 10% FBS, 10 nM insulin (Sigma Aldrich), and 1 μM dexamethasone (Sigma Aldrich), and cultured at a density of 1×10^5 cells/cm^2 in collagen-coated plastic dishes at 37°C under 5% CO_2 in air. After 2 h, the medium was changed to fresh culture medium and cultured for another 20 h, allowing cells to completely attach to the dish bottom. Then, their media were changed again to the experimental media and cultured for the indicated time.

All animal care and experiments conformed to the Guidelines for Animal Experiments of The University of Tokyo and were approved by the Animal Research Committee of The University of Tokyo.

METHOD DETAILS

Plasmid preparation and mutagenesis

Rat genomic DNA was extracted from H4IIE cells and the DNA segment around transcription initiation site of G6Pase gene was amplified by polymerase chain reaction (PCR) using primer set #1 (Table 2) (Xu et al., 2007). The DNA fragment was inserted into the pGL4.11[luc2] vector using In-Fusion HD Cloning Kit (Takara Bio., Kyoto, Japan) to obtain the FL construct for luciferase assay. The initiation codon in the luciferase gene of the vector was eliminated because the G6Pase promoter fragment we inserted contained an initiation codon. The other deletion constructs used for luciferase assay (D1, D2) were prepared in the same way using primer sets #2 and #3, respectively.

To generate FL constructs with inactive HREs, we amplified DNA fragments by PCR, using FL construct as a template and using primer sets #4-7 for IRE, GRE, and CRE mutagenesis, respectively. The obtained fragments were ligated using In-Fusion HD Cloning Kit. Mutations were based on a previous report (Vander Kooi et al., 2005).

RNA extraction and real-time quantitative PCR (qPCR)

Total RNA was extracted from cells using the TRIzol Reagent (Invitrogen,Carlsbad, CA, USA), and cDNA synthesis was carried out using the ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). cDNAs were subjected to qPCR using the THUNDERBIRD SYBR qPCR Mix (Toyobo) and Real-Time PCR system 7500TH (Life Technologies, Carlsbad, CA, USA). For all samples, 18S ribosomal RNA was measured as internal control and used for data normalization. Primer sets used are listed in Table 2.

Luciferase assay

H4IIE cells were seeded in 12-well plates at a density of 4×10^5 cells/well and grown over-night. Cells were transfected with luciferase reporter plasmids using polyethyleneimine. After 24 h, the medium was changed to the Zero medium for a 4 h amino acid starvation. Then the medium was changed again to the experimental media, and the cells were cultured for another 6 h. Luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) and ALVO X3 (PerkinElmer,

Waltham, MA, USA) according to the manufacturers' protocols. As an internal control, *Renilla* luciferase-expressing pRL-SV40 (Promega) was co-transfected.

Metabolome analysis

Samples were prepared based on the Human Metabolome Technologies' (HMT: Yamagata, Japan) protocol. Briefly, H4IIE cells were cultured in Full or Zero medium in 100 mm culture dishes for 24 h at a density of 1.0-5.0×10^6 cells/dish, and their hydrophilic metabolites were extracted using methanol containing the Internal Standard Solution provided by HMT. Extracts were then subjected to ultrafiltration with the Ultra-free MC-PLHCC filter (COMW, 5k: HMT) at $4°C$, $10,000 \times g$, for 2-4 h. Samples were stored at $-80°C$ until they were sent to HMT, where metabolomic analysis using capillary electrophoresis-mass spectrometer was performed. Each metabolite quantity was normalized against the cell number.

To measure urea concentrations in the media, H4IIE cells were cultured in Full or Zero medium, and then the medium was collected. After centrifugation ($1,000 \times g$, 5 min, $4°C$), the supernatant was used to measure their urea concentration using the Urea Assay Kit (Cosmo Bio Inc., Tokyo, Japan), according to the manufacturer's protocol.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data are expressed as the mean $\pm$ standard error of the mean (S.E.M.). Comparisons between two groups were performed using Student's *t*-test. Comparisons among more than two groups were carried out by one-way or two-way analysis of variance (ANOVA). If the p-value obtained from ANOVA was under 0.05, post-hoc tests indicated in each figure legend were performed. A value of $p < 0.05$ was considered statistically significant. All statistical calculations were carried out using JMP® Pro (SAS Institute Inc., Cary, NC, USA). All of the statistical details of experiments can be found in the figure legends.