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Glutamate production from ammonia via glutamate dehydrogenase 2 activity supports cancer cell proliferation under glutamine depletion

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Abstract

Cancer cells rapidly consume glutamine as a carbon and nitrogen source to support proliferation, but the cell number continues to increase exponentially after glutamine is nearly depleted from the medium. In contrast, cell proliferation rates are strongly depressed when cells are cultured in glutamine-free medium. How cancer cells survive in response to nutrient limitation and cellular stress remains poorly understood. In addition, rapid glutamine catabolism yields ammonia, which is a potentially toxic metabolite that is secreted into the extracellular space. Here, we show that ammonia can be utilized for glutamate production, leading to cell proliferation under glutamine-depleted conditions. This proliferation requires glutamate dehydrogenase 2, which synthesizes glutamate from ammonia and α-ketoglutarate and is expressed in MCF7 and T47D cells. Our findings provide insight into how cancer cells survive under glutamine deprivation conditions and thus contribute to elucidating the mechanisms of tumor growth.

Key words:

cancer, ammonia, glutamate dehydrogenase, glutamate, proliferation

Introduction

Glutamine is the major nitrogen source for nonessential amino acids, nucleotides, and hexosamines [1-3]. Although glutamine can be synthesized in most tissues, demand often outpaces supply, and glutamine typically becomes an essential nutrient for proliferating cells [4, 5]. Glutamine is metabolized to glutamate by glutaminase (GLS), which releases the amide nitrogen of glutamine as ammonia [6]. Then, glutamate is converted to α-ketoglutarate (α-KG), an intermediate metabolite in the TCA cycle, by two types of reactions. In one, glutamate is deaminated by glutamate dehydrogenase (GDH), releasing ammonia in the mitochondria. In the other, glutamate is transaminated to produce nonessential amino acids by transaminases in either the mitochondria or cytosol [7].

GDH is a housekeeping gene that is widely conserved in many species. GDH normally catalyzes glutamate to α-KG and ammonia, employing nicotinamide adenine dinucleotide phosphate ($NADP^+$) and nicotinamide adenine dinucleotide (NAD^+) as cofactors. However, GDH can also catalyze reductive amination to produce glutamate from α-KG and ammonia, employing NADPH and NADH as cofactors, depending on the environment [8, 9]. Humans possess two GDH isoforms —GDH1 and GDH2 (encoded by the GLUD1 and GLUD2 genes, respectively)—

that have high sequence similarity. GDH1 is widely expressed in many tissues, and GDH2 is expressed in the brain, testis, embryonic tissue and various cancers [10, 11]. GDH1 is upregulated in human cancers and plays an essential role in redox homeostasis by controlling intracellular α-KG levels [12]. In contrast, the roles of GDH2 in cancer cells remain elusive.

Ammonia is produced during glutamine catabolism, which is the conversion of glutamine to α-KG in mitochondria, and is actively or passively exported from the cells [13]. Ammonia is also an important nitrogen source that is involved in amino acid metabolism, protein synthesis and pH homeostasis [14, 15]. However, the relationship between glutamine metabolism and GDH functions remains unknown.

Here, we show that cancer cells rapidly consume glutamine and secrete ammonia during the growth phase and that glutamine limitation suppresses cancer cell proliferation. Strikingly, this proliferation impairment can be partially overcome by supplementing cells with ammonia, depending on the cellular level of GDH2, which synthesizes glutamate from ammonia and α-KG. Our findings provide insight into how cancer cells survive under glutamine deprivation conditions and contribute to elucidating the mechanisms of cancer cell proliferation.

Materials and Methods

Metabolite extraction and derivatization

Cells were quenched with chilled 80% methanol supplemented with 5 μg of sinapic acid as an internal standard at -80°C for 15 min after washing with ice-cold phosphate-buffered saline (PBS). Then, the cells were harvested. After centrifugation to remove cell debris, the supernatant was freeze-dried. The culture medium was also collected and centrifuged, and the supernatant was freeze-dried. The metabolite levels in the medium were compared with those measured in the control medium not exposed to cells and were then normalized to the cell number to calculate the metabolite consumption/production values.

Samples were dissolved in 40 μl of 20 mg/ml methoxyamine (Sigma) in pyridine (Wako) and incubated for 90 min at 30°C. After incubation, the samples were derivatized with 20 μl of N-methyl-N-trimethylsilyl-trifluoroacetamide (GL Science) for 30 min at 37°C.

Stable isotope-based metabolite tracing

After 24 h or 48 h of incubation with [U-^{13}C]-glucose, [^{15}N]H_4-sulfate or [amide-^{15}N]-glutamine (Cambridge Isotope Laboratories), the metabolites were extracted as described above. Lyophilized samples were dissolved in 30 µl of dimethylformamide (Wako) and derivatized by the addition of 30 µl of N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) plus 1% tert-butylmethylchlorosilane (TMCS) (Cerilliant) at 85°C for 60 min. For natural isotope correction, IsoCor software was used [16]. The isotopic enrichment of ^{13}C and ^{15}N glutamate was assessed by quantifying the abundance of the following ions: *m/z* 432-437 and *m/z* 432-433, respectively.

Further information is provided in Supplementary Methods.

Results

Ammonia utilization stimulates glutamate synthesis and enables cells to proliferate under glutamine-depleted conditions

To understand the role of cellular metabolism during cancer cell proliferation, we initially cultured MCF7 cells and quantified the changes in metabolite levels in the culture medium. We confirmed that as glutamine and glucose levels decreased, lactate production increased in a cell number-dependent manner, consistent with previous reports and with the Warburg effect (Fig. S1). Because MCF7 cells grew even when most of the glutamine was consumed after 72 h of incubation, we investigated whether MCF7 cells could proliferate under glutamine-depleted conditions. As expected, the proliferation rate under these conditions was greatly suppressed relative to that in cells cultured with glutamine (Fig. 1A). Interestingly, we found that in the presence of glutamine the level of ammonia, a by-product of glutamine anaplerosis, in the culture medium was elevated after 24 h of incubation and gradually decreased after 72 h as the glutamine level decreased (Fig. 1B). In addition, glutamine withdrawal suppressed ammonia production as expected (Fig. 1B). From these results, we hypothesized that MCF7 cells could proliferate by utilizing ammonia produced from glutamine when the available glutamine level decreased. To understand whether ammonia plays a critical role in cell proliferation, presumably as a nitrogen donor, we incubated MCF7 cells with or without ammonium sulfate under glutamine-depleted

conditions. The addition of ammonium sulfate partially restored MCF7 cell proliferation in the absence of glutamine (Fig. 1C).

Next, we compared the effect of glutamine and ammonia levels on cellular metabolism. To this end, we performed gas chromatography-mass spectrometry (GC-MS) analysis of intracellular metabolites in the presence or absence of glutamine or ammonia. Glutamine withdrawal dramatically decreased the levels of amino acids, such as glutamine, glutamate, aspartate, alanine, and proline (Table S1). These decreases were presumably a direct result of glutamine withdrawal because these amino acids are synthesized *de novo* from glutamine and were not present in the medium. On the other hand, other amino acid levels were increased, presumably because of the nutritional decrease, suggesting that glutamine withdrawal enhances the uptake of essential amino acids by the general amino acid control pathway [17]. Furthermore, ammonia supplementation significantly increased the glutamate levels in these cells (Fig. 1D). In addition, the amino acids produced from glutamate that were decreased by glutamine depletion, such as aspartate and alanine, were also increased (Table S1).

To evaluate metabolic turnover of the carbon source of glutamate under glutamine deprivation and ammonia addition, we measured glutamate generation from [U-^{13}C glucose] in glutamine-

depleted conditions. The relative amount of ^{13}C-labeled glutamate was increased by ammonia supplementation (Fig. 1E). These results indicate that ^{13}C-labeled glutamate is increased by ammonia addition, which indicates that ammonia induced glutamate production from glucose-derived carbon, likely in the form of α-KG. Because ammonia supplementation stimulates cell proliferation under glutamine-deprived conditions, we investigated whether ammonia acts as a nitrogen donor for glutamate generation. As expected, the added [^{15}N]H_4-sulfate was utilized when glutamine was present and increased the rate of ^{15}N-labeled glutamate in the absence of glutamine relative to that in the presence of glutamine (Fig. 1F), indicating that some glutamate is formed from ammonia when cells are stimulated by glutamine depletion. Thus, MCF7 cells can utilize ammonia as a nitrogen source to generate amino acids, and the ammonia utilization rate for glutamate production is increased by glutamine depletion.

GDH is thought to use glutamate to generate α-KG and ammonia under physiological conditions [18]. However, when the TCA cycle pool is full, GDH generates glutamate from α-KG and ammonia by reductive amination (Fig. 1G). Considering the results of the above-described experiments with ammonia, we hypothesized that GDH might be deeply involved in glutamine depletion and cell proliferation in cancer cells.

Some cancer cells effectively utilize ammonia for cell growth

To determine whether ammonia utilization is a universal phenomenon in cancer cells, we explored the effects of glutamine depletion and ammonia addition on other cancer cell lines. Surprisingly, ammonia supplementation supported cell proliferation in MCF7 and T47D cells but not in other cell lines (Fig. 2A). Furthermore, the addition of glutamate partially restored MCF7, T47D, PC3 and MDA-MB-231 cell proliferation, whereas glutamate addition was merely effective in preventing cell death in MDA-MB-157 and Hs578T cells under glutamine-deprived conditions. Thus, the effects of glutamate supplementation are different among cell lines, possibly because of the glutamine dependency in these cell lines.

To elucidate the different effects of ammonia and glutamate addition under glutamine-depleted conditions, we used GC-MS to analyze the intracellular amino acids in PC3 cells. Ammonia supplementation failed to restore proliferation in PC3 cells, whereas glutamate addition successfully rescued proliferation. Ammonia supplementation had no effect on most of the metabolic differences between MCF7 and PC3 cells under glutamine-depleted conditions (Tables S1 and S2). However, in PC3 cells, the glutamate levels were increased by the glutamate addition

but not by ammonia addition (Fig. 2B). Furthermore, the amount of ^{13}C-labeled glutamate was not increased by ammonia addition, indicating that PC3 cells could not effectively utilize ammonia compared to MCF7 cells (Fig. 1E and 2C). We also confirmed that Hs578T cells showed the same behavior as PC3 cells with regard to glutamate level (Figure 2C).

We next examined the direct evidence that ammonia from glutamine could be utilized to produce glutamate under normal conditions using [amide-^{15}N]-glutamine. The labeled nitrogen in the amide group is released as ammonia by glutaminase (GLS) to generate glutamate. When GDH is the enzyme predominantly responsible for incorporation of ammonia into α-KG, ^{15}N-labeled glutamate will be generated (Fig. 2D). To verify this hypothesis, we measured generated ^{15}N-labeled glutamate during 24 h after the addition of [amide-^{15}N]-glutamine. We detected ^{15}N-labeled glutamate in all the cells, which means that GDH catalyzes not only the oxidative but also the reductive reaction and utilizes ammonia generated from glutamine to generate glutamate. Importantly, we found that the relative amount of labeled glutamate was significantly higher in MCF7 cells than in PC3 and Hs578T cells (Fig. 2E), suggesting that GDH in MCF7 cells plays a predominant role in synthesizing glutamate using ammonia compared with GDH in Hs578T cells.

Thus, we conclude that the difference in the ability of GDH to produce glutamate from ammonia is responsible for ammonia-dependent cell proliferation.

GDH2 is important for ammonia-dependent cell proliferation

To determine the importance of GDH activity for ammonia-dependent cell proliferation, we examined whether GLUD knockdown attenuates cell growth. We first confirmed that transfection of cells with siRNAs for GLUD effectively reduced the protein expression level (Fig. 3A). In the absence of glutamine, GDH knockdown failed to restore cell growth with ammonia supplementation, unlike the control cells (Fig. 3B). In addition, the glutamate levels were not elevated in GLUD knockdown cells compared with control cells following treatment with ammonia (Fig. 3C). These results indicate that GDH plays a crucial role in generating glutamate from ammonia for cell growth when glutamine is depleted. To further investigate this possibility, we performed cell proliferation experiments in which GLUD knockdown cells were treated with glutamate. We observed that glutamate also restored cell proliferation almost as much as ammonia under glutamine-depleted conditions (Fig. 3B).

Because glutamate production from ammonia is required for cell growth upon glutamine depletion, we focused on the involvement of GDH. First, we examined the expression levels of GDH in cells and found no differences in the protein expression levels among cancer cells (Fig. 3D). In addition, withdrawal of glutamine or addition of ammonia had no effect on the GDH protein expression levels in MCF7, PC3 or Hs578T cells (Fig. S2). Therefore, the ability of GDH to use ammonia to support cell growth is independent of the GDH protein level or glutamine depletion.

Unlike other mammals that have a single GDH-encoding gene, humans possess two GDH isoforms. The high similarity between GDH1 and GDH2 makes determining differences in the expression levels of these isoforms by western blotting difficult. Thus, we analyzed the differences in the mRNA levels using droplet digital polymerase chain reaction (ddPCR). Interestingly, the GLUD1 mRNA levels were higher in PC3 and Hs578T cells than in MCF7 and T47D cells (Fig. 3E). On the other hand, the GUDL2 mRNA levels were higher in MCF7 and T47D cells than in PC3 and Hs578T cells (Fig. 3F). Thus, we hypothesized that GDH2 contributes to the glutamate production from ammonia in MCF7 and T47D cells. We demonstrated that GLUD knockdown inhibited glutamate production and proliferation under glutamine-depleted

conditions in MCF7 cells (Fig. 3B and C). Therefore, we aimed to determine whether siRNA targeting GLUD could knockdown both the GLUD1 and GLUD2 isoforms. We analyzed the mRNA levels of both GLUD1 and GLUD2 in MCF7 cells that had been transfected with siRNAs targeting GLUD. As expected, the mRNA levels of both GLUD1 and GLUD2 were attenuated in siRNA-transfected MCF7 cells (Fig. S3).

GLUD2 is sufficient to produce glutamate for proliferation or survival under glutamine-depleted conditions.

We examined whether GLUD2-expressing cells can proliferate in glutamine-depleted conditions with ammonia supplementation. To this end, we established stable GLUD2 expression clones from PC3 and Hs578T cells. We first confirmed the expression of exogenous GLUD2 in these clones by western blot (Fig. 4A) and confirmed that exogenous GLUD2 localized in the mitochondria in the obtained clones (Fig. 4B). Next, we conducted proliferation assays under glutamine-depleted conditions with or without addition of ammonia. As we expected, GLUD2-expressing PC3 clones improved cell growth under glutamine-depleted conditions with ammonia supplementation (Fig. 4C). Although GLUD2-expressing Hs578T clones needed to be

supplemented with α-KG because the cells died under glutamine-deprived conditions, ammonia addition increased the proliferation of GLUD2-expressing Hs578T clones (Fig. S4). To confirm whether overexpressed GLUD2 can utilize ammonia from glutaminolysis to generate glutamate, we measured the ^{15}N-labeled glutamate generated during 24 h after the addition of [amide-^{15}N]-glutamine in GLUD2-expressing PC3 cells compared with PC3 cells harboring an empty vector. The amount of ^{15}N-labeled glutamate production was significantly higher in GLUD2 expressing cells than in mock or original cells (Fig. 4D). Similar results were obtained using Hs578T cells that expressed GLUD2 (Fig. S4).

Discussion

In this study, we showed that cancer cells can survive even under glutamine-limited conditions and that the ability to utilize ammonia enables specific types of cancer cells to partially restore proliferation under glutamine starvation. In such cells, GDH2 can induce increased intracellular levels of glutamate, which is synthesized from ammonia and α-KG. In contrast, in cells expressing low levels of GDH2, ammonia supplementation had no effect on proliferation in the absence of

glutamine. Furthermore, we showed that the proliferation rate of GLUD2-expressing PC3 and Hs578T cells increased under glutamine-depleted conditions by ammonia supplementation.

Most cancer cells exhibit apoptosis or growth arrest when extracellular glutamine is depleted [14, 19]. However, we also observed that although extracellular glutamine was rapidly consumed and almost disappeared after 3 days of incubation some cancer cells were still able to proliferate. This observation challenges the glutamine addiction model of cancer cells. The present report adds yet another dimension to the diverse array of metabolic events that stem from the cellular uptake and metabolism of glutamine in proliferating cells. In mitochondria, glutamine deamination by glutaminase yields ammonia, which is actively or passively exported from the cell [13]. The resulting glutamate is deaminated to α-KG, either by transaminases, which generate amino acids such as alanine or aspartate, or by glutamate dehydrogenase, which generates a second ammonia molecule as a byproduct. Although growing evidence indicates that GDH2 is involved in cellular functions in the central nervous system and testis, its roles in cancer cells remain poorly understood [11, 20]. We observed that the expression levels of GDH2 were altered in cancer cells and that MCF7 and T47D cells, which express high levels of GDH2, were able to proliferate under glutamine-restricted conditions with ammonia supplementation. Thus, GDH2 is

required for glutamate synthesis from ammonia, and its expression may contribute to glutamine insensitivity.

The last question is how cancer cells utilize glutamate produced from ammonia and α-KG to proliferate. Glutamate is associated with many aspects of cell proliferation, for example, as a source material for nonessential amino acid synthesis through the activity of transaminases and as a component of GSH, which allows cells to resist the oxidative stress associated with rapid metabolism and inflammation.

During cancer cell proliferation, lactate and ammonia are secreted into the extracellular space, resulting in low pH and a stressful microenvironment. Thus, tumor cells must reprogram their metabolism in response to their microenvironment [15, 21]. Our findings emphasize the importance of GDH2 activity, which produces glutamate from ammonia, during cell growth under nutrient-limited conditions in which glutamine availability is insufficient.

Acknowledgments

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Figure Legends

Fig. 1. Ammonia utilization stimulates glutamate synthesis and enables cells to proliferate under glutamine-depleted conditions

(A) MCF7 cells were cultured in the presence (+Gln) or absence (-Gln) of glutamine. The medium was not changed during the experiments. The cell number was normalized to the cell number at 0 h. (B) Ammonia secretion by the cultured MCF7 cells in the presence or absence of glutamine. (C) Relative growth of MCF7 cells. Cells were plated in complete culture medium (4 mM glutamine), which was replaced the following day with glutamine-withdrawal medium supplemented with ammonium sulfate (4 mM), in which the cells were incubated for 48 h. (D) The relative glutamate abundance in MCF7 cells under the same conditions as in (C). (E) The relative amount of ^{13}C-incorporated glutamate in MCF7 cells cultured without glutamine and with addition of ammonium sulfate. The relative amount of ^{13}C-incorporated glutamate was calculated by the ratio of ^{13}C incorporation into glutamate applied to the total amount of glutamate shown in (D). (F) Analysis of the ^{15}N incorporation rate into glutamate in MCF7 cells incubated with [^{15}N]H_4-sulfate for 24 h with or without glutamine. (G) Schematic image of the reaction catalyzed by GDH. All experiments were performed in triplicate. The values are expressed as the mean±standard error of the mean (SEM) of three independent experiments. The P-value was

determined using unpaired Student's two-tailed *t*-test (A, B and E) or by ANOVA with Tukey's multiple comparisons post-test (C, D). * $P < 0.05$, *** $P < 0.001$, and **** $P < 0.0001$.

Fig. 2. Some cancer cells effectively utilize ammonia for cell growth

(A) The relative proliferation of each cell line following glutamine depletion and supplementation with ammonium sulfate (4 mM) or glutamate (4 mM). After 7 days of incubation, the growth rate of each cell line was determined by cell counting and normalized to the cell number at time = 0 (i.e., when the medium conditions were applied). The line represents the cell number at time = 0. (B) The relative amounts of glutamate in cancer cells. Cells were plated in complete culture medium (4 mM glutamine), which was replaced the following day with glutamine-depleted medium supplemented with ammonium sulfate (4 mM) or glutamate (4 mM). After 48 h of incubation, intracellular glutamate was measured by GC-MS. (C) The relative amount of ^{13}C-incorporated glutamate in PC3 and Hs578T cells cultured without glutamine and addition of ammonia calculated by the ratio of ^{13}C incorporation into glutamate shown in (B). (D) Schematic image of the GLS reaction that produces ammonia from glutamine and the GDH reaction that incorporates ammonia into α-KG to generate glutamate. (E) The increased levels of ^{15}N-

incorporated glutamate synthesized from ^{15}N-ammonia released from glutamine in MCF7, PC3 and Hs578T cells cultured with [amide-^{15}N]-glutamine in complete culture medium for 24 h. The values are expressed as the mean±SEM of three independent experiments. P-values were determined using unpaired Student's two-tailed *t*-test (C) and by ANOVA with Tukey's multiple comparisons post-test (A, B and E). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$; n.s., not significant.

Fig. 3. GDH2 is important for ammonia-dependent cell proliferation

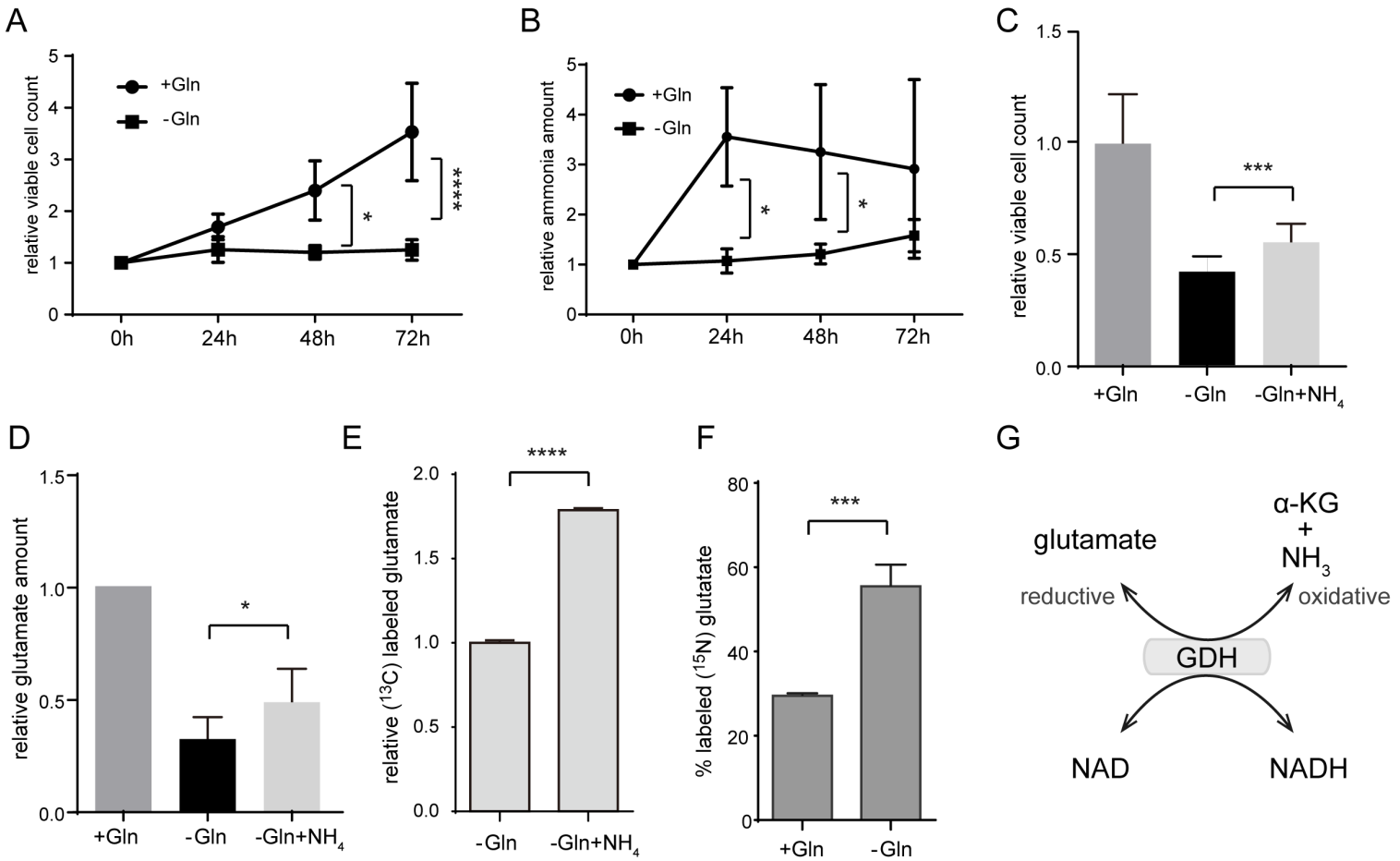
(A) Representative western blot of GDH in MCF7 cells transfected with siRNAs or control siRNA (siNC). β-Actin served as a loading control. (B) The relative cell growth in siGLUD and siControl transfected MCF7 cells. MCF7 cells were transfected with siRNAs and incubated for 24 h in complete medium (4 mM glutamine), which was replaced the following day with glutamine-free medium supplemented with ammonium sulfate (4 mM) or glutamate (4 mM) in which the cells were then incubated for 48 h. (C) Relative glutamate abundance in MCF7 cells under the same conditions as in (B) except for glutamate supplementation. The values are expressed as the mean±SEM of three independent experiments. P-values were determined by ANOVA with Tukey's multiple comparisons post-test (B) or using unpaired Student's two-tailed *t*-test (C). * P

< 0.05, ** P < 0.01, and *** P < 0.001; n.s., not significant. (D) Representative western blot of GDH in each cell line. β-Actin served as the loading control. (E and F) The mRNA level of GLUD1 and GLUD2 in cancer cells was measured by ddPCR. The data presented represent independent experiments performed more than two times.

Fig. 4. GLUD2 is sufficient to utilize ammonia for glutamate production.

(A) Representative western blot of extracts from PC3 cells transfected with empty vector (V14) or expressing DYKDDDDK-tagged GLUD2 (G16 and G23, respectively) using anti-DYKDDDDK-tag antibody. β-Actin served as the loading control. (B) Cells were stained with anti-DYKDDDDK-tag antibody (green), anti-GDH antibody (red) and Mito Tracker Deep Red to show mitochondria (blue). Bar = 20 μm. (C) The relative cell growth of control (V14) or GLUD2-expressing PC3 cells (G16 and G23). The cells were plated in complete culture medium (4 mM glutamine), which was replaced the following day with glutamine-withdrawal medium supplemented with ammonium sulfate (4 mM), in which the cells were incubated for 7 days. The data presented represent independent experiments performed more than 3 times. (D) The increased levels of ^{15}N-incorporated glutamate synthesized from ^{15}N-ammonia released from

glutamine in PC3 (original) and control (V14) cells or in GLUD2-expressing PC3 cells (G16 and G23) cultured with [amide-^{15}N]-glutamine in complete culture medium for 24 h. The values are expressed as the mean±SEM of three independent experiments. The P-value was determined using unpaired Student's two-tailed *t*-test (C) or by ANOVA with Tukey's multiple comparisons post-test (D). * $P < 0.05$, **** $P < 0.0001$, n.s, not significant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B
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24h
48h
72h
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C
relative viable cell count
***
+Gln
-Gln
-Gln+NH4
D
relative glutamate amount
*
+Gln
-Gln
-Gln+NH4
E
relative (13C) labeled glutamate
****
-Gln
-Gln+NH4
F
% labeled (15N) glutatate
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+Gln
-Gln
G
glutamate
α-KG
+
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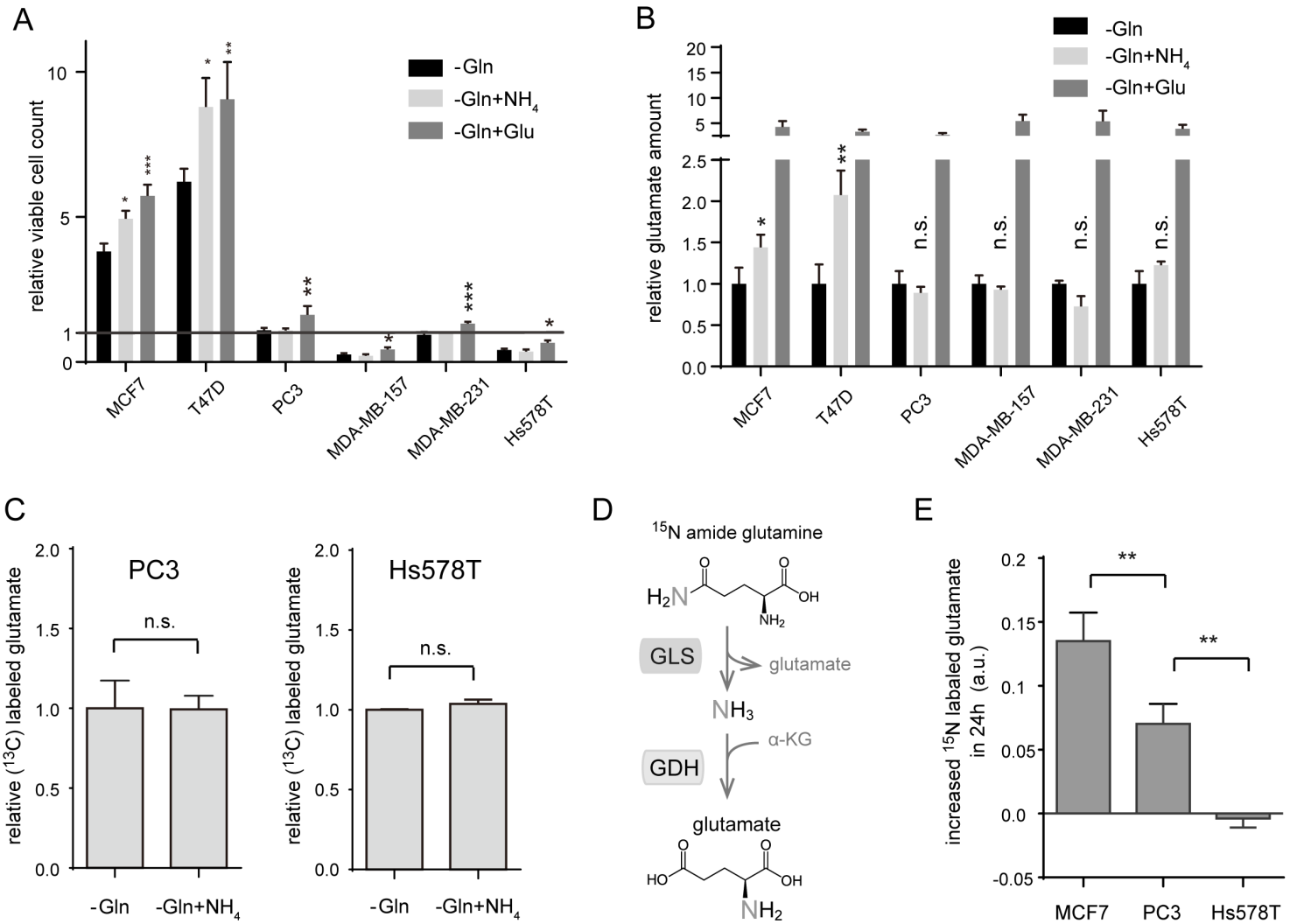
reductive
oxidative
GDH
NAD
NADH

Figure 1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-Gln
-Gln+NH4
-Gln+Glu
MCF7
T47D
PC3
MDA-MB-157
MDA-MB-231
Hs578T
B
relative glutamate amount
n.s.
C
PC3
Hs578T
relative (13C) labeled glutamate
n.s.
-Gln
-Gln+NH4
D
15N amide glutamine
GLS
glutamate
NH3
α-KG
GDH
glutamate
E
increased 15N labaled glutamate in 24h (a.u.)

Figure 2

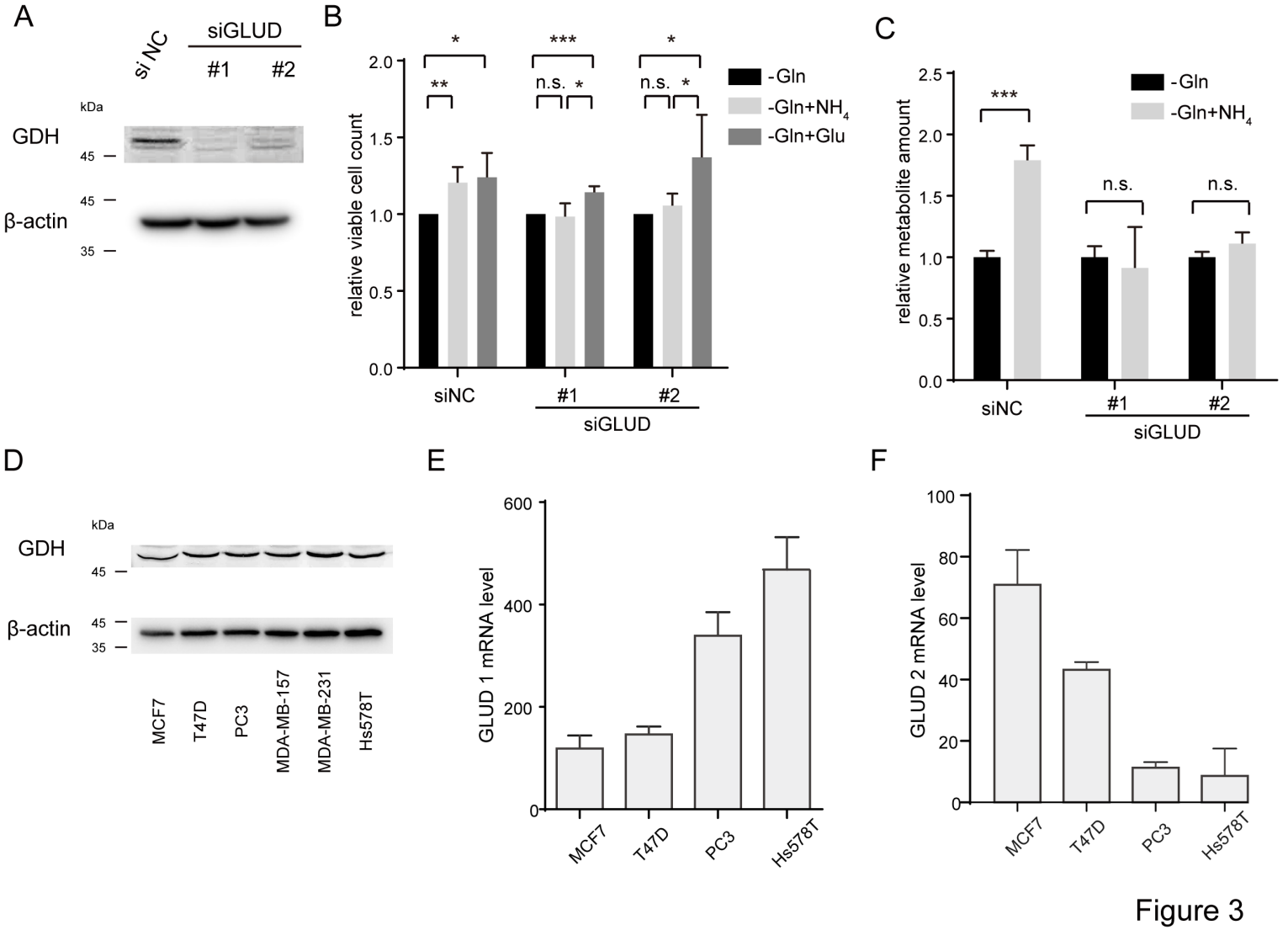
A
si NC
siGLUD
#1
#2
kDa
GDH
45
45
β-actin
35
B
relative viable cell count
2.0
1.5
1.0
0.5
0.0
*
**
***
n.s.
*
*
n.s.
*
-Gln
-Gln+NH4
-Gln+Glu
siNC
#1
#2
siGLUD
C
relative metabolite amount
2.5
2.0
1.5
1.0
0.5
0.0
***
n.s.
n.s.
-Gln
-Gln+NH4
siNC
#1
#2
siGLUD
D
kDa
GDH
45
45
β-actin
35
MCF7
T47D
PC3
MDA-MB-157
MDA-MB-231
Hs578T
E
GLUD 1 mRNA level
600
400
200
0
MCF7
T47D
PC3
Hs578T
F
GLUD 2 mRNA level
100
80
60
40
20
0
MCF7
T47D
PC3
Hs578T

Figure 3

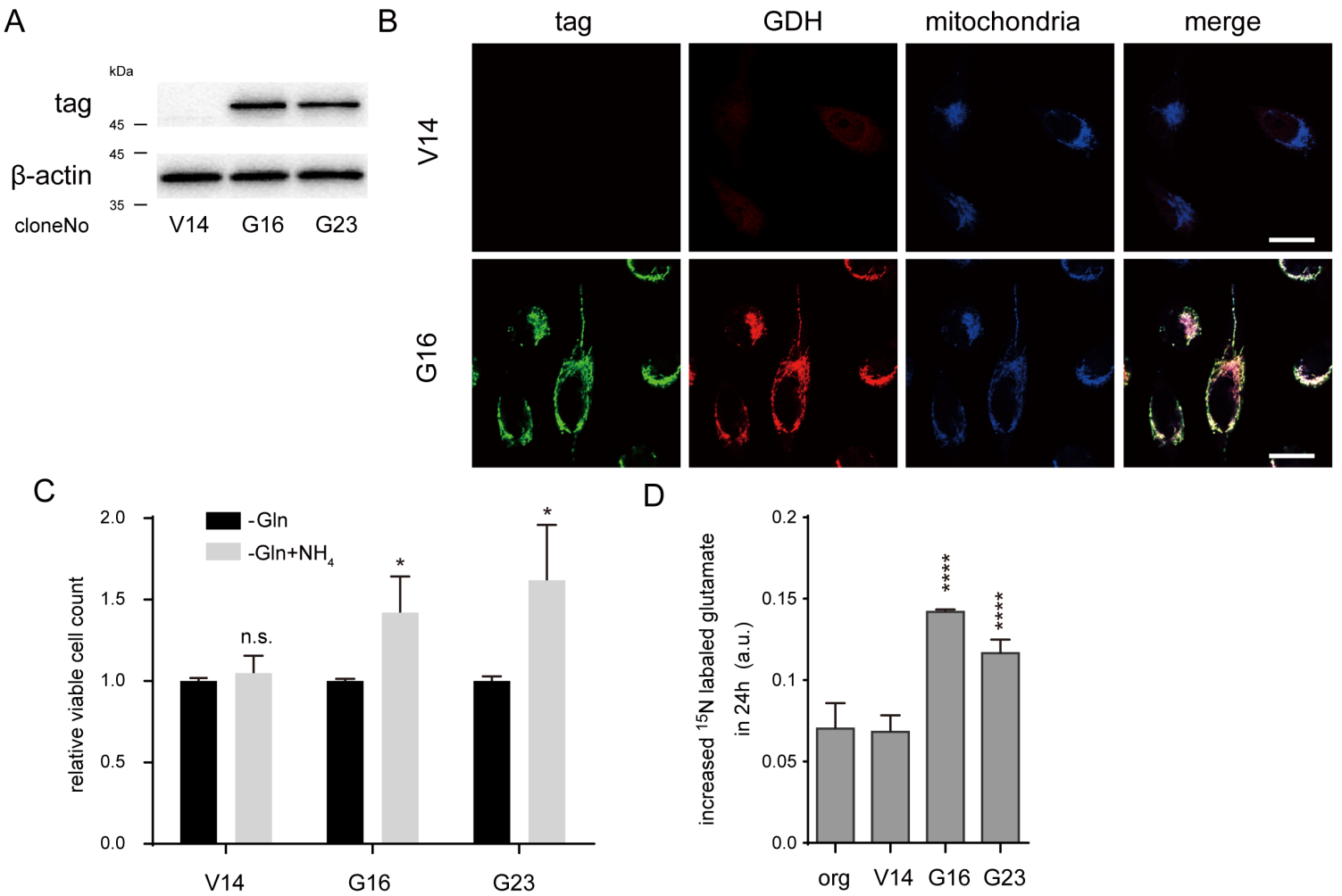
A
kDa
tag
45
45
β-actin
35
cloneNo
V14
G16
G23
B
tag
GDH
mitochondria
merge
V14
G16
C
relative viable cell count
2.0
1.5
1.0
0.5
0.0
-Gln
-Gln+NH4
n.s.
*
*
V14
G16
G23
D
increased 15N labaled glutamate in 24h (a.u.)
0.2
0.15
0.1
0.05
0.0
****
****
org
V14
G16
G23

Figure 4

Supplementary Material

Glutamate production from ammonia via glutamate dehydrogenase 2 activity supports cancer cell proliferation under glutamine depletion

Yukiko Takeuchi, Yasumune Nakayama, Eiichiro Fukusaki and Yasuhiro Irino

Supplementary Methods

Cell culture

MCF7, MDA-MB-231, Hs 578T and MDA-MB-157 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Wako, 044-29767) supplemented with 10% fetal bovine serum (FBS). T47D cells were cultured in DMEM (Sigma, D5030) supplemented with 25 mM glucose, 2 mM glutamine and 10% FBS. PC3 cells were maintained in RPMI 1640 (Wako, 189-02025) medium supplemented with 10% FBS. The cell lines were obtained from the American Type Culture Collection and European Collection of Authenticated Cell Cultures.

For stable isotope-based metabolite tracing experiments and glutamine depletion experiments, cells were incubated in DMEM (Sigma, D5030) with 10% dialyzed FBS supplemented with 25 mM glucose and/or 4 mM (or 2 mM) glutamine, as needed.

GC-MS

GC-MS analysis was performed using a GCMS-QP2010 Ultra mass spectrometer (Shimadzu) with an AOC-20is series injector/autosampler as previously described [1]. To more accurately analyze the

amino acids, TCA cycle components, glucose and lactate, the data were acquired with selected ion monitoring. The ion used to quantify the glutamate level was *m/z* 246. All data were normalized to the protein content.

Western blot analysis

After culture for 48 h under each condition, the cells were washed with cold PBS and extracted with lysis buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.4, 150 mM NaCl, 1% sodium dodecyl sulfate [SDS] and 1% nonyl phenoxypolyethoxylethanol [NP-40]). The following antibodies were used for western blotting: anti-GDH (Cell Signaling Technology, 12793) and anti-β-actin (Sigma, A1978).

RNA interference (RNAi)

siRNAs targeting GDH were purchased from Sigma Genosys (#1, SASI_Hs01_00082304; #2, SASI_Hs01_00082305). Negative control siRNA was purchased from Invitrogen (12935-300). Cells were transfected with siRNAs using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher,

13778) according to the manufacturer's instructions. After 24 h of transfection, the medium was replaced with the experimental medium.

Production of GLUD2-expressing cell lines

Expression vector encoding GLUD2 and empty vector (pcDNA3.1+/C-(K)DYK) were purchased from GenScript. Transfection was conducted using Lipofectamine 2000 transfection reagent (Thermo Fisher, 11668027) according to the manufacturer's instructions. Transfected PC3 and Hs578T cells were selected by adding 400 μg/ml and 300 μg/ml G418 (Nakalai, 09380-86) to the medium, respectively.

Immunofluorescence analysis

Cells grown on coverslips were treated with Mito Tracker Deep Red (Thermo Fisher, M22426) for 20 minutes at 37°C in 5% CO_2 and fixed with ice-cold methanol for 15 minutes. After being blocked with 5% normal goat serum and permeabilized with 0.3% Triton X-100 in PBS for 60 minutes at

room temperature, the cells were incubated with primary antibodies diluted in blocking buffer followed by incubation with secondary antibody in 1% BSA and 0.3% Triton X-100 in PBS.

The following antibodies were used for immunofluorescence: anti-GDH (Cell Signaling Technology, 12793) and anti-DYKDDDDK Peptide (Wako, 044-30951).

Images were taken with an LSM700 system (Zeiss) equipped with a Plan Apochromat VC 63× 1.2 oil immersion lens. Zen (2.3 SP1) software was used for image acquisition. Brightness and contrast were moderately enhanced using Photoshop software across the entire image, and no further image processing was performed.

Ammonia measurement

The levels of ammonia were determined using a commercially available ammonium assay kit (BioAssay Systems, BA_ENH3-100) according to the manufacturer's instructions. The experiment was performed independently three times.

Proliferation assay

Cells were trypsinized, and the cell number was determined by trypan blue exclusion using a Countess® automated cell counter (Thermo Fisher). Each test was conducted using three wells per condition. The experiment was independently performed more than three times.

In GDH knockdown and GLUD2 overexpression experiments, cells were counted using an IN Cell Analyzer 2000 (GE Healthcare) after fixation and 4',6-diamidino-2-phenylindole (DAPI) staining according to the manufacturer's instructions.

ddPCR

Total RNA was isolated from cultured cells using an RNeasy Mini Kit (Qiagen, 74104), and reverse-transcription (RT)-PCR was performed using a PrimeScript II 1st strand cDNA Synthesis Kit (Takara, 6210A) and 1 μg of total RNA. ddPCR assays were performed according to the manufacturer's instructions. Briefly, each 20-μl reaction contained 10 μl of ddPCR Supermix (Bio-Rad, Hercules, CA), 1 μl of primers for GLUD1 (Hs03989560_s1) or GLUD2 (Hs01649931_s1) (Thermo Fisher Scientific), and 3 μl of the prepared cDNA samples. The target mRNA concentrations were calculated

using Poisson statistics. The absolute transcript levels were calculated as copies/μl. The data are presented as the normalized values of three independent experiments.

Statistical analysis

Comparisons were made using unpaired two-tailed Student's *t*-test or one-way analysis of variance (ANOVA) with Tukey's post-test, as indicated. All statistical analyses were performed using GraphPad Prism software. Variations are presented as indicated in the figure legends.

Reference

[1] Y. Nakayama, Y. Tamada, H. Tsugawa, T. Bamba, E. Fukusaki, Novel strategy for non-targeted isotope-assisted metabolomics by means of metabolic turnover and multivariate analysis, Metabolites, 4 (2014) 722-739.

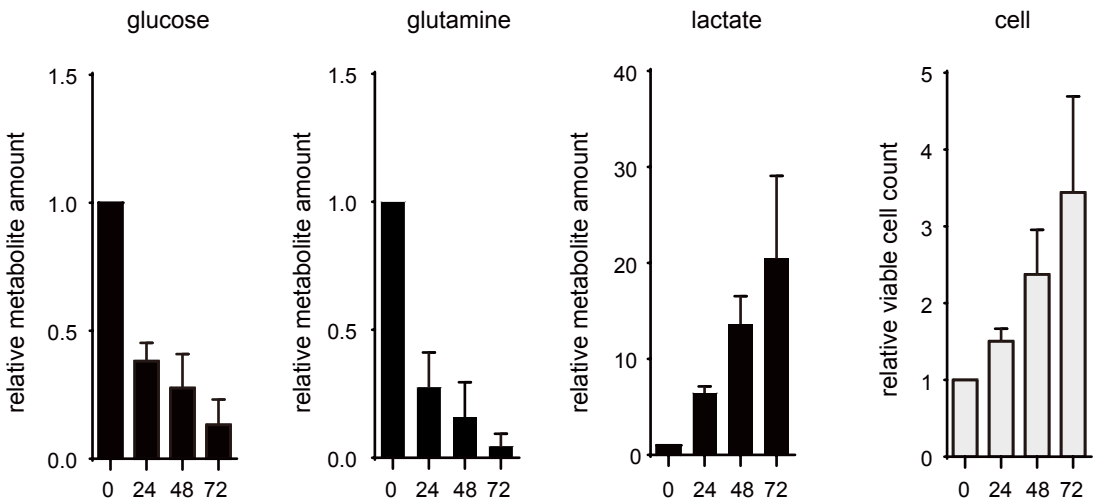

Fig. S1 . The amount of extracellular metabolites in the medium changed during cell proliferation.

MCF7 cells were cultured in complete medium. The glucose, glutamine and lactate levels were measured by GC-MS at each time point (indicated hour in the graphs). The proliferation rate of MCF7 cells was determined by cell counting.

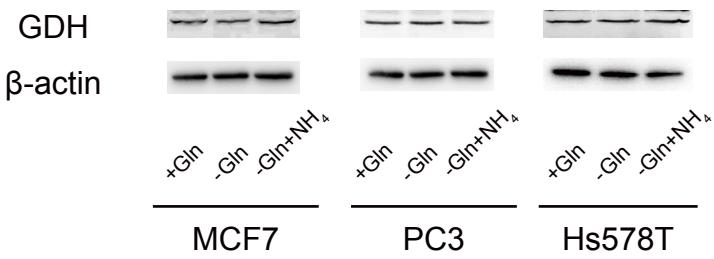

Fig. S2. The GDH protein expression level did not reflect the ammonia-related proliferation.

MCF7 and PC3 cells were cultured with glutamine (+Gln), without glutamine (-Gln), or with ammonia but without glutamine (-Gln+NH4). The cells were harvested after 48 h of incubation under each condition. β-Actin was used as the loading control.

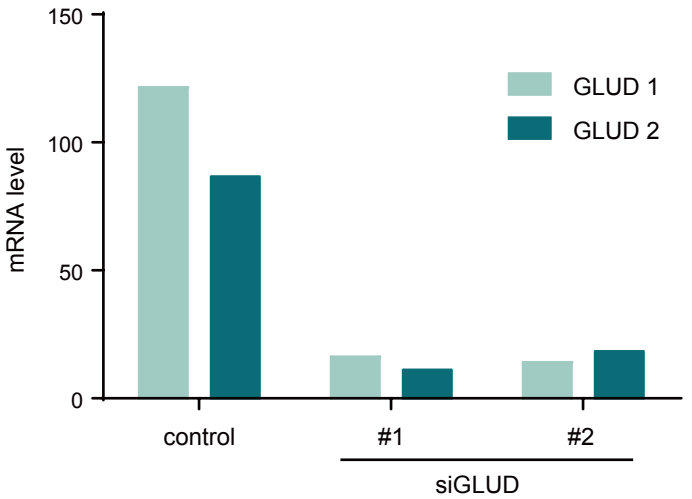

Fig. S3. GLUD mRNA levels in GLUD knockdown MCF7 cells.

MCF7 cells were harvested after 72 h of siRNA transfection, and the total RNA was isolated. The mRNA levels of GLUD1 and GLUD2 were determined by ddPCR.

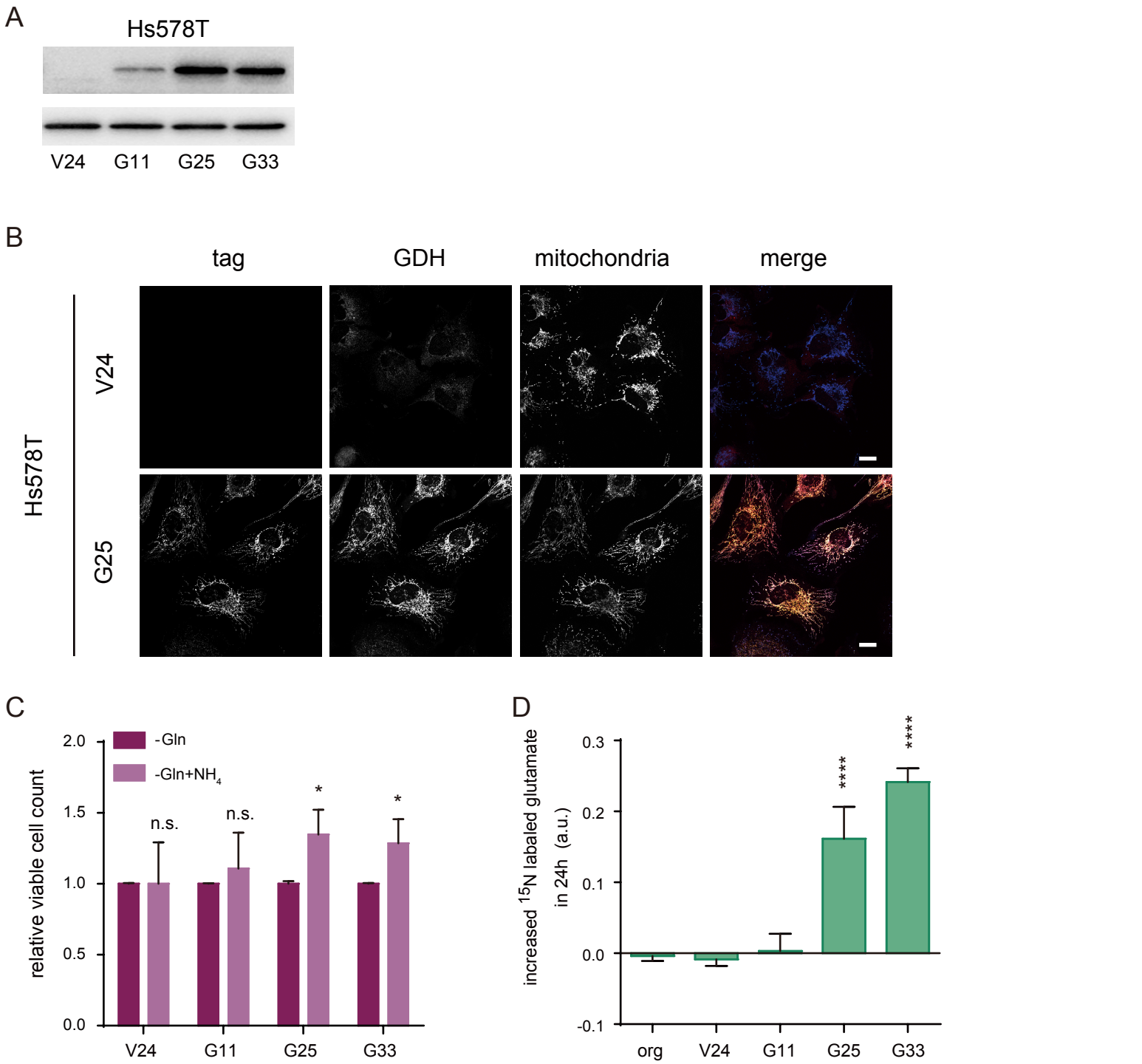

Fig. S4. GLUD2 is sufficient to utilize ammonia for glutamate production in Hs578T cells.

(A) Representative western blot of extracts from Hs578T cells transfected with empty vector (V24) or expressing DYKDDDDK-tagged GLUD2 (G11, G25 and G33, respectively) using anti-DYKDDDDK-tag antibody. β-Actin served as the loading control. (B) Cells were fixed and stained with anti-DYKDDDDK-tag antibody (green), anti-GDH antibody (red) and Mito Tracker Deep Red to show mitochondria (blue). Bar = 20 μm. (C) The relative cell growth of control (V24) or GLUD2-expressing Hs578T cells (G11, G25 and G33). Each Hs578T cell clone was plated in complete culture medium (4 mM glutamine), which was replaced the following day with glutamine-withdrawal medium containing α-KG (2 mM) and supplemented with or without ammonium sulfate (4 mM), and then, the cells were incubated for 7 days. The data presented represent independent experiments performed more than 3 times. The P-value was determined using unpaired Student's two-tailed t-test. * $P < 0.05$, n.s, not significant. (D) The increased levels of 15N-incorporated glutamate synthesized from 15N-ammonia releasedfrom glutamine in Hs578T cells (original) and control (V24) cells or in GLUD2-expressing Hs578T cells (G11, G25 and G33) cultured with [amide-15N]-glutamine in complete culture medium for 24 h. The values are expressed as the mean±SEM of three independent experiments. P-values were determined by ANOVA with Tukey's multiple comparisons post-test. **** $P < 0.0001$.

A

total metabolite	glucose	lactate	citrate	aKG	succinate	fumarate	malate	glutamine	glutamate	aspartate	alanine	proline	glycine	serine	cysteine	tyrosine	leucine	isoleucine	valine	threonine	methionine	lysine	tryptophane	phenylalanine
+Gln	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
-Gln(vs + Gln)	2.43*	1.79*	0.91	**2.56******	0.57****	0.25****	0.24****	0.02****	0.31****	0.09****	0.20****	0.28***	2.68	3.03**	3.14	2.22**	2.27**	2.34**	2.49**	1.82*	3.66*	2.01*	2.34*	2.17**
-Gln + NH4(vs - Gln)	1.66**	1.51	0.90	**1.08******	0.50	0.20	0.27	0.01	**0.43***	0.17*	0.33*	0.30	2.27	2.02	2.30	1.68*	1.82	1.94	2.02	1.58	3.26	1.35**	2.01	1.76

B

^{13}C labeled Glucose		lactate	citrate	aKG	succinate	fumarate	malate	glutamine	glutamate	aspartate	alanine	proline	glycine	serine	cysteine	leucine	isoleucine	valine	threonine	methionine
+ Gln	12C	0.09	0.19	0.47	0.60	0.60	0.58	0.77	0.49	0.55	0.14	0.69	1.00	1.00	0.93	1.00	1.00	0.99	1.00	0.99
	13C	0.91	0.81	0.53	0.40	0.40	0.42	0.23	0.51	0.45	0.86	0.31	0.00	0.00	0.07	0.00	0.00	0.01	0.00	0.01
-Gln	12C	0.11	0.18	0.26	0.38	0.54	0.17	0.31	0.22	0.36	0.35	0.59	1.00	1.00	0.92	1.00	1.00	0.99	1.00	0.99
	13C	0.89	0.82	0.74	0.62	0.46	0.83	0.52	0.78	0.64	0.65	0.41	0.00	0.00	0.08	0.00	0.00	0.01	0.00	0.01
-Gln+NH_4	12C	0.08	0.06	0.06	0.29	0.39	0.11	0.26	0.16	0.16	0.25	0.43	0.99	0.99	0.89	1.00	0.99	0.99	1.00	0.99
	13C	0.92	0.94	0.94	0.71	0.61	0.89	0.49	0.84	0.84	0.75	0.57	0.01	0.01	0.11	0.00	0.01	0.01	0.00	0.01

C

^{15}N labeled Ammonium Sulfate		glutamine	glutamate	aspartate	alanine	proline	glycine	serine	cysteine	tyrosine	leucine	isoleucine	valine	threonine	methionine	lysine	tryptophane	phenylalanine
+Gln	14N	0.73	0.71	0.72	0.73	0.74	1.00	1.00	1.00	1.00	1.00	0.96	0.98	1.00	1.00	1.00	1.00	1.00
	15N	0.27	0.29	0.28	0.27	0.26	0.00	0.00	0.00	0.00	0.00	0.04	0.02	0.00	0.00	0.00	0.00	0.00
-Gln	14N	0.22	0.44	0.57	0.63	0.62	1.00	0.99	1.00	1.00	1.00	0.97	0.98	0.97	0.99	1.00	1.00	1.00
	15N	0.78	0.56	0.43	0.37	0.38	0.00	0.01	0.00	0.00	0.00	0.03	0.02	0.03	0.01	0.00	0.00	0.00

Table S1. Changes in the metabolite levels in MCF7 cells

(A) MCF7 cells were collected after 48 h of incubation with glutamine (+Gln), without glutamine (-Gln), or supplemented with ammonium sulfate without glutamine (-Gln+NH4). The metabolite levels were determined by GC-MS. Each dataset was normalized to the level of metabolites produced under the +Gln condition. The unpaired two-tailed Student's t-test was applied between the +Gln and –Gln group and between the –Gln and -Gln+NH4 group. *P < 0.05, **P < 0.01, *** P < 0.001, and ****P < 0.0001. (B) The cells were collected after 24 h of incubation in [U-13C]-glucose-supplemented medium with glutamine (+Gln) or without glutamine (-Gln). Each figure represents the proportion of non-labeled (12C) or labeled (13C) carbon in the metabolites. (C) The cells were collected after 24 h of incubation in [15N]H4-sulfate-supplemented medium with glutamine (+Gln) or without glutamine (-Gln). Each figure represents the proportion of non-labeled (14N) or labeled (15N) nitrogen in the metabolites. Each analysis was independently conducted more than three times.

A

total metabolite	glucose	lactate	citrate	aKG	succinate	fumarate	malate	glutamine	glutamate	aspartate	alanine	proline	glycine	serine	cysteine	tyrosine	leucine	isoleucine	valine	threonine	methionine	lysine	tryptophane	phenylalanine
+Gln	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
-Gln(vs + Gln)	2.18**	1.66*	0.29****	1.05	0.30****	0.10****	0.08****	0.00****	0.24****	0.04****	0.10****	0.45****	1.71*	2.28**	1.46	1.84	2.09*	2.09*	2.16**	1.73*	2.78**	1.96	2.92	2.24*
-Gln+NH4(vs -Gln)	1.74	1.36	0.61***	0.72	0.29	0.11	0.10	0.00	**0.23**	0.09	0.17	0.48	1.55	1.96	1.16	1.22	2.05	1.90	1.86	1.44	2.35	1.37	1.96	1.87

B

^{13}C labeled Glucose		lactate	citrate	aKG	succinate	fumarate	malate	glutamine	glutamate	aspartate	alanine	proline	glycine	serine	cysteine		leucine	isoleucine	valine	threonine	methionine			
+Gln	12C	0.05	0.15	0.63	0.73	0.74	0.74	0.90	0.70	0.72	0.14	0.86	0.96	0.74	0.99		1.00			1.00	0.96			
	13C	0.95	0.85	0.37	0.27	0.26	0.26	0.10	0.30	0.28	0.86	0.14	0.04	0.26	0.01		0.00			0.00	0.04			
-Gln	12C	0.05	0.10	0.46	0.56	0.83	0.89	0.40	0.43	0.91	0.28	0.83	0.99	0.77	0.99		1.00			1.00	0.99			
	13C	0.95	0.90	0.54	0.44	0.17	0.11	0.60	0.57	0.09	0.72	0.17	0.03	0.23	0.01		0.00			0.00	0.01			
-Gln+NH_4	12C	0.05	0.10	0.39	0.52	0.80	0.77	0.40	0.40	0.86	0.31	0.80	0.99	0.77	0.99		1.00			1.00	0.99			
	13C	0.95	0.90	0.61	0.48	0.20	0.23	0.60	0.60	0.14	0.69	0.20	0.03	0.23	0.01		0.00			0.00	0.01			

C

^{15}N labeled Ammonium Sulfate		glutamine	glutamate	aspartate	alanine	proline	glycine	serine	cysteine	tyrosine	leucine	isoleucine	valine	threonine	methionine	lysine	tryptophane	phenylalanine
+Gln	14N	0.91	0.83		0.84			0.98	0.89	1.00		1.00			1.00	1.00	1.00	1.00
	15N	0.09	0.17		0.16			0.02	0.11	0.00		0.00			0.00	0.00	0.00	0.00
-Gln	14N	0.57	0.65		0.78			0.99	0.88	1.00		1.00			1.00	1.00	1.00	1.00
	15N	0.43	0.35		0.22			0.01	0.12	0.00		0.00			0.00	0.00	0.00	0.00

Table S2. Changes in the metabolite levels in PC3 cells

(A) PC3 cells were collected after 48 h of incubation with glutamine (+Gln), without glutamine (-Gln), or supplemented with ammonium sulfate without glutamine (-Gln+NH4). The metabolite levels were determined by GC-MS. Each dataset was normalized to the metabolite levels produced under the +Gln condition. Unpaired two-tailed Student's t-test was applied between the +Gln and –Gln group and between the –Gln and -Gln+NH4 group. *P < 0.05, **P < 0.01, *** P < 0.001, and ****P < 0.0001. (B) The cells were collected after 24 h of incubation in [U-13C]-glucose-supplemented medium with glutamine (+Gln) or without glutamine (-Gln). Each figure represents the proportion of non-labeled (12C) or labeled (13C) carbon in the metabolites. (C) The cells were collected after 24 h of incubation in [U-15N]-ammonium sulfate-supplemented medium with glutamine (+Gln) or without glutamine (-Gln). Each figure represents the proportion of non-labeled (14N) or labeled (15N) nitrogen in the metabolites. Each analysis was independently conducted more than three times.