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The glutaminase activity of ASNS fuels glutamine metabolism in leukemia

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Author contributions
Concept and experimental design: W.K.C., J.N.W., and P.L.L.; Cell culture work: W.K.C; Development of isotope analysis methodology: L.T, T.D.H, L.A.M. and Y.C.; Acquisition of MS data: L.T., S.A.M, and B.Q.T.; Analysis of MS data: L.T., S.A.M., Y.C. and B.Q.T.; ASNase recombinant proteins: W.K.C., P.L.L., J.N.W., S.B.R. and S.S.; Manuscript writing: W.K.C., L.T., J.N.W., and P.L.L.

Declaration of interests
The authors declare no competing interests.

Data availability
All data generated and/or analyzed during this study are available from the corresponding author upon reasonable request.

Supplemental information
Supplemental Figures S1–S3.

TO THE EDITOR:

Asparagine synthetase (ASNS) converts aspartate to asparagine and deamidates glutamine to glutamate. ASNS is highly responsive to cellular stress, including amino acid limitations and endoplasmic reticulum (ER) stress, and is frequently upregulated in cancers, where it enables cells to synthesize asparagine *de novo*, reducing their dependence on extracellular asparagine [1]. Of clinical relevance, ASNS expression in acute lymphoblastic leukemia (ALL) cells has been correlated with resistance to L-asparaginase (ASNase) treatment [2].

Glutamine, the most abundant amino acid in human blood, is a crucial source of carbon and nitrogen for various biosynthetic processes, including nucleotide synthesis, ATP production, amino acid formation, and maintaining redox balance [3]. Cancer cells, with their high energy and biosynthetic needs, often become reliant on glutamine, using it as a key fuel for the TCA cycle through glutaminase (GLS/GLS2) [3]. Recent research has shown that glutamine is crucial for the metabolism of acute myeloid leukemia (AML) cells, making glutamine metabolism a potential target for AML treatment [4].

Research on ASNS has primarily focused on its role in asparagine synthesis, likely influenced by its nomenclature, while its effects on glutamine deamidation and metabolism remain unclear. This study demonstrates that ASNS functions as a glutaminase, enhancing glutamine metabolism and contributing to leukemia cell resistance against glutaminase inhibitors.

The metabolic pathways involving asparagine, aspartate, glutamine, and glutamate are intricately interconnected, with each serving as precursors for the others and playing significant roles in nitrogen, energy, and redox metabolism[5,6]. Due to limited permeability of aspartate and glutamate into cells [7,8], we hypothesized that glutamine is the primary carbon source for ASNS-mediated intracellular asparagine synthesis. We first used isotopic tracers $^{13}C_4$-asparagine, $^{13}C_4$-aspartate, $^{13}C_5$-glutamine, or $^{13}C_5$-glutamate to evaluate uptake in ASNS-negative RS4;11 and ASNS-positive NALM-6 leukemia cells [9]. We found that while aspartate and glutamate exhibited very limited cellular uptake, nearly 100% of the intracellular asparagine and glutamine pools were labeled with ^{13}C (**Figures 1A & 1B**).

Despite extensive uptake of $^{13}C_4$-asparagine and $^{13}C_5$-glutamine by leukemia cells, the tracing experiments revealed minimal incorporation into glycolytic intermediates, except for glucose-6-phosphate (G6P), in both RS 4;11 and NALM-6 cells (**Supplemental Figure S1**). Furthermore, we observed minimal incorporation of $^{13}C_4$-asparagine into TCA cycle intermediates in both cell lines (**Supplemental Figure S2**). In contrast, incorporation of ^{13}C from $^{13}C_5$-glutamine was most significant in TCA cycle intermediates with ^{13}C enrichment ranging from 50% to 95% (**Figures 1C and 1D**). In addition, we observed notable ^{13}C enrichment derived from $^{13}C_5$-glutamine in both glutamate and aspartate, with ^{13}C enrichment ranging from 80% to 90% (**Figure 1E**). These findings suggest that glutamine serves as a major carbon source for the TCA cycle and synthesis of intracellular aspartate and glutamate, distinguishing it from asparagine, glutamate, and aspartate.

Since cells can use glutamine as both a carbon source for intracellular aspartate synthesis (**Figure 1E**) and a nitrogen source for asparagine synthesis, we hypothesized that ASNS could produce asparagine using only glutamine. When $^{13}C_5$-glutamine was provided in medium lacking asparagine, aspartate, and glutamine, ASNS-positive NALM-6 cells showed over 80% ^{13}C-enrichment in asparagine, while ASNS-negative RS4;11 cells exhibited no enrichment (**Figure 2A**). This confirms that cells can synthesize asparagine through ASNS and glutamine alone, without the requirement of extracellular aspartate.

To explore the broader effects of ASNS on metabolism, we stably transfected an ASNS-expression vector into RS4;11 cells to generate RS4;11_ASNS. A luciferase expression vector was used to generate a negative control, RS4;11_LUC. Western blot analysis confirmed successful transfection of the ASNS-expression vector (**Figure 2B**). ASNS overexpression made RS4;11_ASNS cells resistant to the glutaminase-deficient $ASNase^{Q59L}$, an ASNase variant that is ineffective against ASNS-positive cancer cells [10], and decreased sensitivity to $ASNase^{WT}$ (**Supplemental Figure S3A**). It also increased ^{15}N

incorporation into extracellular and intracellular asparagine from $^{15}N_1$-glutamine (**Supplemental Figure S3B**), while no labeling occurred in RS4;11_LUC cells (**Supplemental Figure S3B**). These findings confirm the functionality of transfected ASNS.

We next tested whether ASNS mediates asparagine synthesis from glutamine and glutamine-derived aspartate. In RS4;11_ASNS cells, $^{13}C_5$-glutamine tracing showed higher ^{13}C enrichment in asparagine, aspartate, and glutamate compared to RS4;11_LUC cells, suggesting that ASNS enhances glutamine conversion into these amino acids (**Figure 2C**).

Glutamine metabolism drives the TCA cycle and nucleotide synthesis in many cell types. We observed that ASNS also increased ^{13}C incorporation into TCA cycle intermediates by 10 to 30% (**Figure 2D**) and pyrimidine nucleotides by about 30% (**Figure 2E**), with undetectable effects on purine nucleotides (**Figure 2E**). Additionally, knockdown of GOT1, a key enzyme mediating aspartate synthesis from glutamine via the TCA cycle, decreased the conversion of glutamine to aspartate by 30% (**Supplemental Figure 3C**). These results indicate that ASNS promotes glutamate-driven TCA cycling, aspartate production, and pyrimidine synthesis.

To validate those results, we used shRNAs to stably knock down ASNS in ASNS-positive NALM-6 cells to generate ASNS-KD NALM-6_shRNA3 and NALM-6_shRNA4 cell lines. A scramble shRNA served as a negative control (NALM-6_shCtrl) (**Figure 2F**). ASNS-KD increased sensitivity to $ASNase^{WT}$ and made previously resistant cells sensitive to $ASNase^{Q59L}$ (**Supplemental Figure S3D**). It also decreased $^{13}C_5$-glutamine incorporation into aspartate by 20%, glutamate by 10% (**Supplemental Figure S3E**), TCA cycle metabolites by > 10% (**Figure 2G**) and pyrimidine nucleotides by 20-30% (**Figure 2H**) but had no effect on purine nucleotides (**Supplemental Figure S3F**). These findings confirm that ASNS promotes glutamine metabolism and nucleotide synthesis.

Glutamine conversion to glutamate is crucial for cancer cell biosynthesis. To test whether ASNS acts as a glutaminase, we treated RS4;11_ASNS, RS4;11_LUC, and NALM-6 cells with CB-839, an inhibitor of glutaminase (GLS), and traced $^{13}C_5$-glutamine incorporation into TCA cycle and nucleotide metabolites. CB-839 treatment significantly decreased ^{13}C enrichment in TCA metabolites (**Figures 3A**) and pyrimidine nucleotides (**Figures 3B**) in RS4;11_LUC and NALM-6 cells. However, RS4;11_ASNS cells exhibited resistance to the inhibitory effects of CB-839 on $^{13}C_5$-glutamine incorporation into those metabolites (**Figures 3A & 3B**). These observations indicate that ASNS mediates resistance to the glutaminase inhibitor CB-839.

To explore how ASNS impacts on other glutamine-derived metabolites linked to cancer, we examined 2-hydroxyglutarate (2-HG) and proline, which contribute to altered cancer metabolism and metastasis[11,12]. After administering $^{13}C_5$-glutamine to RS4;11_LUC, RS4;11_ASNS, and NALM-6 cells, the ASNS-positive cells exhibited greater ^{13}C enrichment in 2-HG and proline (**Figure 3C**). CB-839 treatment decreased ^{13}C-enriched glutamate, aspartate, 2-HG, and proline, but ASNS overexpression partially rescued those effects (**Figures 3D**). These results suggest that ASNS may play a key role in positively modulating cancer metabolism and progression.

ASNS is well-known for its role in asparagine synthesis and resistance to ASNase therapy in leukemia [13]. In this study, we highlight its underappreciated glutaminase activity in the context of cancer metabolism. We show that this activity supports key metabolic processes, including reductive carboxylation, TCA cycling, nucleotide and amino acid synthesis, and oncometabolite production (**Figure 3E**), thereby enhancing leukemia cell glutamine metabolism. Notably, ASNS also contributes to resistance against the glutaminase inhibitor CB-839, potentially acting as a bypass mechanism to sustain glutamine dependent metabolic flux under therapeutic pressure. These findings help explain its frequent upregulation in cancers and point to its broader role in tumor growth, metabolic adaptation, drug resistance, and metastasis [1].

ASNase is ineffective against ASNS-positive leukemia cells [10]. Our data show that leukemia cells can use glutamine carbon to produce aspartate for asparagine synthesis, with ASNS enhancing this process through the TCA cycle. This explains

why depleting extracellular asparagine alone fails to eliminate ASNS-positive cells—resistance is mediated by ASNS and glutamine. These findings underscore the potential of therapeutic strategies that co-target ASNS, asparagine (e.g. with ASNase), and/or glutamine.

Our findings also tie into the well-known preference of cancer cells to import extracellular glutamine. Potential explanations for that phenomenon include: 1) glutamine levels in plasma are considerably higher than those of glutamate; 2) glutamine demonstrates much greater solubility than glutamate; 3) glutamate transport is more energy-demanding than glutamine transport [14]; and 4) glutamate is a crucial neurotransmitter, so maintaining stable blood levels is essential for optimal central nervous system function [15]. ASNS, therefore, is well-positioned to take advantage of the cancer cell preference for glutamine.

The glutaminase activity of ASNS differs from that of GLS and GLS2, which produce ammonia that can easily accumulate to toxic levels. As a cytoplasmic enzyme, ASNS provides asparagine and glutamate for protein synthesis without generating ammonia. Additionally, asparagine supports the import of other amino acids, promoting cellular growth and metabolism (**Figure 3E**).

Given that many cancers depend on glutamine metabolism, targeting GLS has been a promising therapeutic strategy. However, the recently discontinued clinical development of the GLS inhibitor CB-839 underscores the complexity of targeting glutamine metabolism in cancer [16]. Our findings suggest that ASNS presents an additional target that could potentially increase the efficacy of GLS inhibition alone. Overall, targeting ASNS alone or in combination with other metabolic inhibitors presents a promising approach for development of new therapeutic strategies for the treatment of blood cancers and potentially other cancer types.

Although our study establishes a mechanism through which ASNS regulates glutamine metabolism through its glutaminase activity, this may represent only one aspect of a broader and more complex regulatory network. Other amidotransferases that use glutamine as a nitrogen donor likely contribute to glutamine metabolism as well. Several such enzymes participate in nucleotide biosynthesis, including PPAT, PFAS, CAD, and CTPS1/CTPS2, while others such as GFPT1/2 and NADSYN1 are involved in amino sugar and NAD^+ biosynthesis, respectively. Given their glutamine-deaminating functions, it would be valuable in future studies to examine whether those enzymes influence glutamine flux and whether their activity as a glutaminase contributes to metabolic reprogramming in cancer cells.

References

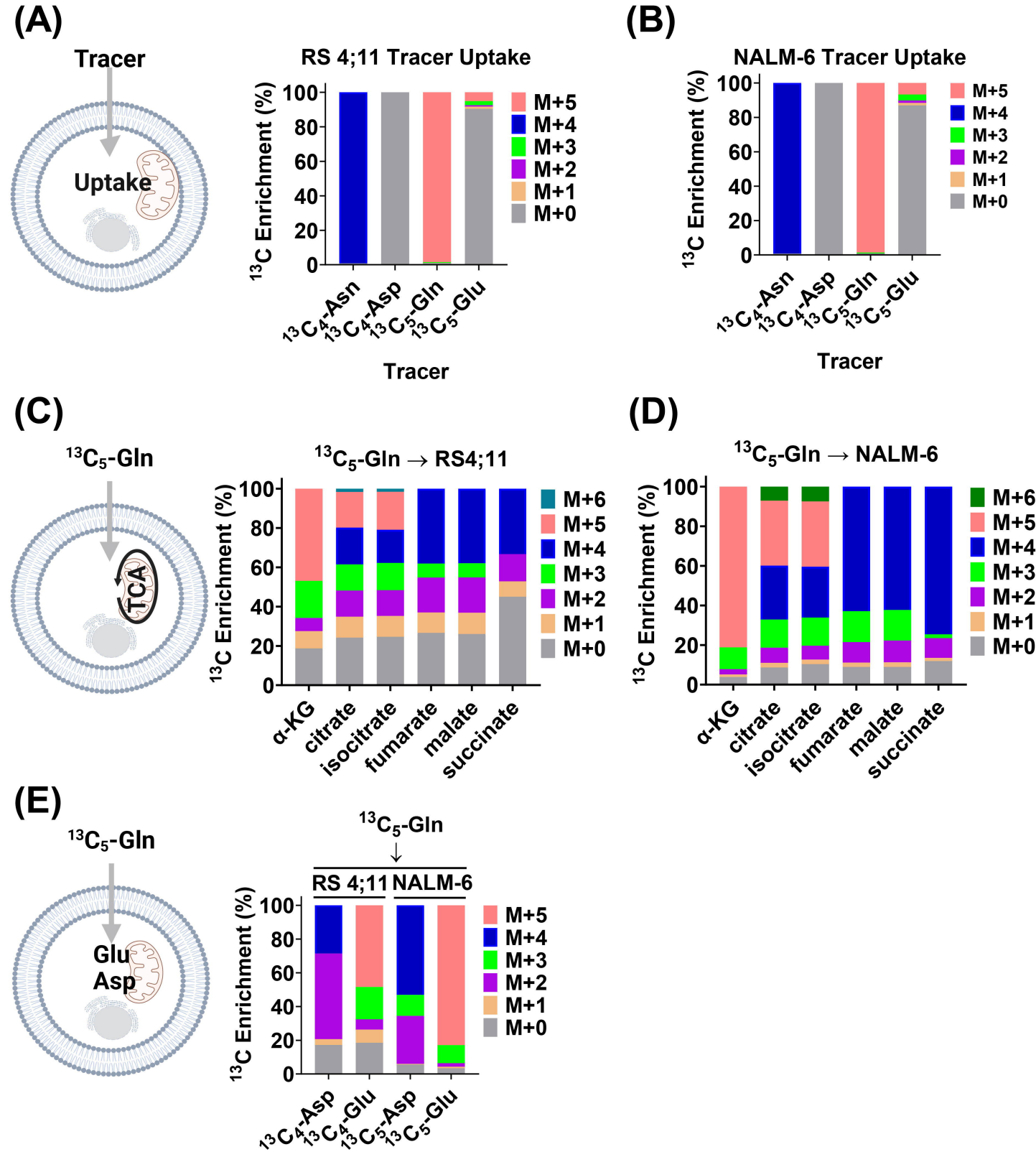
1. Balasubramanian MN, Butterworth EA, Kilberg MS. Asparagine synthetase: regulation by cell stress and involvement in tumor biology. Am J Physiol Endocrinol Metab. 2013;304(8):E789-799.
2. Richards NG, Kilberg MS. Asparagine synthetase chemotherapy. Annu Rev Biochem. 2006;75:629-654.
3. Zhang J, Pavlova NN, Thompson CB. Cancer cell metabolism: the essential role of the nonessential amino acid, glutamine. EMBO J 2017;36(10):1302-1315.
4. Xiao Y, Hu B, Guo Y, et al. Targeting Glutamine Metabolism as an Attractive Therapeutic Strategy for Acute Myeloid Leukemia. Curr Treat Options Oncol. 2023;24(8):1021-1035.
5. Huang H, Vandekeere S, Kalucka J, et al. Role of glutamine and interlinked asparagine metabolism in vessel formation. EMBO J. 2017;36(16):2334-2352.
6. Pavlova NN, Hui S, Ghergurovich JM, et al. As Extracellular Glutamine Levels Decline, Asparagine Becomes an Essential Amino Acid. Cell Metab. 2018;27(2):428-438.e5.
7. Garcia-Bermudez J, Baudrier L, La K, et al. Aspartate is a limiting metabolite for cancer cell proliferation under hypoxia and in tumours. Nat Cell Biol. 2018;20(7):775-781.
8. Kanai Y, Clemencon B, Simonin A, et al. The SLC1 high-affinity glutamate and neutral amino acid transporter family. Mol Aspects Med. 2013;34(2-3):108-120.
9. Chan WK, Lorenzi PL, Anishkin A, et al. The glutaminase activity of L-asparaginase is not required for anticancer activity against ASNS-negative cells. Blood. 2014;123(23):3596-3606.
10. Chan WK, Horvath TD, Tan L, et al. Glutaminase Activity of L-Asparaginase Contributes to Durable Preclinical Activity against Acute Lymphoblastic Leukemia. Mol Cancer Ther. 2019;18(9):1587-1592.
11. Du X, Hu H. The Roles of 2-Hydroxyglutarate. Front Cell Dev Biol. 2021;9:651317.
12. D'Aniello C, Patriarca EJ, Phang JM, Minchiotti G. Proline Metabolism in Tumor Growth and Metastatic Progression. Front Oncol. 2020;10:776.
13. Chen SH. Asparaginase Therapy in Pediatric Acute Lymphoblastic Leukemia: A Focus on the Mode of Drug Resistance. Pediatr Neonatol. 2015;56(5):287-293.
14. Scalise M, Pochini L, Galluccio M, Console L, Indiveri C. Glutamine Transport and Mitochondrial Metabolism in Cancer Cell Growth. Front Oncol. 2017;7:306.
15. Zhou Y, Danbolt NC. Glutamate as a neurotransmitter in the healthy brain. J Neural Transm (Vienna) 2014;121(8):799-817.
16. Tannir NM, Agarwal N, Porta C, et al. Efficacy and Safety of Telaglenastat Plus Cabozantinib vs Placebo Plus Cabozantinib in Patients With Advanced Renal Cell Carcinoma: The CANTATA Randomized Clinical Trial. JAMA Oncol. 2022;8(10):1411-1418.

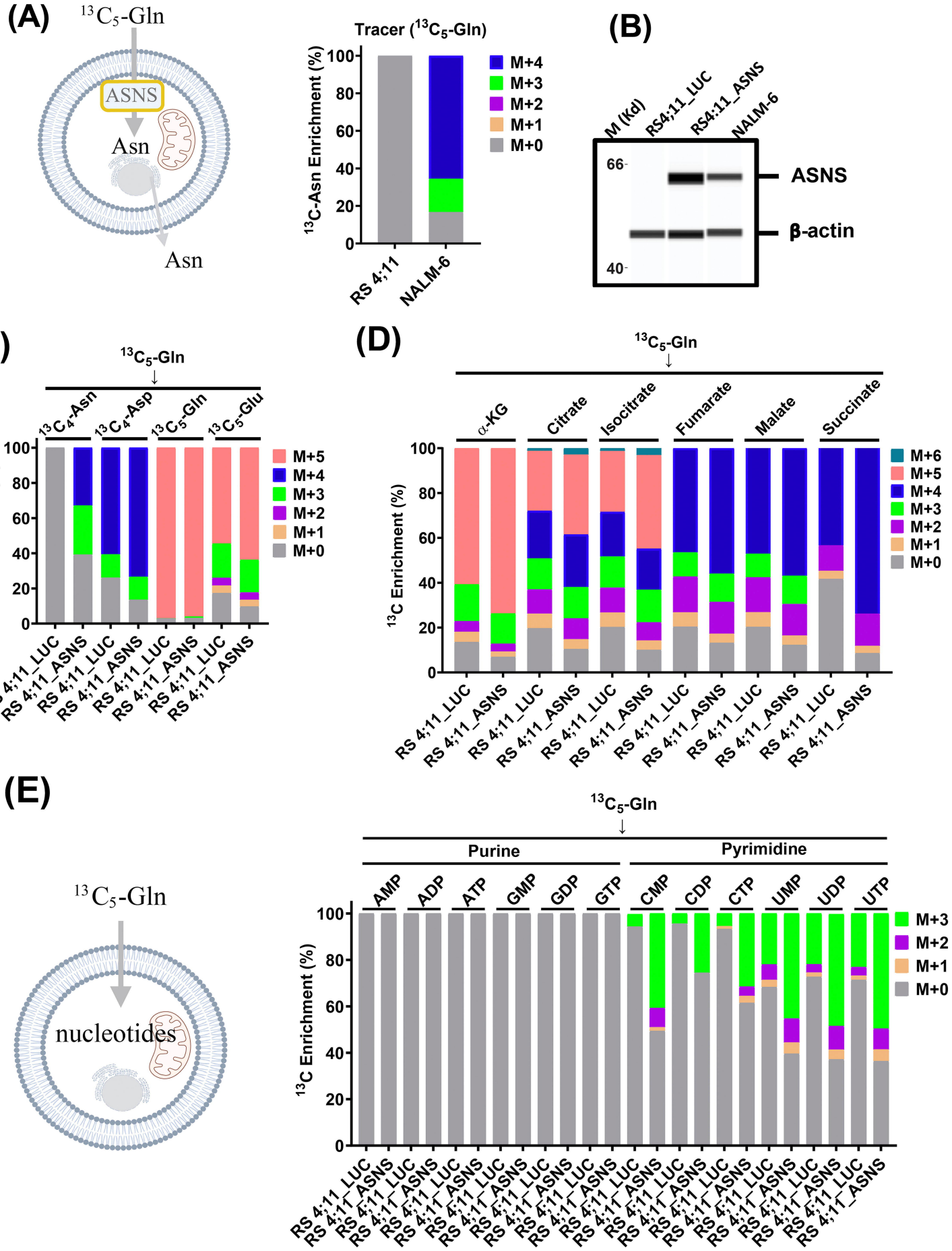
Figure legends

Figure 1: Intracellular aspartate and glutamate originate primarily from glutamine. (**A**) RS4;11 and (**B**) NALM-6 cells efficiently transport asparagine and glutamine from the extracellular compartment to the intracellular compartment, but not aspartate and glutamate. Indicated ^{13}C tracers were added to RPMI-1640 cell culture medium lacking the corresponding ^{12}C metabolite. (**C**) RS4;11 and (**D**) NALM-6 cells efficiently convert $^{13}C_5$-glutamine to TCA cycle intermediates. (**E**) RS4;11 and NALM-6 cells efficiently convert $^{13}C_5$-GLN to stable isotope labeled aspartate and glutamate. All isotope tracing experiments were conducted with a 24-h labeling period. Each experiment was performed twice, with a representative result shown.

Figure 2: Asparagine synthetase (ASNS) enhances glutamine metabolism. (**A**) NALM-6 but not RS 4;11 cells efficiently convert $^{13}C_5$-glutamine to stable isotope labeled asparagine. (**B**) Western blot analysis of ASNS levels in RS4;11 cells stably transfected with an ASNS expression plasmid (RS4;11_ASNS) or a luciferase expression plasmid (RS4;11_LUC). ASNS-expressing NALM-6 cells served as a positive control and RS4;11_LUC was a negative control. β-actin protein levels were used as a loading control. (**C**) RS4;11 cells expressing ASNS (RS4;11_ASNS), but not the control cells expressing luciferase (RS 4;11_LUC), efficiently convert $^{13}C_5$-glutamine to stable isotope labeled asparagine, aspartate, and glutamate. (**D, E**) RS4;11 cells expressing ASNS (RS4;11_ASNS), but not the control cells expressing luciferase (RS4;11_LUC), efficiently convert $^{13}C_5$-glutamine to TCA cycle intermediates (**D**) and pyrimidines (**E**). (**F**) Western blot analysis of ASNS levels in NALM-6 cells stably transfected with *ASNS* shRNA expression plasmids (NALM-6_shASNS.3 and NALM-6_shASNS.4) or a scramble shRNA expression plasmid (NALM-6_shCtrl) as a negative control. β-actin protein levels were used as a loading control. (**G, H**) Depletion of ASNS decreases conversion efficiency from $^{13}C_5$-glutamine to TCA cycle intermediates and pyrimidines in NALM-6 cells depleted of ASNS, as described in (**F**). All tracer experiments were conducted with a 24-h labeling period. Each experiment was performed twice, with a representative result shown.

Figure 3: Asparagine synthetase (ASNS) counteracts the effects of the glutaminase inhibitor CB-839 on glutamine metabolism. Expression of ASNS partially rescues the conversion of $^{13}C_5$-GLN to TCA cycle intermediates (**A**) and pyrimidines (**B**) in RS4;11 cells, which is inhibited by CB-839 treatment. (**C**) Restoring expression of ASNS in RS4;11 cells increase the conversion of $^{13}C_5$-glutamine to 2-hydroxyglutarate and proline, while ASNS knockdown in NALM-6 cells decreases this conversion. (**D**) Conversion of $^{13}C_5$-glutamine to 2-hydroxyglutarate, proline, aspartate and glutamate in RS4;11 cells and NALM-6 cells is inhibited by CB-839 and partially rescued by ASNS expression. (**E**) Working model of the biochemical network modulated by ASNS as described in the Discussion section. Cells (described in **Figure 2**) were treated with 10 μM CB-839 or DMSO (vehicle control). All tracer experiments were conducted with a 24-h labeling period. Each experiment was performed twice, with a representative result shown. Data in (A), (B), and (D) are presented as mean ± standard deviation (n=3).


(A)
Tracer
Uptake
RS 4;11 Tracer Uptake
^{13}C Enrichment (%)
100
80
60
40
20
0
M+5
M+4
M+3
M+2
M+1
M+0
$^{13}C_4$-Asn
$^{13}C_4$-Asp
$^{13}C_5$-Gln
$^{13}C_5$-Glu
Tracer
(B)
NALM-6 Tracer Uptake
^{13}C Enrichment (%)
M+5
M+4
M+3
M+2
M+1
M+0
$^{13}C_4$-Asn
$^{13}C_4$-Asp
$^{13}C_5$-Gln
$^{13}C_5$-Glu
Tracer
(C)
$^{13}C_5$-Gln
TCA
$^{13}C_5$-Gln → RS4;11
^{13}C Enrichment (%)
M+6
M+5
M+4
M+3
M+2
M+1
M+0
α-KG
citrate
isocitrate
fumarate
malate
succinate
(D)
$^{13}C_5$-Gln → NALM-6
^{13}C Enrichment (%)
M+6
M+5
M+4
M+3
M+2
M+1
M+0
α-KG
citrate
isocitrate
fumarate
malate
succinate
(E)
$^{13}C_5$-Gln
Glu
Asp
$^{13}C_5$-Gln
RS 4;11
NALM-6
^{13}C Enrichment (%)
M+5
M+4
M+3
M+2
M+1
M+0
$^{13}C_4$-Asp
$^{13}C_4$-Glu
$^{13}C_5$-Asp
$^{13}C_5$-Glu


(A)
$^{13}C_5$-Gln
ASNS
Asn
Asn
Tracer ($^{13}C_5$-Gln)
^{13}C-Asn Enrichment (%)
M+4
M+3
M+2
M+1
M+0
RS 4;11
NALM-6
(B)
M (Kd)
RS4;11_LUC
RS4:11_ASNS
NALM-6
66-
40-
ASNS
β-actin
(C)
$^{13}C_5$-Gln
$^{13}C_4$-Asn
$^{13}C_4$-Asp
$^{13}C_5$-Gln
$^{13}C_5$-Glu
^{13}C Enrichment (%)
M+5
M+4
M+3
M+2
M+1
M+0
RS 4;11_LUC
RS 4;11_ASNS
(D)
$^{13}C_5$-Gln
α-KG
Citrate
Isocitrate
Fumarate
Malate
Succinate
^{13}C Enrichment (%)
M+6
M+5
M+4
M+3
M+2
M+1
M+0
RS 4;11_LUC
RS 4;11_ASNS
(E)
$^{13}C_5$-Gln
nucleotides
$^{13}C_5$-Gln
Purine
Pyrimidine
AMP
ADP
ATP
GMP
GDP
GTP
CMP
CDP
CTP
UMP
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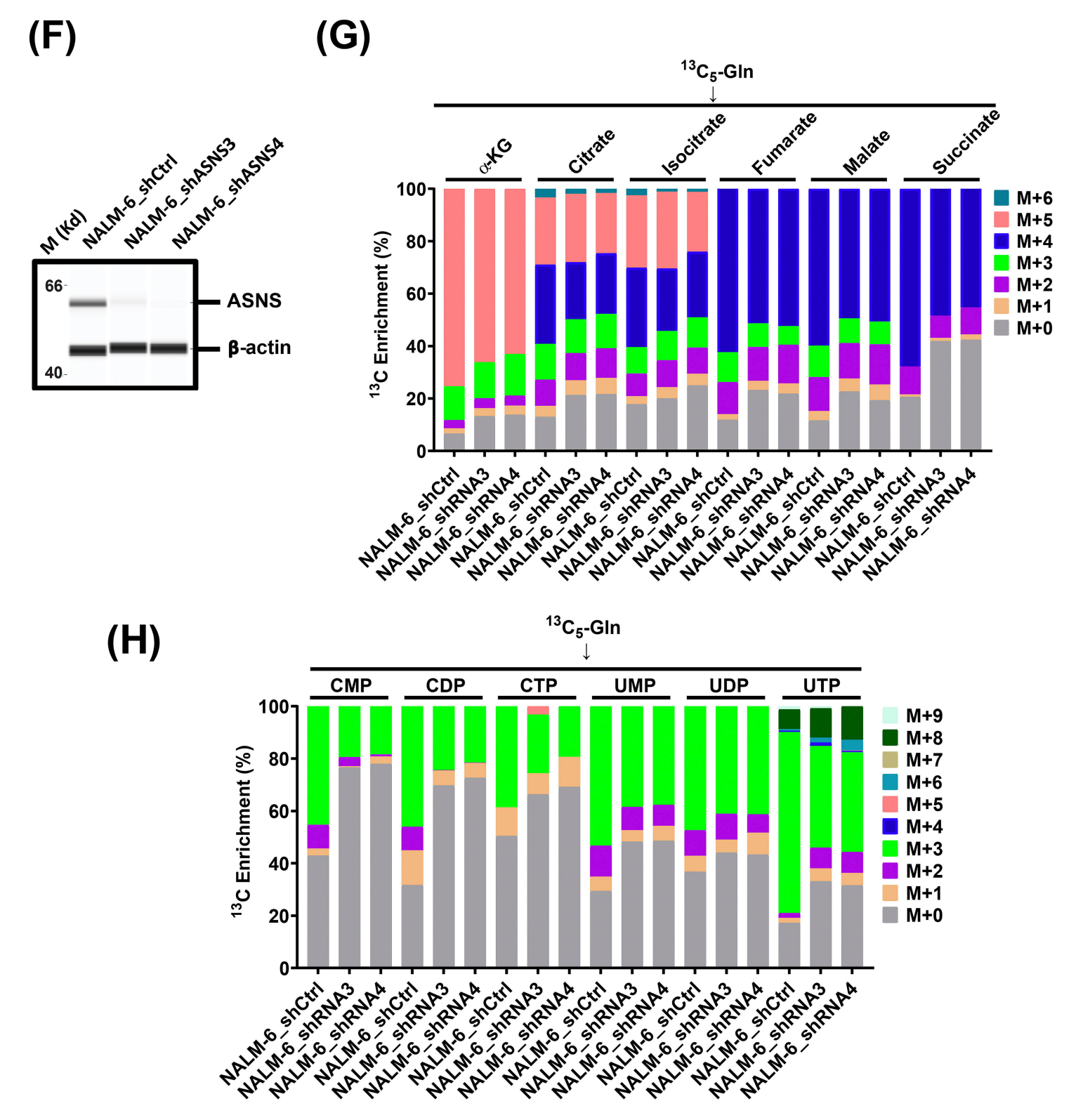
RS 4;11_LUC
RS 4;11_ASNS


(F)
M (Kd)
NALM-6_shCtrl
NALM-6_shASNS3
NALM-6_shASNS4
66
40
ASNS
β-actin
(G)
$^{13}C_5$-Gln
α-KG
Citrate
Isocitrate
Fumarate
Malate
Succinate
^{13}C Enrichment (%)
M+6
M+5
M+4
M+3
M+2
M+1
M+0
NALM-6_shCtrl
NALM-6_shRNA3
NALM-6_shRNA4
(H)
$^{13}C_5$-Gln
CMP
CDP
CTP
UMP
UDP
UTP
^{13}C Enrichment (%)
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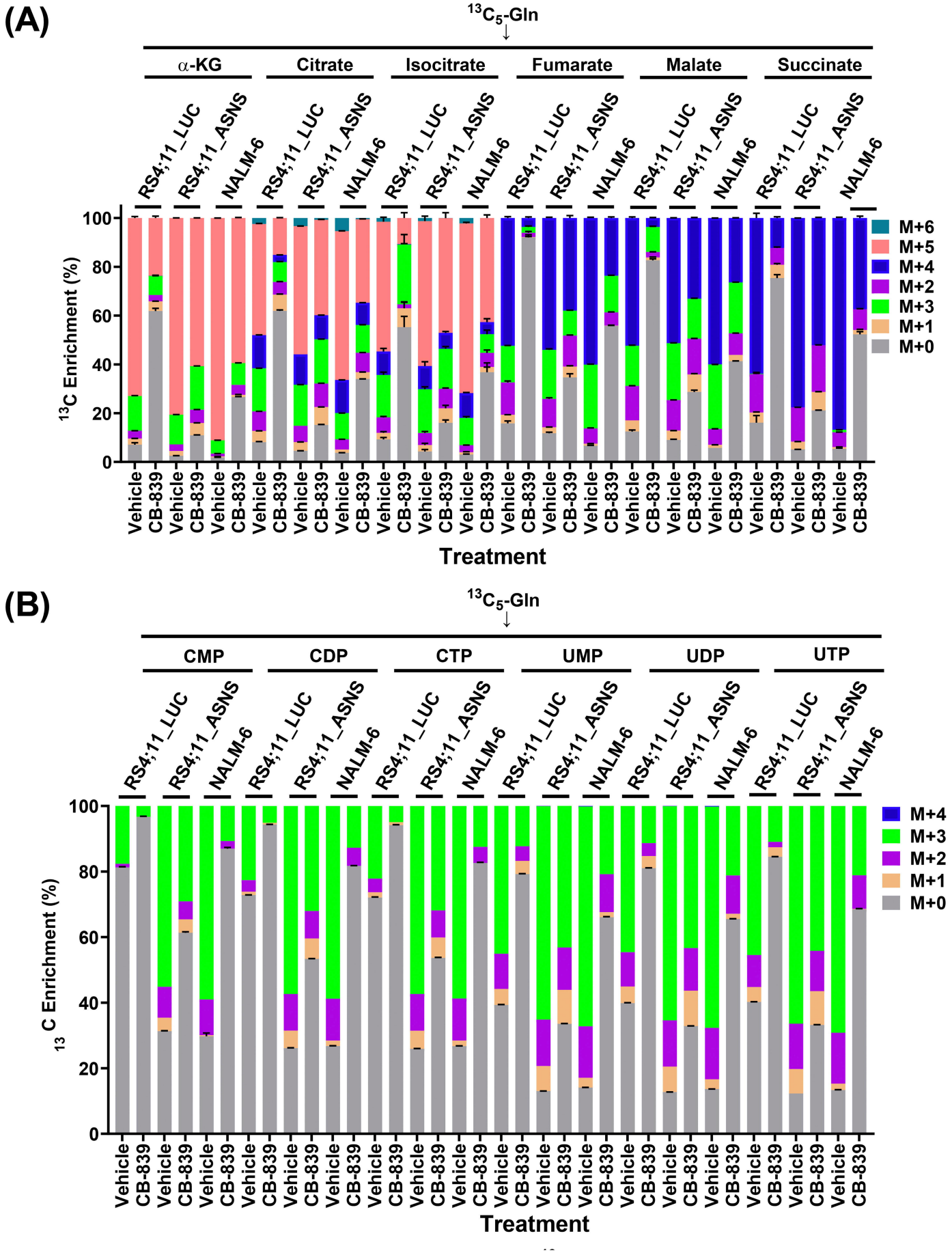
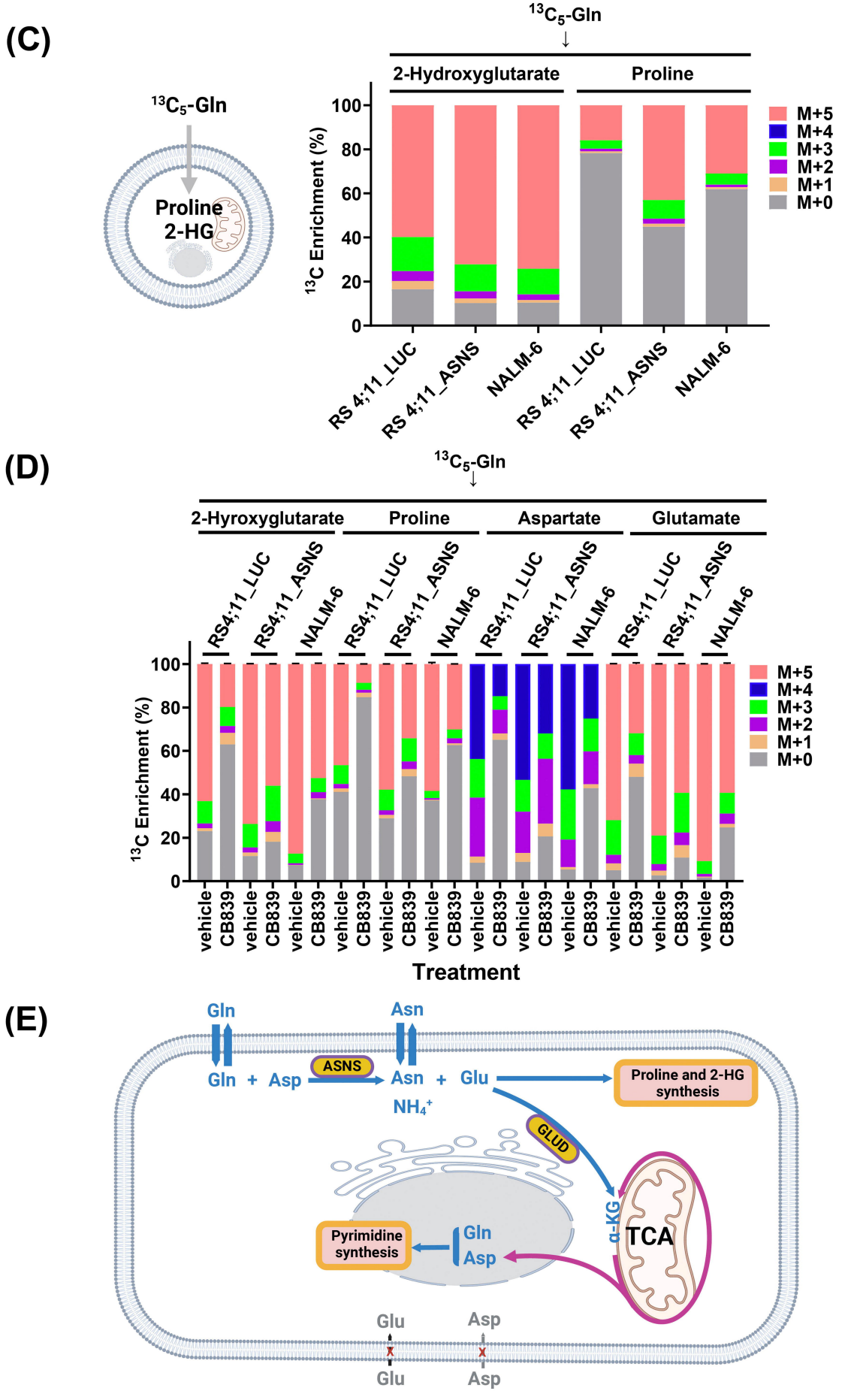
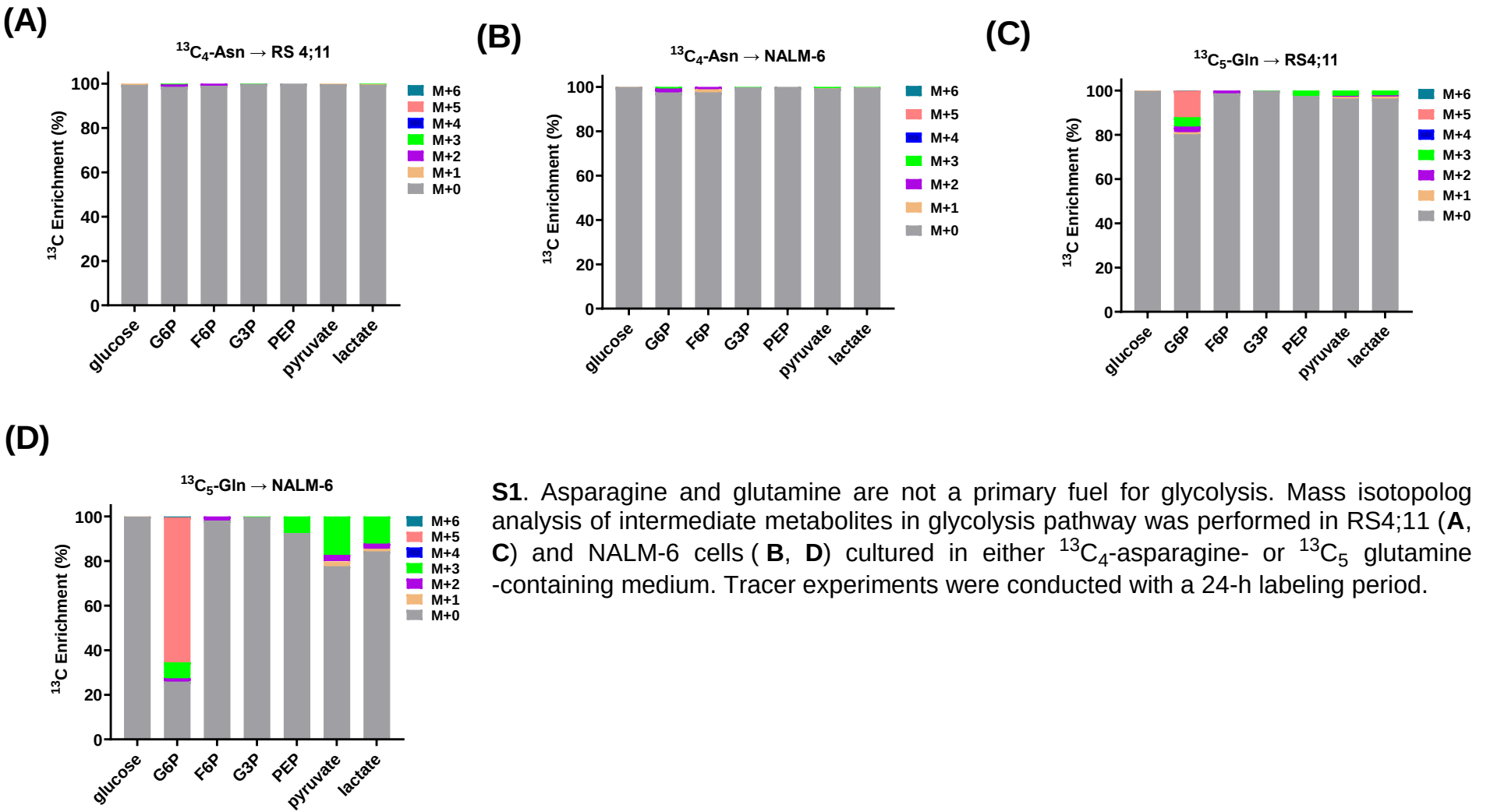
M+8
M+7
M+6
M+5
M+4
M+3
M+2
M+1
M+0


(A)
13C5-Gln
α-KG
Citrate
Isocitrate
Fumarate
Malate
Succinate
RS4;11_LUC
RS4;11_ASNS
NALM-6
13C Enrichment (%)
M+6
M+5
M+4
M+2
M+3
M+1
M+0
Vehicle
CB-839
Treatment
(B)
13C5-Gln
CMP
CDP
CTP
UMP
UDP
UTP
RS4;11_LUC
RS4;11_ASNS
NALM-6
13C Enrichment (%)
M+4
M+3
M+2
M+1
M+0
Vehicle
CB-839
Treatment


(C)
$^{13}C_5$-Gln
Proline
2-HG
$^{13}C_5$-Gln
2-Hydroxyglutarate
Proline
^{13}C Enrichment (%)
M+5
M+4
M+3
M+2
M+1
M+0
RS 4;11_LUC
RS 4;11_ASNS
NALM-6
(D)
$^{13}C_5$-Gln
2-Hyroxyglutarate
Proline
Aspartate
Glutamate
^{13}C Enrichment (%)
vehicle
CB839
Treatment
(E)
Gln
Asn
Gln + Asp
ASNS
Asn + Glu
NH_4^+
Proline and 2-HG synthesis
GLUD
α-KG
TCA
Pyrimidine synthesis
Gln
Asp
Glu
Asp

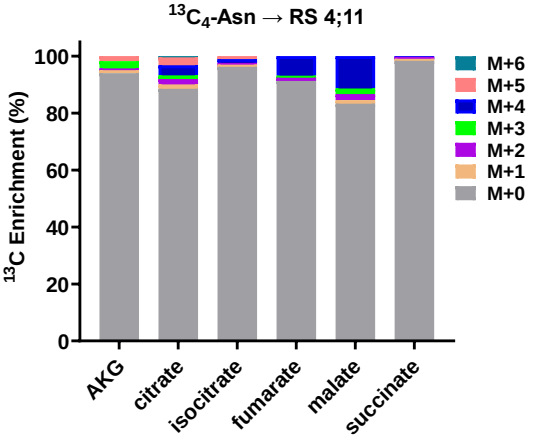
S1



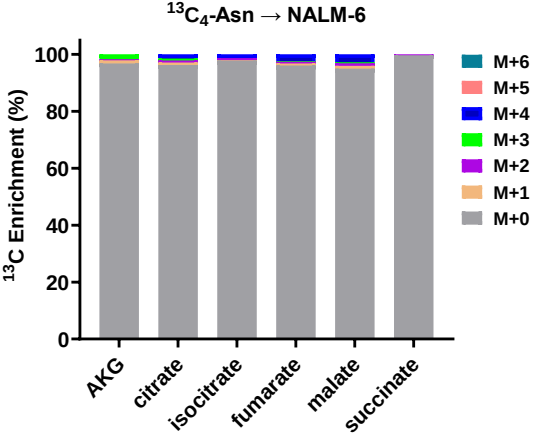
S1. Asparagine and glutamine are not a primary fuel for glycolysis. Mass isotopolog analysis of intermediate metabolites in glycolysis pathway was performed in RS4;11 (**A**, **C**) and NALM-6 cells (**B**, **D**) cultured in either $^{13}C_4$-asparagine- or $^{13}C_5$ glutamine -containing medium. Tracer experiments were conducted with a 24-h labeling period.

S2

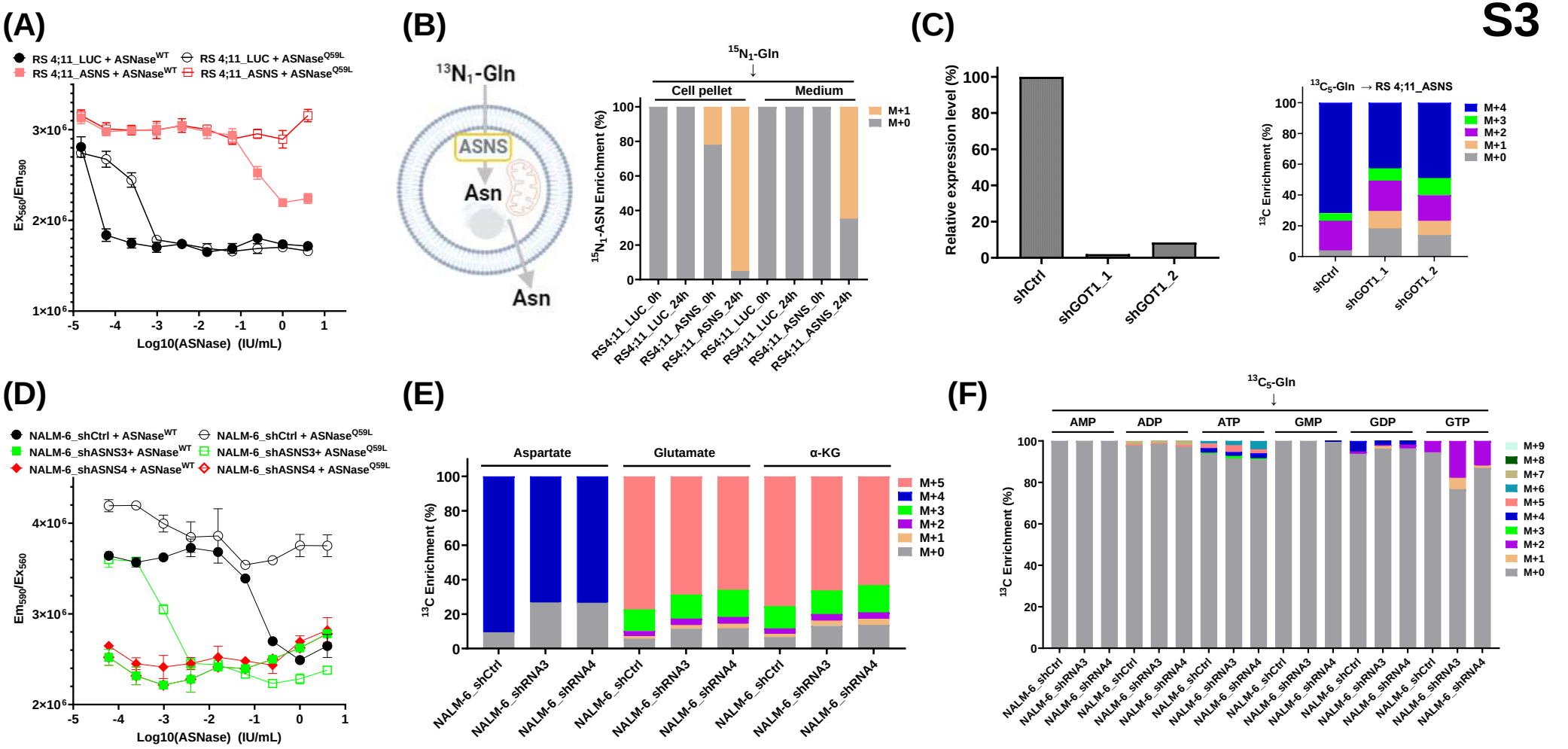
(A)



(B)



S2. Asparagine is not a primary fuel for TCA cycel. (**A**, **B**) Mass isotopolog analysis of intermediate metabolites in TCA cycle was performed in RS4;11 (**A**) and NALM-6 cells (**B**) cultured in $^{13}C_4$-asparagine-containing medium. Tracer experiments were conducted with a 24-h labeling period.



S3. ASNS-overexpressing RS 4:11 cell line and ASNS-knockdown NALM-6 cell lines. (**A**) RS4;11_LUC and RS4;11_ASNS cells in **Figure 2B** were seeded in 96-well plates and incubated overnight, then treated with indicated concentrations of E coli ASNase^{WT} or ASNase^{Q59L} mutant for 72 hours. Cell viability was assayed with CellTiter-Blue using fluorescence excitation at 560 nm and emission at 590 nm. (**B**) RS4;11 cells expressing ASNS (RS4;11_ASNS) but not the control cells expressing luciferase (RS4;11_LUC) efficiently synthesize ASN using $^{15}N_1$-GLN as a nitrogen donor. Cell pellet and media samples were collected at the indicated times. Time zero measurements were made on samples collected immediately after the tracer was administered to the cells. (**C**) Depletion of GOT1 decreases conversion efficiency from $^{13}C_5$-glutamine to aspartate. Left panel: To knock down GOT1 expression, RS 4;11_ASNS cells were infected with shGOT1 lentiviral supernatants for 48 hours, followed by selection with puromycin at a final concentration of 1 µg/mL for 3 days. Total RNA was extracted from the cell pellets. RNA was used for reverse transcription to cDNA, and the resulting cDNA was then used for digital PCR to quantify the relative mRNA expression levels of GOT1 normalized to β-actin, utilizing the QuantStudio™ Absolute Q™ Digital PCR System. Right panel: Mass isotopolog analysis of aspartate was performed in the cells in (**A**) after puromycin selection. $^{13}C_5$-glutamine was used as a tracer.(**D**) CellTiter-Blue assay was performed on the cells in **Figure 2F** with experiment setup as in panel (**A**). (**E**) Depletion of ASNS decreases conversion efficiency from $^{13}C_5$-glutamine to aspartate, glutamate and α-KG in NALM-6 cells depleted of ASNS, as described in **Figure 2F**. (**F**) ASNS does not modulate purine synthesis. Mass isotopolog analysis of purine nucleotides was performed in indicated NALM-6 cells cultured in $^{13}C_5$-glutamine-containing medium. All tracer experiments were conducted with a 24-h labeling period.