Figure S1

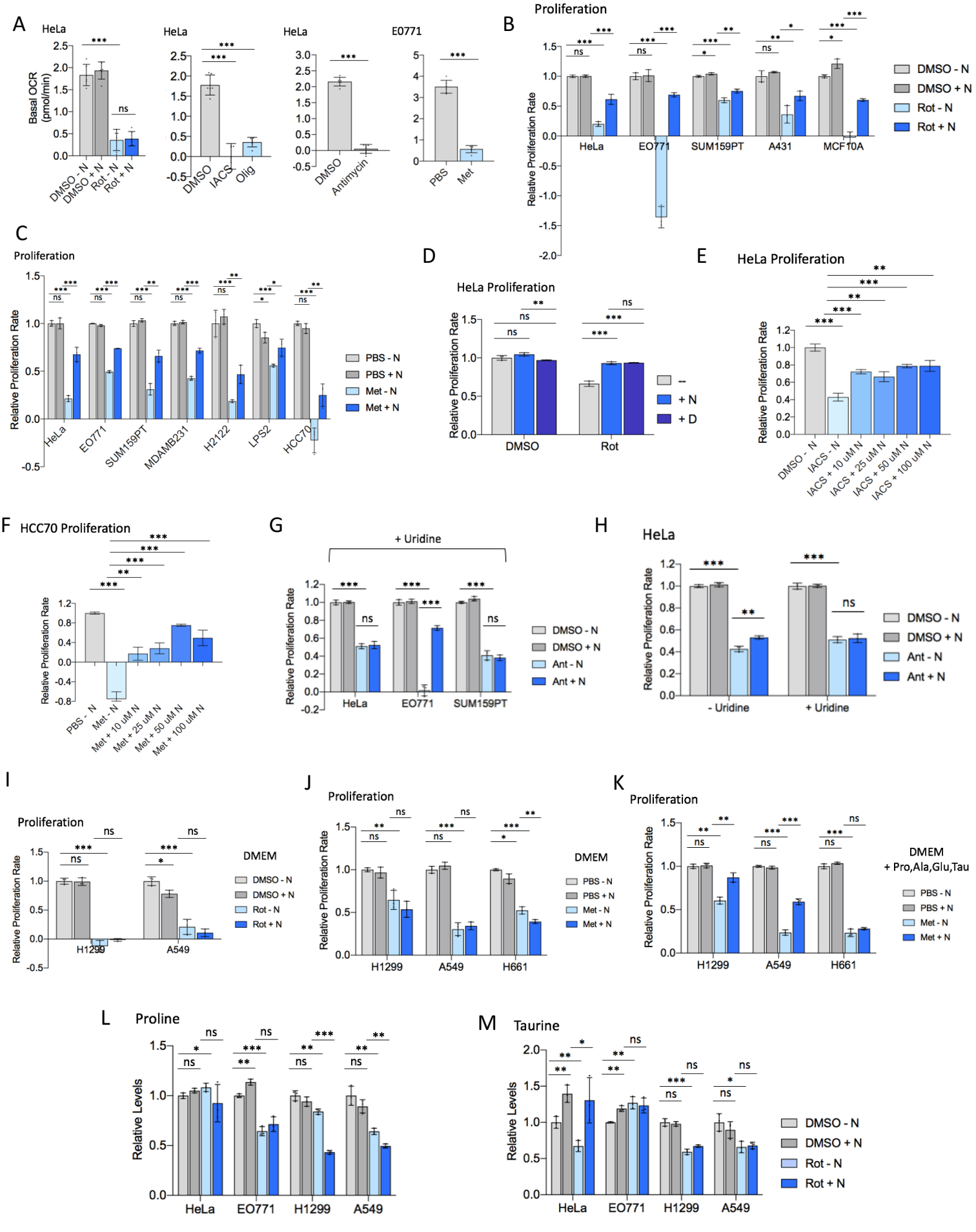

A
HeLa
Basal OCR (pmol/min)
DMSO - N
DMSO + N
Rot - N
Rot + N
HeLa
DMSO
IACS
Olig
HeLa
DMSO
Antimycin
E0771
PBS
Met
B
Proliferation
Relative Proliferation Rate
HeLa
EO771
SUM159PT
A431
MCF10A
DMSO - N
DMSO + N
Rot - N
Rot + N
C
Proliferation
Relative Proliferation Rate
HeLa
EO771
SUM159PT
MDAMB231
H2122
LPS2
HCC70
PBS - N
PBS + N
Met - N
Met + N
D
HeLa Proliferation
Relative Proliferation Rate
DMSO
Rot
– N
+ D
E
HeLa Proliferation
Relative Proliferation Rate
DMSO - N
IACS - N
IACS + 10 uM N
IACS + 25 uM N
IACS + 50 uM N
IACS + 100 uM N
F
HCC70 Proliferation
Relative Proliferation Rate
PBS - N
Met - N
Met + 10 uM N
Met + 25 uM N
Met + 50 uM N
Met + 100 uM N
G
+ Uridine
Relative Proliferation Rate
HeLa
EO771
SUM159PT
DMSO - N
DMSO + N
Ant - N
Ant + N
H
HeLa
Relative Proliferation Rate
- Uridine
+ Uridine
DMSO - N
DMSO + N
Ant - N
Ant + N
I
Proliferation
Relative Proliferation Rate
H1299
A549
DMEM
DMSO - N
DMSO + N
Rot - N
Rot + N
J
Proliferation
Relative Proliferation Rate
H1299
A549
H661
DMEM
PBS - N
PBS + N
Met - N
Met + N
K
Proliferation
Relative Proliferation Rate
H1299
A549
H661
DMEM
+ Pro,Ala,Glu,Tau
PBS - N
PBS + N
Met - N
Met + N
L
Proline
Relative Levels
HeLa
EO771
H1299
A549
M
Taurine
Relative Levels
HeLa
EO771
H1299
A549
DMSO - N
DMSO + N
Rot - N
Rot + N

Figure S1. Asparagine enables proliferation in the context of ETC inhibition. Related to Figure 1.

(A) Basal oxygen consumption rates (OCR) for the indicated cell line in the presence or absence of 0.1 mM exogenous asparagine and the indicated ETC inhibitor at concentrations listed in Supplementary Table I.

(B) Relative proliferation rate of indicated cell lines with rotenone (Rot) or DMSO treatment in the presence or absence of 0.1 mM exogenous asparagine (N).

(C) Relative proliferation rate of indicated cell lines with metformin (Met) or vehicle control (PBS) treatment in the presence or absence of 0.1 mM exogenous asparagine (N).

(D) Relative HeLa cell proliferation rate with rotenone or DMSO treatment in the presence or absence of 0.1 mM exogenous asparagine (N) or 20 mM aspartate.

(E) Relative HeLa cell proliferation rate with IACS-010759 (IACS) or vehicle control (DMSO) treatment in the presence of varying concentrations of asparagine (N).

(F) Relative HCC70 cell proliferation rate with metformin (Met) or vehicle control (PBS) treatment in the presence of varying concentrations of asparagine (N).

(G) Relative proliferation rate of indicated cell lines with DMSO or antimycin A (Ant). Experiments were performed in the presence of 50 ug/ml uridine.

(H) Relative HeLa cell proliferation rate with DMSO or antimycin A (Ant) in the presence or absence of 50 ug/ml uridine.

(I) Relative proliferation rate of indicated cell lines with rotenone (Rot) or DMSO treatment in standard pyruvate-free DMEM in the presence or absence of 0.1 mM exogenous asparagine (N).

(J) Relative proliferation rate of indicated cell lines with metformin (Met) or PBS treatment in standard pyruvate-free DMEM in the presence or absence of 0.1 mM exogenous asparagine (N).

(K) Relative proliferation rate of indicated cell lines with metformin or PBS treatment in pyruvate-free DMEM supplemented with 0.4 mM proline, 0.5 mM alanine, 0.1 mM glutamate, and 0.15 mM taurine in the presence or absence of 0.1 mM exogenous asparagine (N).

(L-M) Relative levels of intracellular proline (L) and taurine (M) in the indicated cell line 6 hours post-treatment with rotenone or DMSO in standard pyruvate-free DMEM in the presence or absence of 0.1 mM exogenous asparagine (N). Data are mean +/- s.d. (n = 3 independent experiments). P value determined by unpaired two-tailed t-test: *p<0.05; **p<0.01; ***p<0.001; ns, not significant.

Figure S2

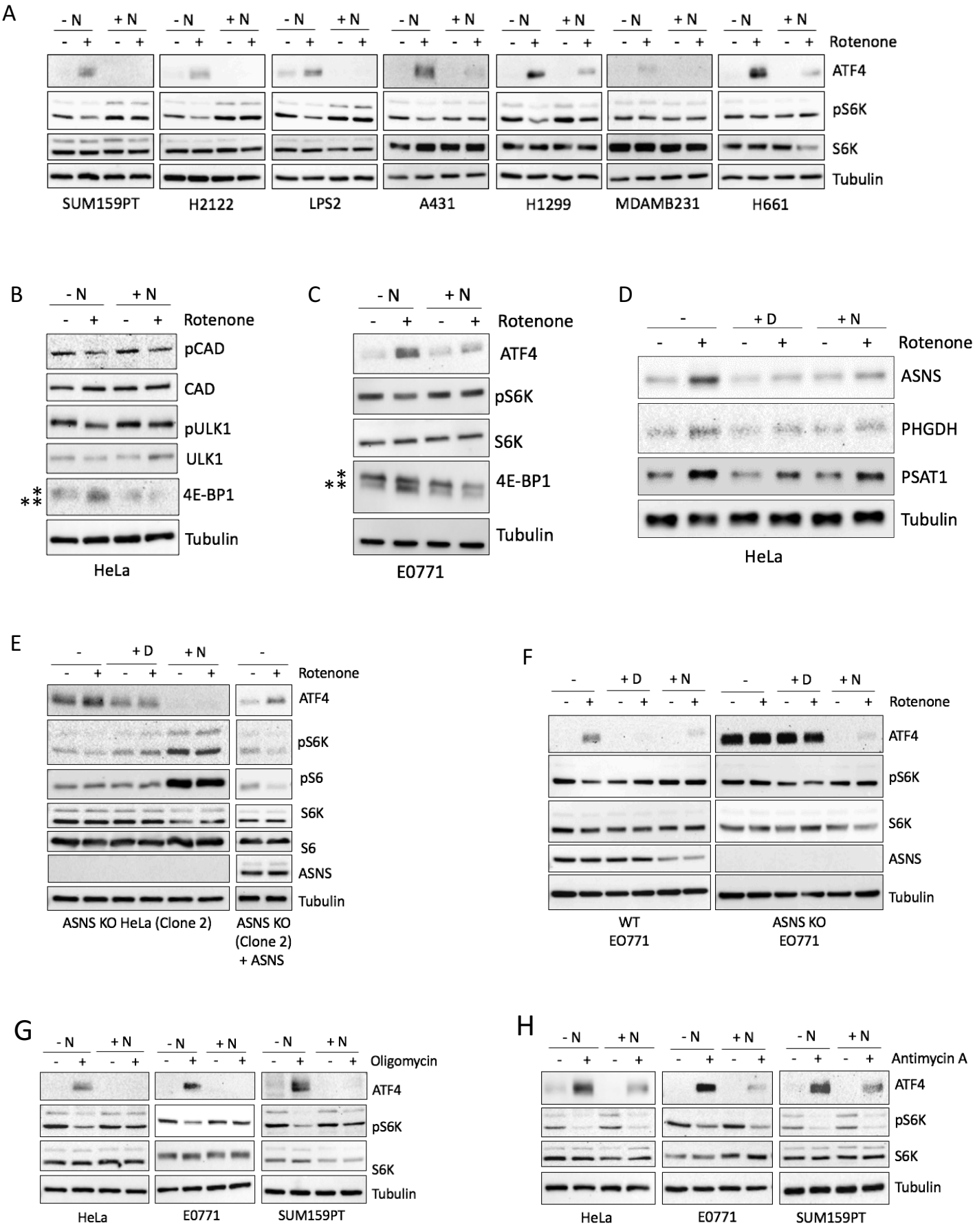

A
- N
+ N
Rotenone
ATF4
pS6K
S6K
Tubulin
SUM159PT
H2122
LPS2
A431
H1299
MDAMB231
H661
B
pCAD
CAD
pULK1
ULK1
4E-BP1
Tubulin
HeLa
C
ATF4
pS6K
S6K
4E-BP1
Tubulin
E0771
D
+ D
ASNS
PHGDH
PSAT1
Tubulin
HeLa
E
pS6
S6
ASNS
ASNS KO HeLa (Clone 2)
ASNS KO (Clone 2) + ASNS
F
WT EO771
ASNS KO EO771
G
Oligomycin
HeLa
E0771
SUM159PT
H
Antimycin A

Figure S2. Asparagine restores ATF4 and mTORC1 activities with ETC inhibition. Related to Figure 3.

(A) Immunoblot of lysates 6 hours post-rotenone (rot) (50 nM) or DMSO treatment. Lysates were immunoblotted for ATF4, mTORC1 activation marker phospho-Thr389 S6K, total S6K, and tubulin.

(B) Immunoblot of HeLa cell lysates 6 hours post-rotenone (rot) (50 nM) or DMSO treatment. Lysates were immunoblotted for phospho-CAD (Ser1859), total CAD, phospho-ULK1 (Ser757), total ULK1, 4E-BP1 (*phosphorylated; **unphosphorylated), and tubulin.

(C) Immunoblot of E0771 cell lysates 6 hours post-rotenone (rot) (50 nM) or DMSO treatment. Lysates were immunoblotted for ATF4, phospho-Thr389 S6K, total S6K, 4E-BP1 (*phosphorylated; **unphosphorylated), and tubulin.

(D) Immunoblot of HeLa cell lysate 48 hours post-rotenone (50 nM) treatment in the presence or absence of 20 mM aspartate (D) or 0.1 mM asparagine (N). Lysates were immunoblotted for ATF4 target genes ASNS, PHGDH, and PSAT1, as well as tubulin.

(E) Left, Immunoblot of HeLa ASNS KO (clone 2; see Figure 3C for ASNS KO clone 1) lysates 6 hours post-treatment with 50 nM rotenone or DMSO in the presence or absence of 20 mM aspartate (D) or 0.1 mM asparagine (N); Right, ASNS was restored in HeLa ASNS KO cells with CMV-driven ectopic expression. Immunoblot shows lysates 6 hours post-treatment with 50 nM rotenone or DMSO in unsupplemented DMEM.

(F) Immunoblot of E0771 WT or ASNS KO lysates 3 hours post-treatment with 50 nM rotenone or DMSO in the presence or absence of 0.1 mM asparagine (N) or 20 mM aspartate (D).

(G-H) Immunoblot of HeLa, E0771, and SUM159PT cell lysate 6 hours post treatment with oligomycin (G) or antimycin A (H) at concentrations listed in Supplementary Table I and in the presence or absence of 0.1 mM asparagine.

Figure S3

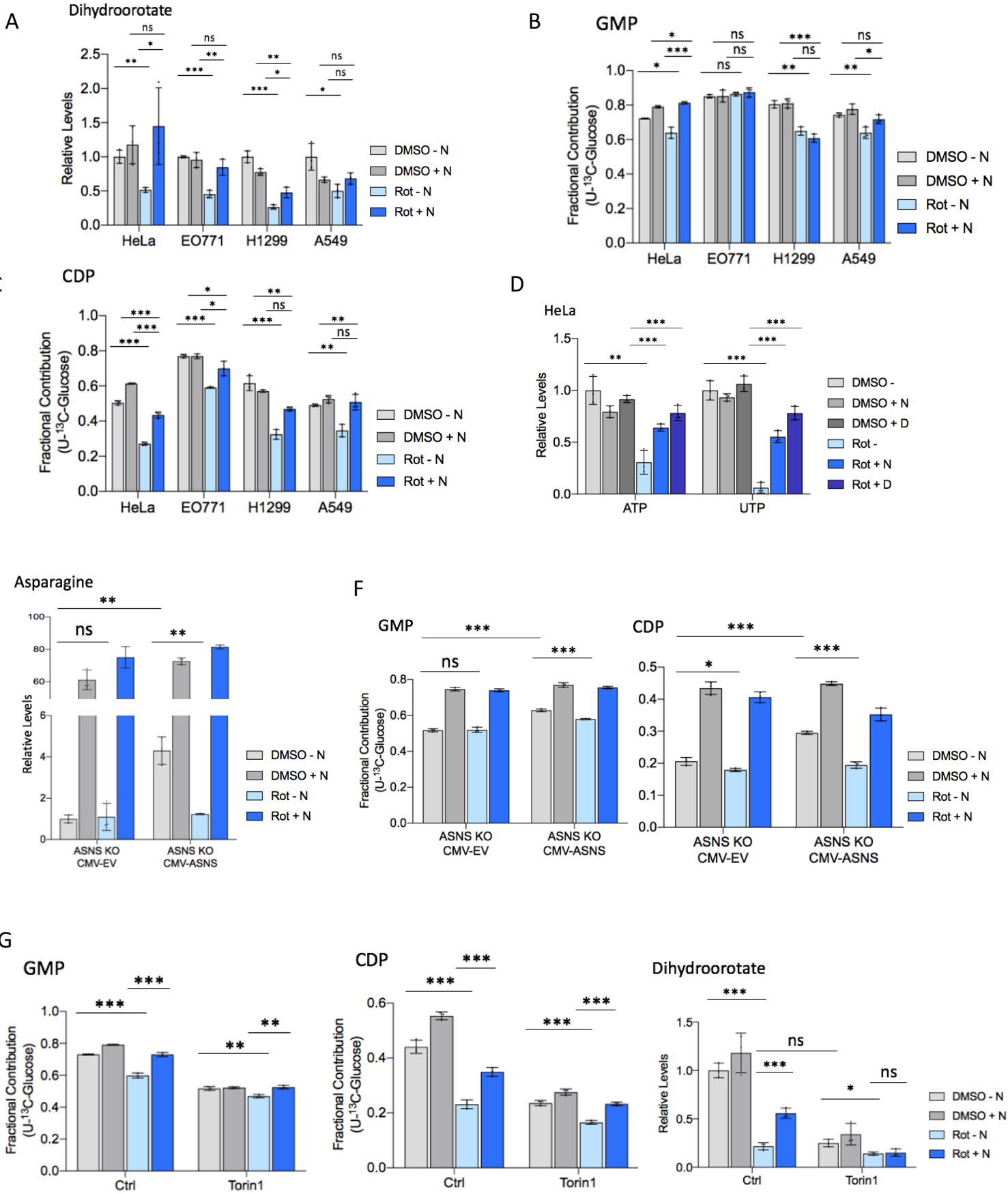

A
Dihydroorotate
Relative Levels
HeLa
EO771
H1299
A549
DMSO - N
DMSO + N
Rot - N
Rot + N
B
GMP
Fractional Contribution (U-13C-Glucose)
C
CDP
D
HeLa
Relative Levels
ATP
UTP
DMSO -
DMSO + D
Rot -
Rot + D
E
Asparagine
ASNS KO CMV-EV
ASNS KO CMV-ASNS
F
GMP
CDP
G
GMP
CDP
Dihydroorotate
Ctrl
Torin1

Figure S3. Asparagine restores nucleotide synthesis in context of ETC inhibition. Related to Figure 4.

(A) Relative levels of pyrimidine precursor dihydroorotate in the indicated cell line 6 hours post-treatment with rotenone or DMSO presence or absence of 0.1 mM exogenous asparagine.

(B-C) Fractional contribution of U-13C-glucose to GMP (B) and CDP (C) in the indicated cell line 6 hours post-treatment with rotenone or DMSO presence or absence of 0.1 mM exogenous asparagine. Medium was replaced with DMEM containing 10 mM U-13C-glucose at the same time as rotenone treatment.

(D) Relative levels of intracellular ATP and UTP in HeLa cells 48 hours post-treatment with 50 nM rotenone or DMSO in the presence or absence of 0.1 mM asparagine (N) or 20 mM aspartate (D).

(E) Relative levels of asparagine in ASNS knockout HeLa cells stably expressing ASNS (CMV-ASNS) or empty vector (CMV-EV) 6 hours post-treatment with rotenone or DMSO presence or absence of 0.1 mM exogenous asparagine, as in B-C.

(F) Fractional contribution of U-13C-glucose to GMP and CDP in ASNS knockout HeLa cells stably expressing ASNS (CMV-ASNS) or empty vector (CMV-EV) 6 hours post-treatment with rotenone or DMSO presence or absence of 0.1 mM exogenous asparagine, as in B-C.

(G) Fractional contribution of U-13C-glucose to GMP and CDP and relative levels of dihydroorotate in HeLa cells 6 hours post-treatment with rotenone or DMSO in the presence or absence of 0.1 mM exogenous asparagine and in the presence of DMSO (Ctrl) or 250 nM Torin1, as in B-C. Data are mean +/- s.d. (n = 3 independent experiments). P value determined by unpaired two-tailed t-test: *$p<0.05$; **$p<0.01$; ***$p<0.001$; ns, not significant.

Figure S4

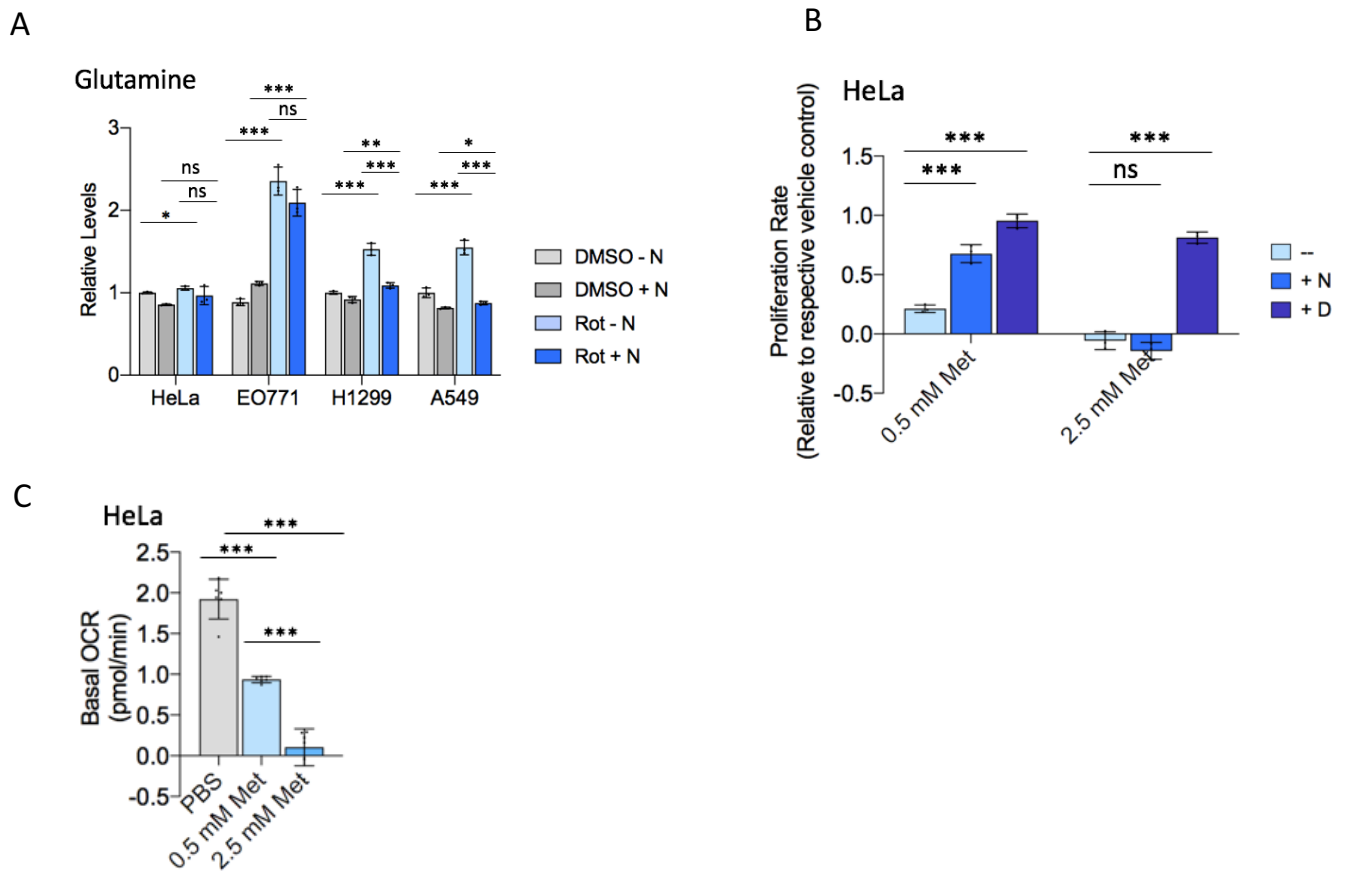


Figure S4. Asparagine versus aspartate auxotrophy with ETC inhibition reflects degree of respiration impairment. Related to Figure 4.

(A) Relative levels of intracellular glutamine in the indicated cell line 6 hours post-treatment with rotenone or DMSO presence or absence of 0.1 mM exogenous asparagine (N).

(B) HeLa cell proliferation rate with either 0.5 or 2.5 mM metformin (Met) treatment in the presence or absence of 0.1 mM exogenous asparagine (N) or 20 mM aspartate, relative to PBS control proliferation in unsupplemented DMEM.

(C) HeLa cell oxygen consumption rate (OCR) 6 hours post-treatment with PBS, 0.5, or 2.5 mM metformin. Data are mean +/- s.d. (n = 3 independent experiments). P value determined by unpaired two-tailed t-test: *p<0.05; **p<0.01; ***p<0.001; ns, not significant.

Figure S5

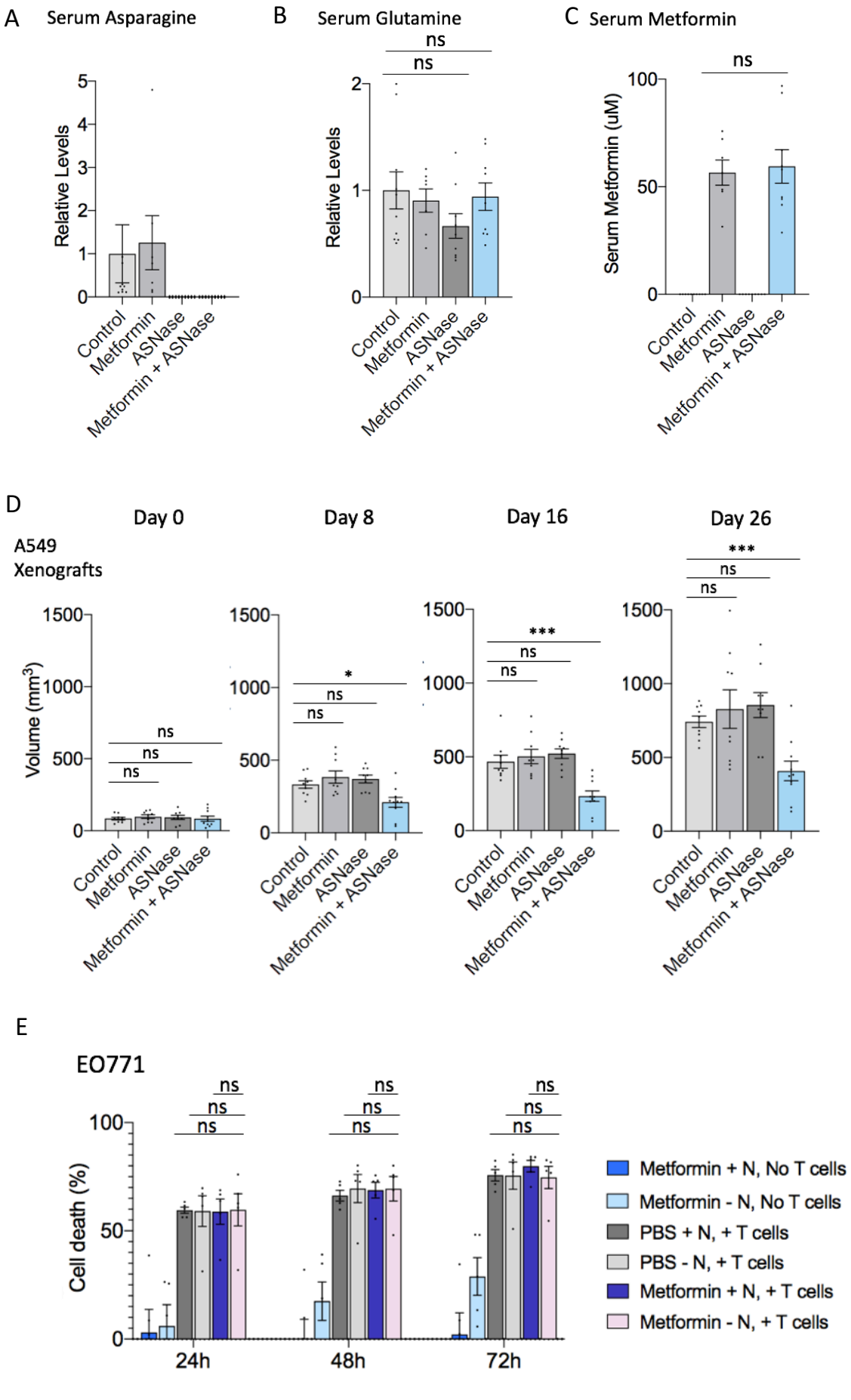

A
Serum Asparagine
Relative Levels
Control
Metformin
ASNase
Metformin + ASNase
B
Serum Glutamine
Relative Levels
ns
C
Serum Metformin
Serum Metformin (uM)
D
Day 0
Day 8
Day 16
Day 26
A549
Xenografts
Volume (mm3)
*
***
E
EO771
Cell death (%)
24h
48h
72h
Metformin + N, No T cells
Metformin - N, No T cells
PBS + N, + T cells
PBS - N, + T cells
Metformin + N, + T cells
Metformin - N, + T cells

Figure S5. Combining metformin with asparaginase inhibits tumor growth. Related to Figure 5.

(A-C) Serum asparagine (A), glutamine (B) and metformin (C) levels from mice treated with metformin, asparaginase (ASNase), the combination, or vehicle controls. n = 7-10.

(D) Tumor volume (mm3) of A549 subcutaneous tumor xenografts at days 0, 8, 16, and 26 post-initiation of treatment with metformin (250 mg/kg/day), asparaginase (ASNase) (5 IU/kg), the combination, or vehicle controls as determined by caliper measurements. n = 9-10.

(E) Cell death of E0771 cells with metformin treatment in the presence of absence of 0.1 mM exogenous asparagine (N) and in the presence (grey and purple bars) or absence (blue bars) of OT1 T cells, n = 5.

Data are mean +/- s.e.; P value determined by unpaired two-tailed t-test: *p<0.05; **p<0.01; ***p<0.001; ns, not significant.

Figure S6

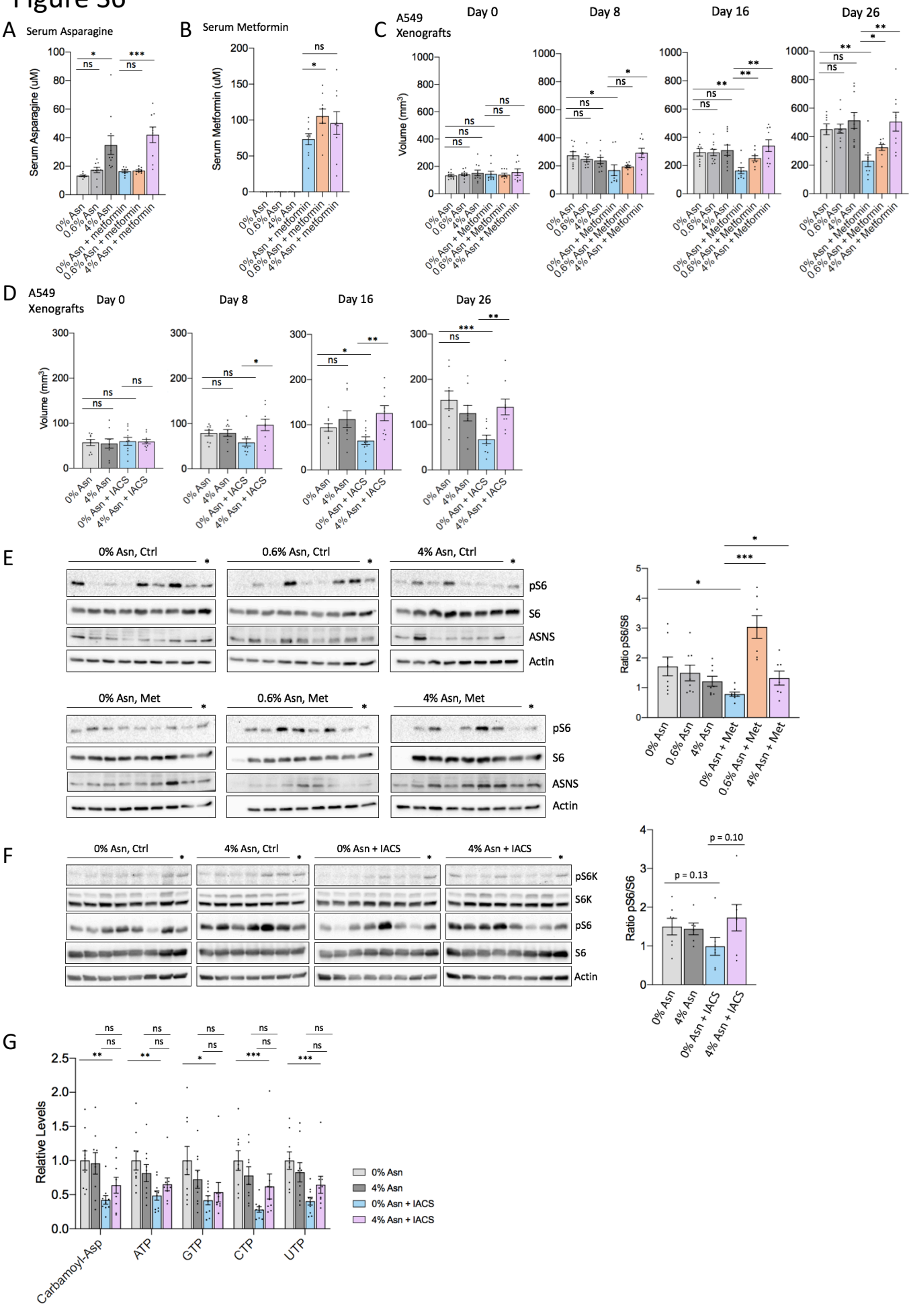

A
Serum Asparagine
Serum Asparagine (uM)
0% Asn
0.6% Asn
4% Asn
0% Asn + metformin
0.6% Asn + metformin
4% Asn + metformin
B
Serum Metformin
Serum Metformin (uM)
C
A549 Xenografts
Day 0
Day 8
Day 16
Day 26
Volume (mm3)
0% Asn + Metformin
0.6% Asn + Metformin
4% Asn + Metformin
D
A549 Xenografts
Day 0
Day 8
Day 16
Day 26
Volume (mm3)
0% Asn + IACS
4% Asn + IACS
E
0% Asn, Ctrl
0.6% Asn, Ctrl
4% Asn, Ctrl
pS6
S6
ASNS
Actin
0% Asn, Met
0.6% Asn, Met
4% Asn, Met
Ratio pS6/S6
0% Asn + Met
0.6% Asn + Met
4% Asn + Met
F
0% Asn, Ctrl
4% Asn, Ctrl
0% Asn + IACS
4% Asn + IACS
pS6K
S6K
pS6
S6
Actin
Ratio pS6/S6
p = 0.10
p = 0.13
G
Relative Levels
Carbamoyl-Asp
ATP
GTP
CTP
UTP
0% Asn
4% Asn
0% Asn + IACS
4% Asn + IACS

Figure S6. Combining metformin with dietary asparagine restriction inhibits tumor growth. Related to Figure 6.

(A-B) Serum asparagine (A) and metformin (B) levels from mice treated with or without metformin and diets containing 0%, 0.6%, or 4% asparagine (Asn).

(C) Tumor volume (mm3) of A549 subcutaneous tumor xenografts at days 0, 8, 16, and 26 post-initiation of treatment with or without metformin and diets containing 0%, 0.6%, or 4% asparagine (Asn).

(D) Tumor volume (mm3) of A549 subcutaneous tumor xenografts at days 0, 8, 16, and 26 post-initiation of treatment with or without IACS-010759 (IACS) and diets containing 0% or 4% asparagine (Asn).

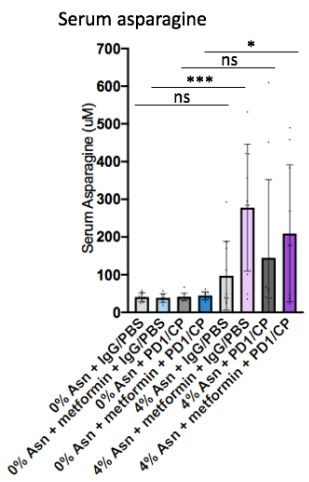
(E) Immunoblot of A549 tumor lysates from mice treated with or without metformin and fed a diet containing 0%, 0.6%, or 4% asparagine. Each lane contained lysate from individual tumors within each treatment group. Lysates were immunoblotted for mTORC1 activation marker phospho-Ser235/6 S6, total S6, ASNS, and actin. The asterisk (*) indicates a reference lysate (taken from the largest of the “4% Asn, Ctrl” group) that was run on each gel. Quantification is shown to the right.

(F) Immunoblot of A549 tumor lysates from mice treated with or without IACS-010759 (IACS) and fed a diet containing 0% or 4% asparagine. Each lane contained lysate from individual tumors within each treatment group. Lysates were immunoblotted for mTORC1 activation marker phospho-Ser235/6 S6, total S6, ASNS, and actin. The asterisk (*) indicates a reference lysate (taken from the largest of the “4% Asn, Ctrl” group) that was run on each gel. Quantification is shown to the right

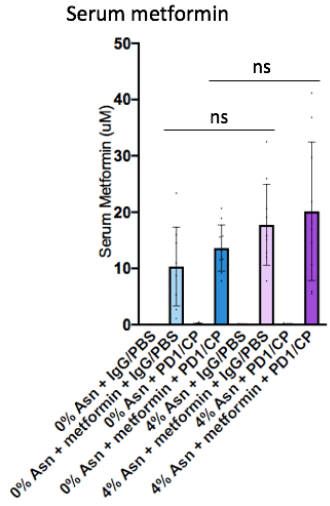
(G) Relative levels of tumor carbamoyl-aspartate and nucleotides at endpoint from mice with or without IACS-010759 (IACS) and fed a diet containing 0% or 4% asparagine. For (A-D) and (G), data are mean +/- s.e. *p<0.05; **p<0.01; ***p<0.001; ns, not significant.

Figure S7

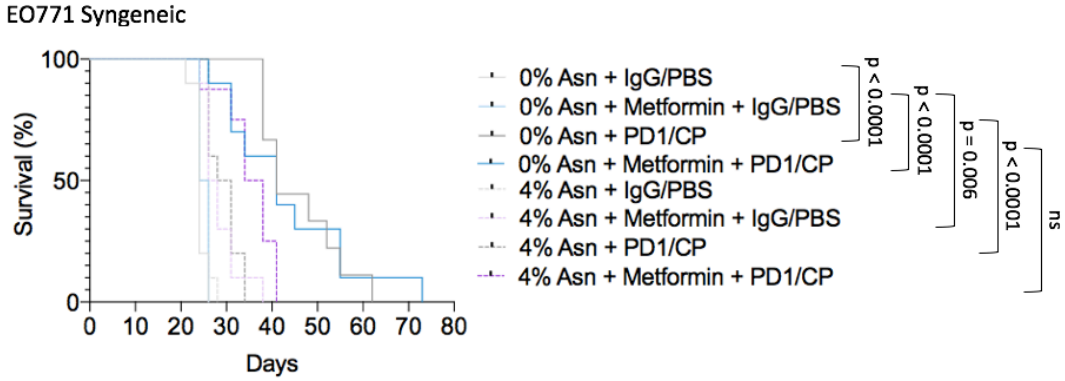
A



B



C



D

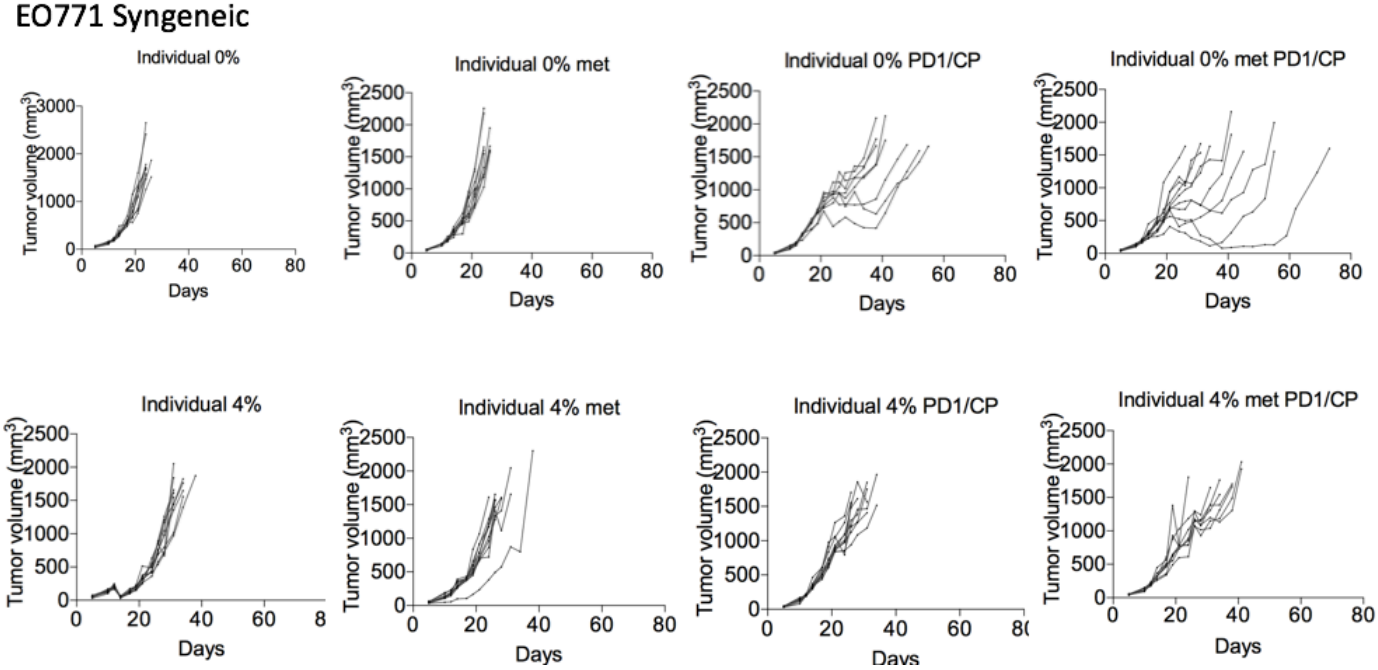


Figure S7. Dietary asparagine restriction synergizes with PD1 inhibition and chemotherapy. Related to Figure 6.

(A-B) Endpoint serum asparagine (A) and metformin (B) from mice harboring syngeneic E0771 mammary tumors and treated with or without metformin and/or a combination of inhibitory PD1 antibody and cyclophosphamide (CP) and fed a diet containing 0% or 4% asparagine (Asn), n = 10.

(C) Kaplan-Meier survival curve indicating percentage of mice with syngeneic E0771 mammary tumors surviving with the indicated treatments, n = 10.

(D) Individual tumor growth curves of E0771 tumors with the indicated treatment. For (A-B) data are mean +/- s.e. For (C), P-value was calculated by Mantel-Cox test. *p<0.05; **p<0.01; ***p<0.001; ns, not significant.

Table S1: Cell line-specific conditions for ETC inhibition. Related to Figure 1.

Cell line	Serum*	Added AAs Required	Metformin (mM)	Rotenone (nM)	IACS-010759 (nM)	Antimycin A (nM)	Oligomycin (nM)
HeLa	Dialyzed FBS	-	0.5	50	25	25	100
EO771	Dialyzed FBS	-	5	50	50	25	100
SUM159PT	Non-dialyzed FBS	-	1	50	10	250	100
MDAMB231	Dialyzed FBS	-	2.5	50			
H2122	Dialyzed FBS	-	0.5	50			
LPS2	Dialyzed FBS	-	0.1	50			
HCC70	Dialyzed FBS	-	2.5				
A431	Dialyzed FBS	-		50			
A549	Dialyzed FBS	Pro, Ala, Glu, Tau	0.5	10			
H1299	Dialyzed FBS	Pro, Ala, Glu, Tau	1	50			
MCF10A	Non-dialyzed HS	-		50			
H661	Dialyzed FBS	-	2.5	50			
HeLa ATF4 KO & CRISPR Ctrl	Non-dialyzed FBS	-		25			

* Dialyzed FBS was used to eliminate asparagine from the culture medium except in cell lines that do not proliferate with dialyzed FBS.