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Supplemental Information

Cytosolic Aspartate Availability Determines Cell Survival When Glutamine Is Limiting

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SUPPLEMENTARY FIGURE LEGENDS

Figure S1 (Related to Figure 1, 2). AGC1 expression supports increased proliferation, NAD+/NADH ratio, and aspartate levels

(A) Schematic showing the canonical Malate-Aspartate Shuttle (MAS).

(B) mRNA expression of MAS components in (left) human cell lines from the Cancer Cell Line Encyclopaedia (CCLE) or (right) in proliferating transformed or non-transformed mouse cell lines determined using qPCR (compared to TfIIb) (n=1).

(C) Proliferation rate of control (NTC) and AGC1 knockdown LLC1 cells (KD1 and KD2). Fold change in viable cell number is presented as percent of non-targeted control (NTC) cells (n=3)

(D) Intracellular NAD+/NADH ratio was determined in pyruvate free DMEM (n=5) for control LLC1 cells (grey) and AGC1-KD1 LLC1 cells (red) described in panel C. Mean ± SEMs are shown.

(E) Pyruvate to lactate ratio was determined for the same cells in panel D grown in pyruvate-free media using GCMS (n=3).

(F) Cellular aspartate and asparagine levels for the same cells in panel D in standard DMEM without pyruvate as measured using GCMS (n=3).

(G) (Top) Proliferation rate of control (C2C12 + Vehicle) and AGC1-overexpressing (C2C12 + mAGC1) C2C12 cells in media without pyruvate. Final cell counts were normalized to initial cell number before transfection (n=3). (Bottom) AGC1 protein expression by Western blot with β-actin expression shown as a loading control.

(H-I) AMP/ATP ratio, NAD+/NADH ratio and Aspartate levels measured in C2C12 cells that (H) transiently overexpressed mouse AGC1 (as in G) or (I) had stable knockdown of AGC1 (n=3).

(J) Aspartate (Asp), asparagine (Asn), alanine (Ala), and glutamate (Glu) levels in C2C12 cells that individually express 2 independent non-targeting shRNA controls (NTC1 and NTC2) or 3 independent shRNAs targeting AGC1 (KD1, KD2 or KD3). n=2 for NTC1, n=1 for all other conditions.

(K) Proliferation/survival rate of control (C2C12 NTC) or AGC1 knockdown cells (C2C12 AGC1-KD1) transfected with empty vector (EV) or with human AGC1 (hAGC1) as indicated. Cells were cultured in vehicle control (DMSO), 1 μM CB-839, or pyruvate-free DMEM media with 0.1mM glutamine (0.1mM Gln) as indicated, and fold change in viable cell number after two days is shown (n=3)

(L) The percent annexin V and/or propidium iodide (PI) positive control (NTC) and AGC1-KD C2C12 and LLC1 cells cultured for 24h in 4mM glutamine or 0.1mM glutamine as indicated was determined by flow cytometry as shown (n=3).

(M) Cleaved caspase 3 protein expression as determined by Western Blot in control (N) or AGC1-knockdown (A) C2C12 and LLC1 cells cultured for 24h in the presence of 4mM glutamine or 0.1mM glutamine without or with 10mM aspartate as indicated is shown. β-actin expression is also shown as a loading control. Data are representative of n=3 experiments.

All panels show mean ± SD unless indicated otherwise. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure S2 (Related to Figure 3). Glucose and glutamine utilization is minimally affected by AGC1 loss

(A) Relative labeled(^{13}C)/unlabeled(^{12}C) aspartate (Asp) ratio of control (NTC) or AGC1-knockdown (AGC1-KD) C2C12 or LLC1 cells cultured in media with 5mM [U^{13}C]glucose (Glc) or 2mM [U^{13}C]glutamine (Gln) for 12 hours as determined by NMR spectroscopy (n=5).

(B) Labeled (^{13}C) glucose (Glc) uptake and lactate (Lac) release by control (NTC) or AGC1-knockdown (AGC1-KD) C2C12 or LLC1 cells after culture for 12 hours in media with 5mM [U^{13}C]glucose as determined by NMR spectroscopy (n=5).

(C) Labeled (^{13}C) glutamine (Gln) uptake and glutamate (Glu) release by control (NTC) or AGC1-knockdown (AGC1-KD) C2C12 or LLC1 cells after culture for 12 hours in media with 2mM [U^{13}C]glutamine as determined by NMR spectroscopy (n=5).

(D) Schematic showing how Erastin inhibits glutamate export.

(E) Proliferation rate of control (NTC) or AGC1-knockdown (KD1) C2C12 cells cultured in 1μM CB-839 and with varying concentrations of Erastin (n=1).

(F-G) Steady state labelling of the indicated metabolites when (F) C2C12 or (G) LLC1 cells without (+) or with (-) AGC1 knockdown are cultured for 24 hours in the presence of [U^{13}C]glutamine or [U^{13}C]glucose as noted (aspartate (Asp), citrate (Cit), glutamate (Glu), alpha-ketoglutarate (a-KG), malate (Mal), and succinate (Suc)) (n=3).

(H, K) Heatmap showing relative total pool sizes of the indicated metabolites when control (NTC) or AGC1-knockdown (KD) (H) C2C12 and (K) LLC1 cells are cultured in full DMEM-Pyr media for 24h. Data were normalized to cell number and the median relative change compared to NTC are shown (n=3).

(I, L) Heatmap showing relative levels of the indicated metabolite that increase in media after control (NTC) or AGC1-knockdown (I) C2C12 and (L) LLC1 cells are cultured in full DMEM-Pyr for 24 hours. Data normalized to cell number; median relative changes compared to NTC are shown (n=3).

(J, M) Heatmap showing relative levels of the indicated metabolites that decreased in media when control (NTC) or AGC1-knockdown (J) C2C12 and (M) LLC1 cells are cultured in full DMEM-Pyr for 24 hours. Data normalized to cell number; median relative changes compared to NTC are shown (n=3).

All panels show mean ± SEM unless indicated otherwise.

Figure S3 (Related to Figure 4). Cytosolic aspartate delivery improves proliferation/survival when glutamine is limiting

(A) Survival/proliferation rate of control (NTC) and AGC1-knockdown (AGC1-KD) C2C12 cells cultured in pyruvate-free DMEM containing 0.1mM glutamine in the presence and absence of 5mM aspartate as indicated (n=3) mean ± SEM.

(B) Survival/proliferation rate of control (NTC) and ACG1-KD C2C12 cells expressing the plasma membrane aspartate transporter SLC1A3 cultured in pyruvate-free DMEM containing 0.1mM glutamine in the presence and absence of 0.15mM aspartate as indicated (n=3).

(C) (Left) AGC1 protein expression as determined by Western blot (with Vinculin expression as a loading control). (Center) survival/proliferation rate of control (EV, black bars) and CRISPR/Cas9-mediated AGC1 deleted (AGC1 KO, pink bars) C2C12 cells cultured in 4mM glutamine (Gln) with or without 1μM CB-839, or 0.1mM glutamine, with or without 20mM aspartate (Asp) as indicated. (Right) GCMS-measured aspartate levels and pyruvate/lactate (Pyr/Lac) ratio of control (EV) and CRISPR/Cas9-mediated AGC1 deleted C2C12 cells as indicated (n=3).

(D) Schematic showing of the compartment-specific impact of CB-839 on cytosolic or mitochondrial glutamate production and the TCA cycle.

(E) Survival/proliferation rate of control (NTC) and AGC1-KD C2C12 cells cultured in pyruvate-free DMEM containing 4mM or 0.1mM glutamine (Gln) in the presence or absence of 2.5mM sodium pyruvate (Pyr), 5mM aspartate (Asp), 5mM glutamate (Glu), or 2.5mM alanine (Ala) as indicated (n=3).

All panels show mean ± SDs unless indicated otherwise.

Figure S4 (Related to Figure 5). Levels of TCA metabolites and non-essential amino acids in glutamine limiting conditions and following rescue with different substrates

A) GCMS analysis of TCA cycle intermediates and non-essential amino acids (normalized to cell number) from control (N) and AGC1-knockdown (A) C2C12 or LLC1 cells cultured in 4mM glutamine (4mM Q) or 0.1mM glutamine (0.1mM Q) or 4mM glutamine (4mM Q) with 1μM CB-839 for 24h in the presence and absence of 20mM aspartate (Asp), 2mM dimethylaKG (daKG), 2mM dimethlymalate (dMal), or 2mM sodium pyruvate (Pyr) as indicated. Levels shown are relative to control cells cultured in 4mM glutamine (Q) (n=3, means are shown).

B) GCMS analysis of TCA cycle intermediates and non-essential amino acids (normalized to cell number) from control (N) and AGC1-knockdown (A) C2C12 or LLC1 cells cultured with 0.1mM glutamine (0.1mM Q) or with 1μM CB-839 for 24h in the presence and absence of 20mM aspartate (Asp), 2mM dimethylaKG (daKG), 2mM dimethlymalate (dMal), or 2mM sodium pyruvate (Pyr) as indicated. Levels shown are relative to control cells cultured in 0.1mM glutamine or +1μM CB-839 (n=3, means are shown).

C) GCMS analysis of relative aspartate levels (normalized to cell number) from control (NTC) and AGC1-knockdown (AGC1KD1) C2C12 or LLC cells cultured in 4mM glutamine (Q), with 0.1mM glutamine, or with 1μM CB-839 for 24h in the presence and absence of 20mM aspartate (Asp), 2mM dimethylaKG (daKG), or 2mM sodium pyruvate (Pyr) as indicated. (n=3, means are shown).

D) GCMS analysis of relative asparagine levels (normalized to cell number) from control (NTC) and AGC1-knockdown (AGC1KD1) C2C12 or LLC1 cells cultured in 4mM glutamine (Q), with 0.1mM glutamine, or with 1μM CB-839 for 24h in the

presence and absence of 20mM aspartate (Asp), 2mM dimethylaKG (daKG), or 2mM sodium pyruvate (Pyr) as indicated. (n=3, means are shown).

Figure S5 (Related to Figure 6). Cytosolic aspartate is limiting for nucleotide biosynthesis in glutamine-limited conditions

(A) Schematic showing of N-acetlyaspartate synthesis via the enzyme NAT8L.

(B) GCMS analysis of endogenous intracellular N-acetylaspartate (NAA) in control (NTC) and AGC1-knockdown (AGC1KD1) C2C12 and LLC1 cells as indicated. NAA levels in LLC1 cells engineered to express NAT8L to produce NAA is shown as a control. (nd. = not detected) (n=3)

(C) mRNA expressions of genes involved in amino acid metabolism from control (+AGC1) or AGC1-KD (-AGC1) C2C12 or LLC1 cells cultured for 24h in 4mM glutamine (+Gln) or 0.1mM Gln (-Gln) as indicated. Shown is the median fold change in expression of the indicated gene compared to expression in control (NTC) cells cultured in 4mM glutamine (n=3).

(D) Schematic showing how aspartate undergoes transaminations (Got1/2) to support amino acid biosynthesis, provide carbon to the TCA cycle, or oxidize NADH. To produce aspartate, TCA substrates or amino acids also require transamination. Transamination to produce or consume aspartate is inhibited by AOA or by knockdown of aspartate transaminases (siGot1/2).

(E) Proliferation/survival rates of control (NTC) and AGC1-KD C2C12 cells cultured in media with 0.1mM glutamine (Gln) in the presence or absence of 20mM aspartate (Asp), 1mM or 0.1mM of a mixture of non-essential amino acids containing serine, glycine, alanine, aspartate, asparagine, proline, and glutamate (NEAA), 2mM dimethylalpha-ketoglutarate (daKG), with or without 0.3mM AOA as indicated is shown in the left two panels. Shown in the right panel is proliferation/survival rates of

control (NTC) or AGC1-KD C2C12 cells, cultured in 0.1mM glutamine in the presence or absence of 20mM aspartate (Asp), 2mM dimethylalpha-ketoglutarate (daKG), or 2mM sodium pyruvate (Pyr), with or without control siRNA or siRNA targeting Got1 and Got2 (siGot1/2) as indicated. (n=3)

(F) Proliferation/survival rates of control (NTC) and AGC1-KD LLC1 and AL1376 cells cultured in media with 4mM glutamine with the indicated concentration of CB-839 and a 0.1mM mixture of non-essential amino acids, in the presence or absence of 20mM aspartate, 2mM dimethylalpha-ketoglutarate (daKG), 2mM sodium pyruvate with or without 0.3mM AOA (for LLC1 cells) or 1mM AOA (for AL1376 cells) as indicated. (n=3)

(G) Proliferation/survival rate of control (NTC) or ACG1-knockdown (AGC1KD) C2C12 cells cultured in media without (DMSO) or with 1μM CB-839, in the absence or presence of 10mM aspartate (Asp), 10mM asparagine (Asn), or 4mM of both serine and glycine (Ser/Gly) as indicated. (n=5)

(H) (Left) Tracing map showing the relationship between labeled aspartate carbon and the labeling of TCA cycle intermediates. (Right) Relative enrichment of the most abundant TCA intermediate isotopomers that were found to be labeled from [U-^{13}C]aspartate when control (NTC) or AGC1-knockdown (AGC1KD1) C2C12 cells expressing the aspartate/glutamate transporter SLC1A3 were cultured in media containing [U-^{13}C]aspartate and 0.1mM glutamine. Data shown for each species were normalized to enrichment of M+4 aspartate (n=3)

(I) (Left) Tracing map showing the relationship between labeled glutamate carbon and the labeling of TCA cycle intermediates. (Right) Relative enrichment of the most abundant TCA intermediate isotopomers that were found to be labeled from [U-^{13}C]glutamate when control (NTC) or AGC1-knockdown (AGC1KD1) C2C12 cells expressing the aspartate/glutamate transporter SLC1A3 were cultured in media containing [U-

^{13}C]glutamate and 0.1mM glutamine. Data shown for each species were normalized to enrichment of M+5 glutamate (n=3)

(J) Schematic showing how Aspartate contributes to both purine and pyrimidine biosynthesis. R5P, ribose-5-phosphate; Asp, asparate; Fum, fumarate; IMP, inosine monophosphate.

(K) Proliferation/survival rates of control (NTC) and AGC1-KD LLC1 and AL1376 cells cultured in 4mM glutamine with 1μM (LLC1) or 5μM (AL1376) CB-839 in the presence and absence of a mixture of nucleotide precursors (200μM hypoxanthine, 200μM adenine, 200μM guanine, 100μM thymine and 400μM uridine) as indicated. (n=3).

All figures denote mean ± SDs unless indicated otherwise.

Figure S6 (Related to Figure 7). AGC1 knockdown reduces LLC1 allograft tumor growth

(A) Tumor volume over time of allografts generated from control (NTC) and two independent AGC1-knockdown (AGC1-KD1 and AGC1-KD2) LLC1 cell lines implanted into the flanks of C56BL/6 mice (n≥7).

(B) Tumor weights of the LLC1 allografts described in A at the experiment endpoint (day17).

(C) Tumor volume over time of allografts generated from control (NTC) and AGC1-knockdown (AGC1KD1) LLC1 cells implanted into the flanks of C56BL/6 mice that are dosed with vehicle or 200mg/kg/twice daily of CB-839 starting on day 0 as indicated (n≥6).

(D) Relative levels of the indicated non-essential amino acids and TCA intermediates (normalized to valine) from the tumors described in C. Data are presented as relative change compared to vehicle treated NTC control (n≥5), medians are shown.

(E) Tumor volume over time of allografts generated from control (NTC) and AGC1-knockdown (AGC1KD1) AL1376 cells implanted into the flanks of C56BL/6 mice that are dosed with vehicle or 200mg/kg/twice daily of CB-839 as indicated (n≥6). Dosing of vehicle or CB-839 was started on day 16 as indicated.

(F) Growth of tumors derived from the AL1376 cells described in E in C56BL/6 mice flanks that are dosed with vehicle or 200mg/kg/twice daily CB-839 as indicated starting on day 0 (n≥6).

All figures denote mean ± SEMs unless indicated otherwise.

Figure S7 (Related to Figure 7). AGC1 is expressed in mouse and human tumors

(A) Representative immunohistochemistry staining for AGC1 in the indicated human cancer tissues.

(B) Representative immunohistochemistry for AGC1 expression in normal and cancerous human pancreas tissue sections (n=5). AGC1 expression is observed in pancreatic islets [above left], and ductal carcinoma [right; above and below] but not in normal duct [below left].

(C) AGC1 expression assessed by Western blot in normal mouse pancreas and lung tissue as well as in $Kras^{G12D}$, $p53^{-/-}$ lung and pancreatic tumors arising in genetically engineered mouse models involving these organs as indicated.

Figure S1, related to Figure 1,2

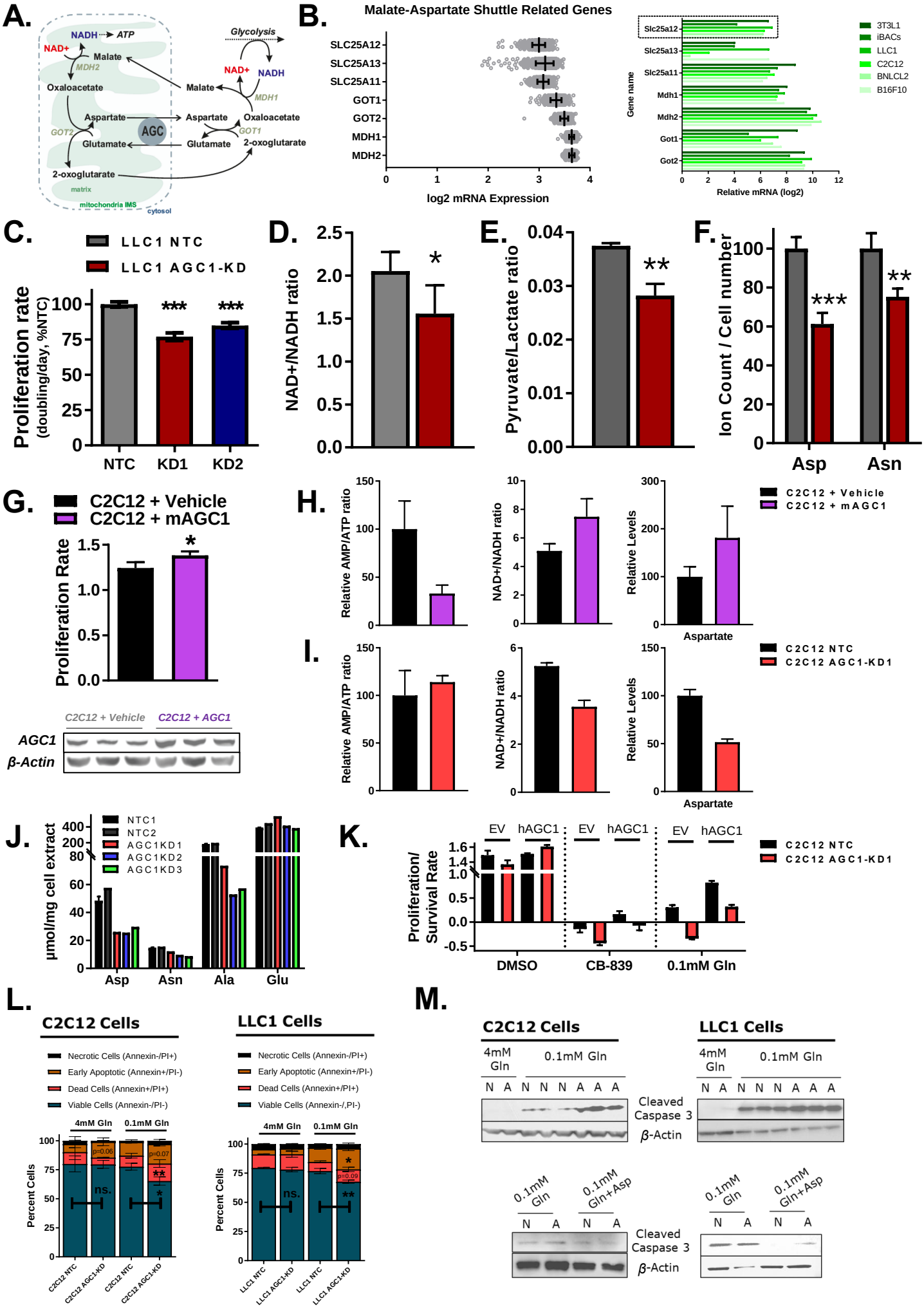
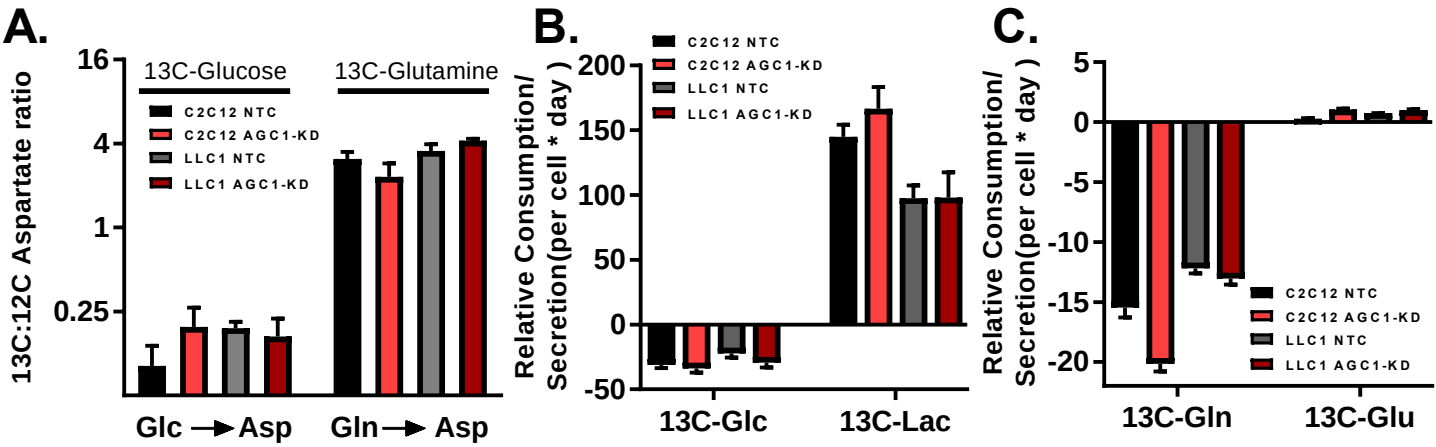
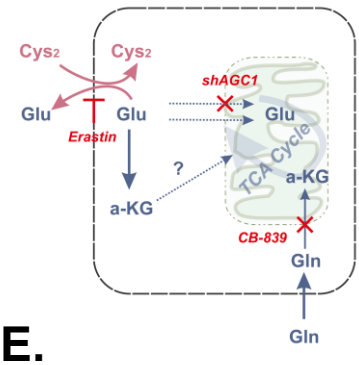
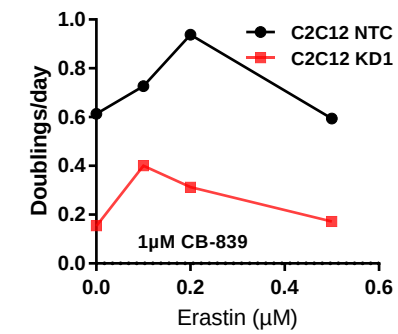
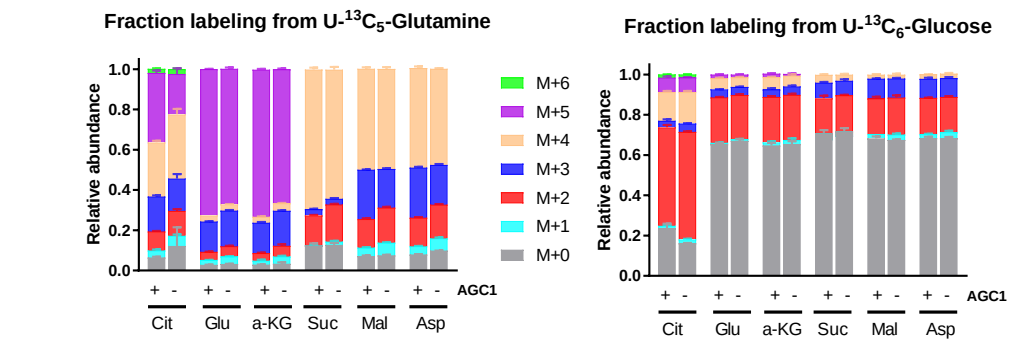


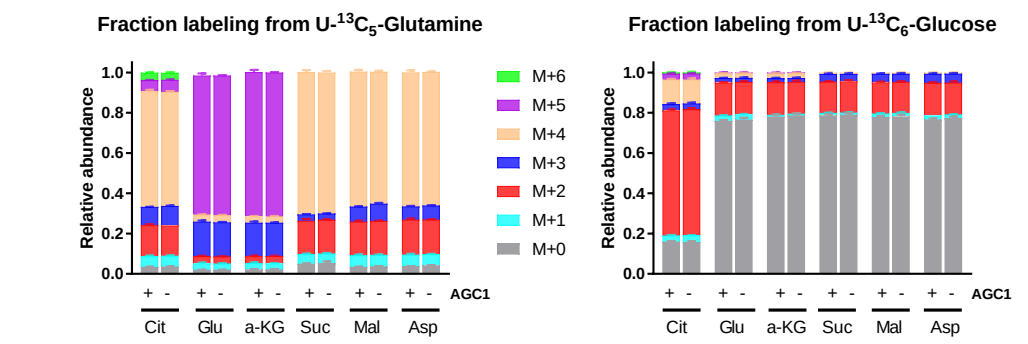
Figure S2, Related to Figure 3

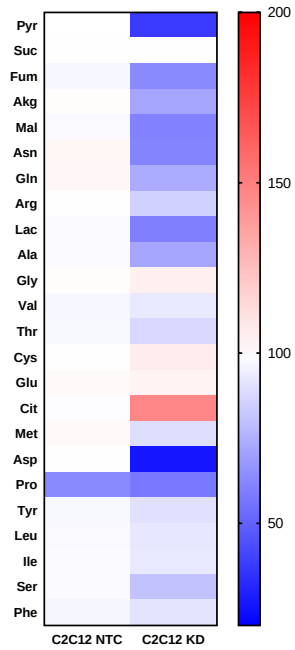


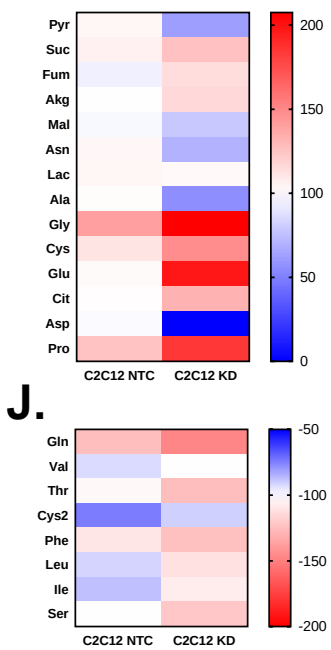


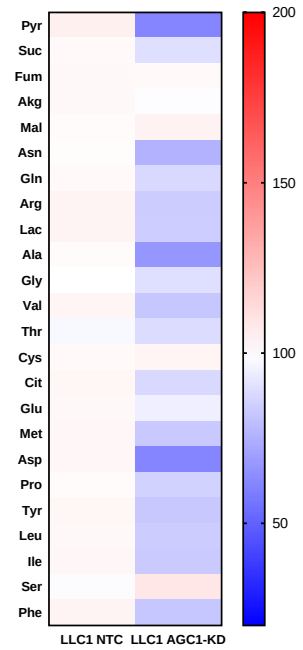












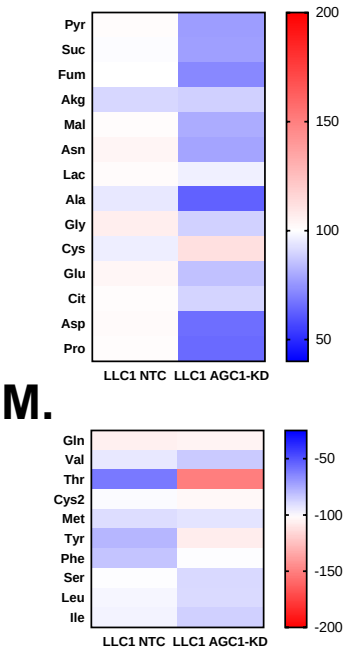
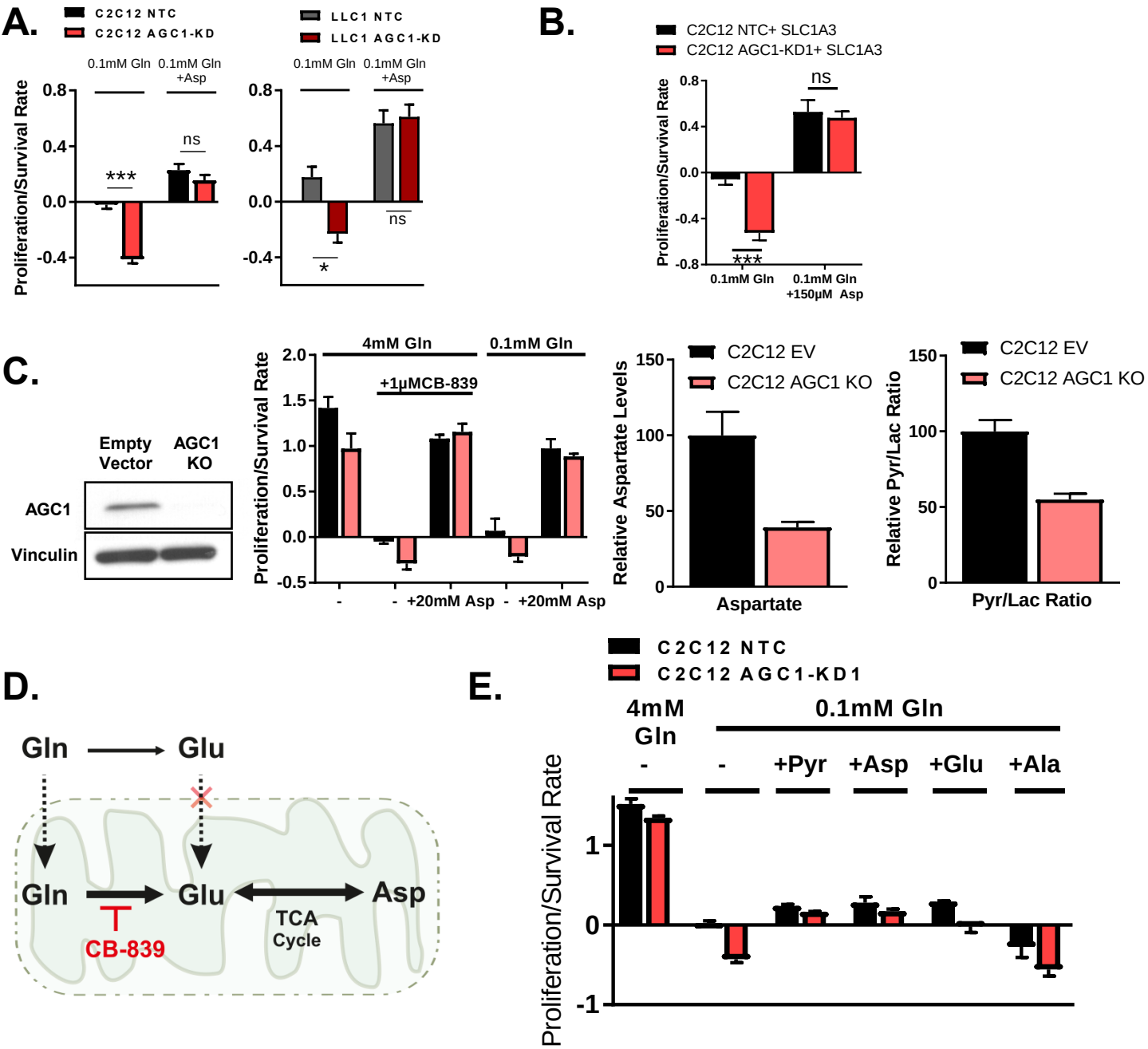


Figure S3, Related to Figure 4



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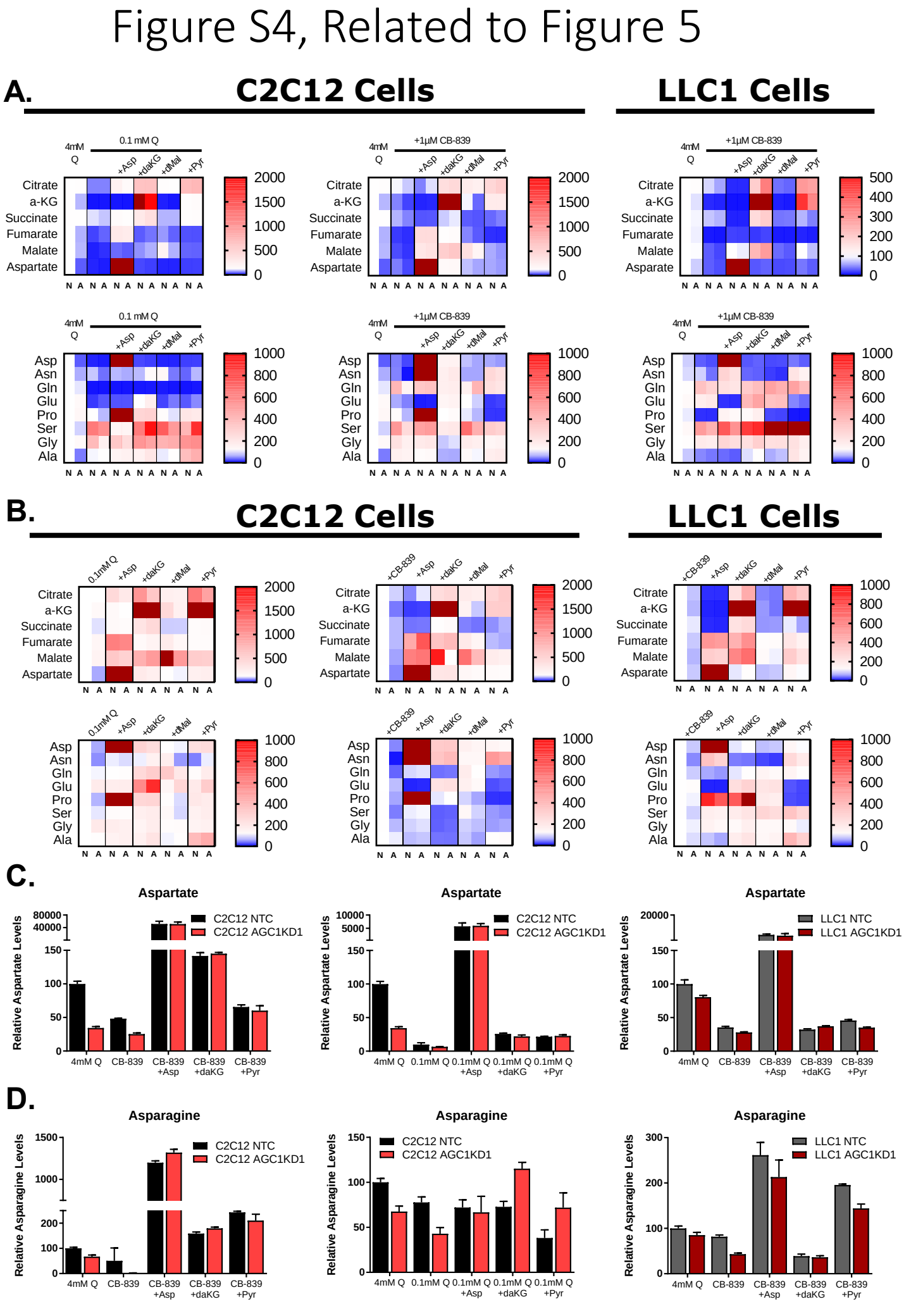

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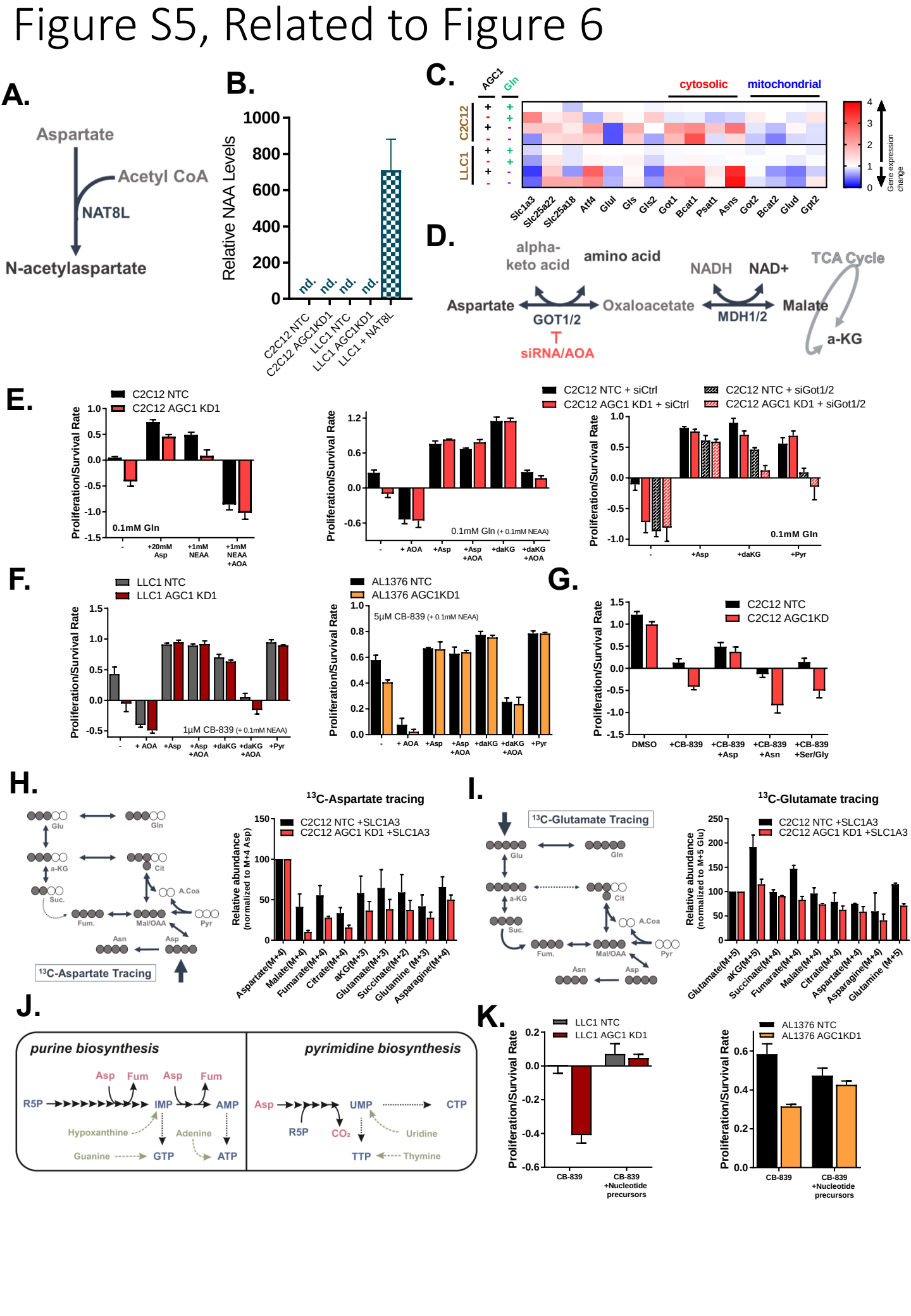

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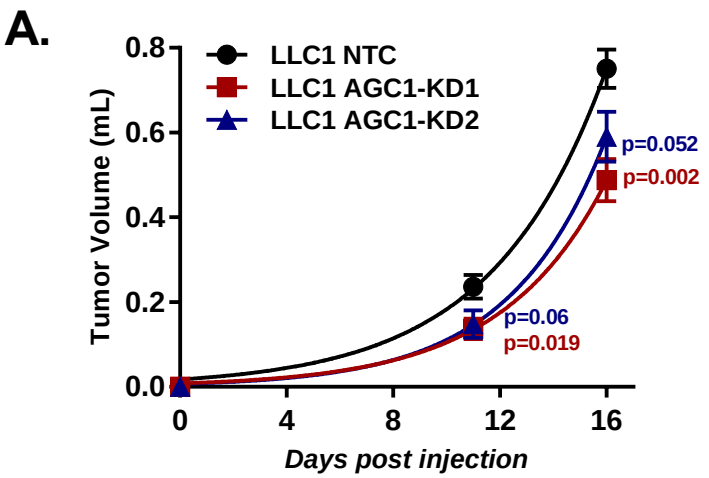

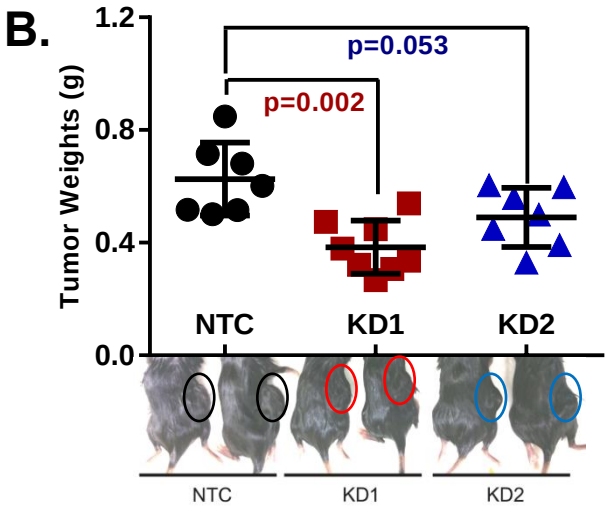

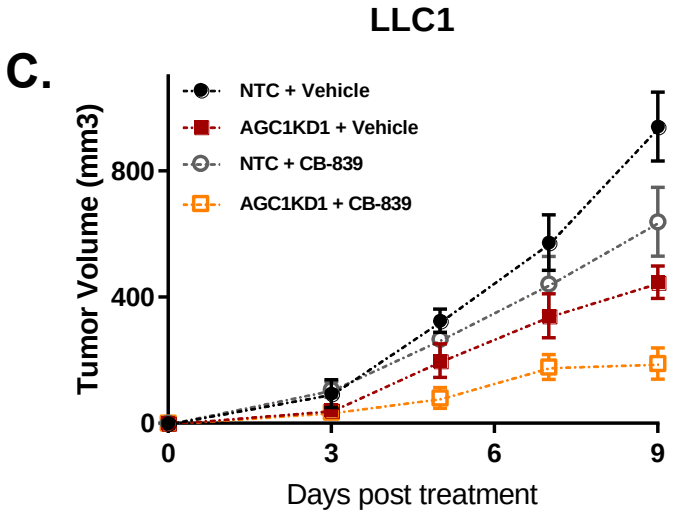

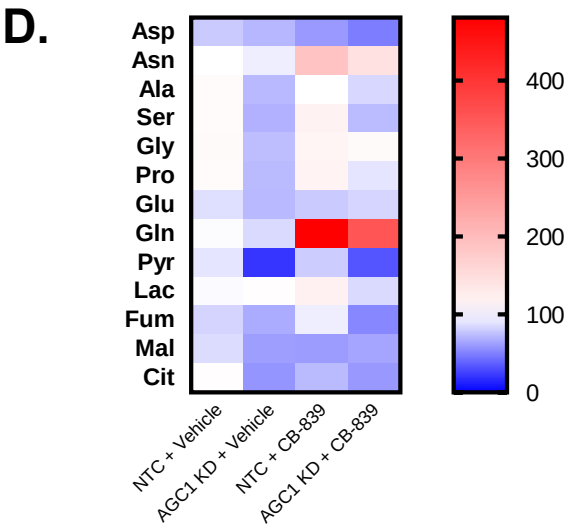

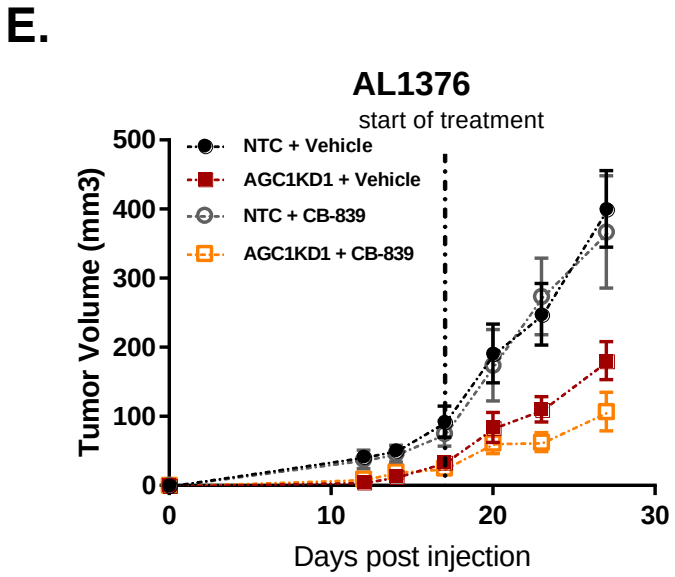

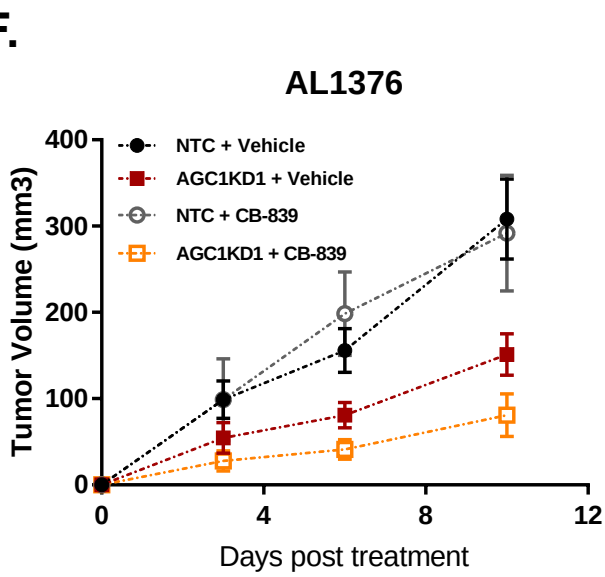

Figure S7, Related to Figure 7