Aspartate is an endogenous metabolic limitation for tumour growth

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Defining the metabolic limitations of tumour growth will help to develop cancer therapies. Cancer cells proliferate slower in tumours than in standard culture conditions, indicating that a metabolic limitation may restrict cell proliferation in vivo. Aspartate synthesis can limit cancer cell proliferation when respiration is impaired; however, whether acquiring aspartate is endogenously limiting for tumour growth is unknown. We confirm that aspartate has poor cell permeability, which prevents environmental acquisition, whereas the related amino acid asparagine is available to cells in tumours, but cancer cells lack asparaginase activity to convert asparagine to aspartate. Heterologous expression of guinea pig asparaginase 1 (gpASNase1), an enzyme that produces aspartate from asparagine, confers the ability to use asparagine to supply intracellular aspartate to cancer cells in vivo. Tumours expressing gpASNase1 grow at a faster rate, indicating that aspartate acquisition is an endogenous metabolic limitation for the growth of some tumours. Tumours expressing gpASNase1 are also refractory to the metabolic limitation for the growth of some tumours. Tumours expressing gpASNase1 grow at a faster rate, indicating that aspartate acquisition is an endogenous metabolic limitation for the growth of some tumours. Tumours expressing gpASNase1 are also refractory to the metabolic limitation for the growth of some tumours. Tumours expressing gpASNase1 grow at a faster rate, indicating that aspartate acquisition is an endogenous metabolic limitation for the growth of some tumours. Tumours expressing gpASNase1 grow at a faster rate, indicating that aspartate acquisition is an endogenous metabolic limitation for the growth of some tumours.

These findings suggest that therapeutic aspartate suppression could be effective to treat cancer.

How cancer-associated metabolic pathways support cell proliferation has been extensively studied in culture; however, environmental differences between tumours and cell culture can result in the use of alternative pathways in vivo. Thus, understanding the metabolic limitations of tumour cells in vivo is critical to translate the growing understanding of cancer metabolism and to help to develop cancer therapies. The production of the amino acid aspartate can be a metabolic limitation for cancer cell proliferation in some contexts. Inhibition of the mitochondrial electron transport chain impairs the regeneration of electron acceptors and suppresses both aspartate synthesis and cell proliferation. Cancer cells in tumours are exposed to lower oxygen levels than cells in culture, and electron transport chain inhibitors can impair tumour growth in some contexts. These findings raise the possibility that asparagine metabolism may constrain cancer cell proliferation in vivo.

The transport of aspartate into most mammalian cells is inefficient, with millimolar concentrations of aspartate needed to restore the proliferation of cells when electron transport is impaired. Because aspartate levels are low in the circulation, we investigated other approaches to raise aspartate levels in cells in a physiological tissue context. Although most mammalian cells lack a known asparaginase activity, the enzymatic activity that converts asparagine to aspartate, such an activity is found in some organisms. We reasoned that because the level of asparagine is more abundant in the circulation, providing cancer cells with an asparaginase activity may be a method to provide aspartate to the cells in tumours.

Human cells have two genes that encode products with homology to asparaginase enzymes from other organisms, but these gene products have not shown robust asparaginase activity and alternative enzymatic functions have been proposed. However, it has been hypothesized that asparaginase activity might be activated in some contexts. Thus, we first examined whether asparaginase could functionally contribute to the aspartate pool in cells. Providing U-13C-labelled asparagine to cells contributed to the intracellular asparagine pool when supplied at micromolar concentrations; however, U-13C-labelled asparagine only labelled the intracellular asparagine pool when provided at millimolar concentrations. Overexpression of the glial transporter solute carrier family 1 member 3 (SLC1A3), which can transport aspartate, allows labelling of intracellular asparagine from micromolar levels of labelled extracellular asparagine, confirming that aspartate is relatively impermeable to cells without this transporter. To confirm that these labelling differences reflect intracellular amino acid pools, we examined the incorporation of labelled asparagine into protein over 24 hours and found that the label from asparagine incorporated into protein at lower extracellular concentrations than the label from aspartate. Exogenous asparagine also increased intracellular asparagine levels when provided at micromolar levels, whereas the addition of 10 mM aspartate was needed to even slightly increase aspartate levels in cells. The expression of SLC1A3 enabled exogenous asparagine to raise intracellular pools at lower aspartate concentrations, which is consistent with asparagine being relatively impermeable to non-SLC1A3-expressing cells. In fact, extracellular aspartate only marginally contributes to intracellular aspartate levels, whereas extracellular asparagine contributes considerably to intracellular asparagine levels, across several cancer cell lines cultured in the presence of 1 mM U-13C-labelled asparagine or asparagine (Supplementary Fig. 1c–f). Taken together, these data are consistent with previous studies suggesting that many cancer cells can take up asparagine, but not aspartate.

Although these data indicate a difference in the ability of physiological levels of aspartate and asparagine to enter cells, we also determined whether these permeability characteristics applied to their...
release from cells. To test this, we measured the extracellular release of metabolites into saline with and without the detergent saponin (Fig. 1e). Saponin can form pores when complexed to cholesterol in the cell membrane, a process that takes approximately 5 minutes, causing the release of cytosolic small molecules by diffusion. We reasoned that permeable metabolites would equilibrate rapidly.
with extracellular saline, whereas impermeable metabolites would only equilibrate after the cytoplasmic membrane was permeabilized with saponin. Lactate, a molecule known to rapidly equilibrate across cell membranes, showed no difference in concentration in extracellular saline with or without saponin, whereas glutamate, a molecule known to be concentrated in cells and not readily exported without co-import of another amino acid, showed saponin-specific metabolite release (Supplementary Fig. 1g). Aspartate, but not asparagine, also shows saponin-specific metabolite release, further arguing that, unlike asparagine, aspartate is not readily transported across cell membranes (Fig. 1f). Despite the ability to take up asparagine, none of the cancer cells tested displayed robust conversion of $^{13}$C-asparagine to $^{13}$C-aspartate (Fig. 1g). These data confirm that asparagine, but not aspartate, is permeable to cell membranes at physiological concentrations and that these cancer cells lack the ability to convert asparagine to aspartate (Fig. 1h).

We hypothesized that if cells were able to convert asparagine to aspartate, extracellular asparagine might be able to contribute to intracellular aspartate levels (Fig. 2a). Guinea pigs are relatively unique among mammals in possessing asparaginase activity in their blood$^9$. The protein responsible for this activity, guinea pig asparaginase 1 (gpASNase1), has only recently been identified$^5$. We confirmed that recombinant gpASNase1 has asparaginase activity (Supplementary Fig. 2a). When epitope-tagged gpASNase1 is expressed in 143B cells (Fig. 2b), an increase in cellular asparagine activity is observed (Supplementary Fig. 2b). The expression of gpASNase1 results in a minor proliferation defect compared to empty vector (ev) control cells in media lacking asparagine and has no effect on proliferation in media with asparagine (Fig. 2c). As asparagine depletion can lead to cell death in certain contexts$^{20–22}$, we determined whether gpASNase1 expression could negatively affect cells when asparagine synthesis was impaired. Indeed, short hairpin RNA (shRNA)-mediated knockdown of asparaginase synthetase (ASNS) does not affect cell viability when asparagine is available, but synergizes with gpASNase1 to kill cells cultured without asparagine (Supplementary Fig. 2c–e). These data confirm that asparagine depletion can impair viability and argue that these cells have sufficient endogenous ASNS capacity to avoid detrimental effects of asparagine consumption by gpASNase1.

To determine whether gpASNase1 expression changes nutrient utilization, we examined asparagine levels in the media over time for gpASNase1-expressing and control cells. Whereas control cells net produce asparagine, gpASNase1 cells consume asparagine (Fig. 2d). When cells are cultured in media containing $^{13}$C-labelled asparagine, gpASNase1-expressing cells exhibit increased aspartate levels, with a substantial fraction of the aspartate derived from asparagine (Fig. 2e). Asparagine-derived aspartate is also incorporated into known asparagine-utilizing pathways, including the tricarboxylic acid (TCA) cycle and pyrimidine synthesis (Supplementary Fig. 2f,g). Cells expressing gpASNase1 had increased levels of some asparagine-derived metabolites compared to control cells (Supplementary Fig. 2h), but consistent with previous work$^{–4}$, asparagine supplementation did not affect the intracellular NAD$^+$/NADH ratio (Supplementary Fig. 2i). These data indicate that gpASNase1 expression confers the ability to convert environmental asparagine into intracellular aspartate and downstream products of aspartate metabolism.

The expression of gpASNase1 is predicted to restore proliferation to asparagine-limited cells when asparagine is available. As glutamine is a major source of intracellular asparate for most cells in culture$^{26–28}$, we examined whether gpASNase1 provides an orthogonal source of asparagine and decreases glutamine utilization (Fig. 3a). Indeed, providing asparagine decreased anaplerotic glutamate consumption (glutamine consumed−glutamate released) in gpASNase1-expressing cells, but in not control cells, and had no effect on other major nutrient fluxes (Fig. 3b and Supplementary Fig. 3a,b).

Asparagine also restores the proliferation of gpASNase1-expressing cells, but not control cells, in glutamine-limited conditions (Fig. 3c). Inhibiting mitochondrial respiration decreases aspartate synthesis, making cells dependent on alternative electron acceptors or supplementation with supraphysiological concentrations of asparagine$^{–4,10,28,29}$ (Fig. 3d). We confirmed that rotenone, an inhibitor of mitochondrial electron transport chain complex I, inhibits the proliferation of control and gpASNase1 cells in the absence of asparagine and that supplementation with 20mM aspartate restores proliferation (Fig. 3e). Asparagine supplementation restores gpASNase1-expressing cell proliferation, but not control cell proliferation, to rotenone-treated cells, consistent with gpASNase1 expression allowing asparagine to support aspartate levels (Fig. 3e). Supplementation with nucleotide precursors that bypass the demand for aspartate to support nucleotide synthesis can also partially restore rotenone-treated cell proliferation.
gpASNase1 expression allows extracellular asparagine to support proliferation under conditions where aspartate is limiting. a, A schematic indicating the relationship between glutamine (GLN) metabolism and aspartate/asparagine metabolism and the TCA cycle. α-KG, α-ketoglutarate; OAA, oxaloacetic acid. b, Anaplerotic glutamine consumption (glutamine consumption rate – glutamate release rate) from media when 143B gpASNase1 cells are cultured in the presence or absence of 1mM asparagine as indicated. c, The proliferation rate of 143B cells expressing empty vector (ev) or gpASNase1 when cultured in 0.1mM α-KG in the presence or absence of 1mM asparagine as indicated. d, A schematic depicting the requirement for electron acceptors to produce aspartate. e, The proliferation rate of 143B cells expressing ev or gpASNase1, with or without 20mM aspartate or 1mM asparagine as indicated. f, The proliferation rate of 143B cells expressing ev or gpASNase1 when cultured in hypoxia (0.8% O2), with or without 20mM aspartate or 1mM asparagine as indicated. The values denote the mean ± s.e.m. Sample size (n) = 3 independent biological replicates from a single representative experiment (b, c, e, f). The values denote the mean ± s.e.m. Sample size (n) = 3 independent biological replicates from a single representative experiment (b, c, e, f).
Expression of gpASNase1 increases tumour growth rate and causes metformin insensitivity. a. The concentrations of asparagine and aspartate in mouse plasma. b. Fractional labelling of intracellular aspartate from 143B cells expressing empty vector (ev) or gpASNase1 when cultured with the indicated concentrations of U-13C asparagine for 3 hours. The vertical dotted line indicates average asparagine concentration measured in mouse plasma. Treatment was repeated once with similar results. c. The proliferation rate of 143B cells expressing ev or gpASNase1, with or without rotenone (120 nM), and cultured in media with the indicated concentrations of asparagine. d. The volumes of tumours derived from 143B cells expressing ev or gpASNase1 measured over time as indicated. e. Tumour volumes 13 days after implantation of 143B cells expressing ev or gpASNase1 as indicated. f. Tumours dissected 13 days after implantation of 143B cells expressing ev and gpASNase1 from a separate experiment than (e). The relative aspartate/asparagine ratio from tumours derived from 143B cells expressing ev or gpASNase1 as indicated. h. The measurement of labelled M4-4 malate in tumours derived from AL1376 cells expressing ev or gpASNase1 following two boluses of either vehicle (saline) or 30 mg·mL−1 U-13C asparagine. i. Assessment of tumour volume over time for tumours derived from 143B cells expressing ev or gpASNase1 in mice that were treated with either vehicle (water) or 1 g per kg metformin once by oral gavage. Treatment was initiated in mice with size-matched tumours after reaching 45 mm3. The P values were calculated by unpaired, one-tailed t-test (c-e,g,i). The values denote the mean ± s.e.m. Sample size (n) = 7 independent biological replicates from a single representative experiment (a); n = 3 independent biological replicates from a single representative experiment (b,c); 10 mice were injected per genotype, with 3 injections per mouse (d-f), yielding n = 27 (ev) and n = 28 (gpASNase1); n = 5 measurements from independent tumours from each genotype (g); n = 6 (vehicle) or n = 9 (bolus) independent tumour samples from each genotype (h). For i, 5 mice were used per group harbouring 3 injection sites each; when tumours reached ≥45 mm3, they were put on study, occasionally resulting in multiple tumours per mouse going on study, yielding n = 6 (ev vehicle and ev metformin), n = 7 (gpASNase1 vehicle) and n = 8 (gpASNase1 metformin). The source data for b, d and i are available in Supplementary Table 1.

Fig. 4a: The concentrations of asparagine and aspartate in mouse plasma.
Fig. 4b: Fractional labelling of intracellular aspartate from 143B cells expressing empty vector (ev) or gpASNase1 when cultured with the indicated concentrations of U-13C asparagine for 3 hours.
Fig. 4c: The proliferation rate of 143B cells expressing ev or gpASNase1, with or without rotenone (120 nM), and cultured in media with the indicated concentrations of asparagine.
Fig. 4d: The volumes of tumours derived from 143B cells expressing ev or gpASNase1 measured over time.
Fig. 4e: Tumour volumes 13 days after implantation of 143B cells expressing ev or gpASNase1.
Fig. 4f: Tumours dissected 13 days after implantation of 143B cells expressing ev and gpASNase1 from a separate experiment.
Fig. 4g: The relative aspartate/asparagine ratio from tumours derived from 143B cells expressing ev or gpASNase1.
Fig. 4h: The measurement of labelled M4-4 malate in tumours derived from AL1376 cells expressing ev or gpASNase1 following two boluses of either vehicle (saline) or 30 mg·mL−1 U-13C asparagine.
Fig. 4i: Assessment of tumour volume over time for tumours derived from 143B cells expressing ev or gpASNase1 in mice that were treated with either vehicle (water) or 1 g per kg metformin once by oral gavage.

Expression of gpASNase1 increases tumour growth in HCT116-derived and AL1376-derived tumours, but not in AsPC-1-derived tumours, suggesting that some cancer cells are aspartate limited in tumours but that aspartate is not limiting the growth of all tumours.

We tested whether gpASNase1 expression affects the growth of tumours derived from cancer cells of different tissue origins and genotypes. We expressed gpASNase1 and observed conversion of asparagine to aspartate in gpASNase1-expressing HCT116, AL1376 and AsPC-1 cells (Supplementary Fig. 4e,f). The expression of gpASNase1 increased tumour growth in HCT116-derived and AL1376-derived tumours, but not in AsPC-1-derived tumours, suggesting that some cancer cells are aspartate limited in tumours but that aspartate is not limiting the growth of all tumours (Supplementary Fig. 4g). In all cases, increases in the intratumoural aspartate/asparagine ratio were observed in gpASNase1 tumours and there were no significant differences in the rate of tumour formation (Supplementary Fig. 4h,i). The expression of gpASNase1 altered several metabolite levels in tumours, although the most consistent

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change was decreased asparagine levels (Supplementary Fig. 4j). As observed in culture, gpASNase1 expression did not alter NAD+ levels in tumours (Supplementary Fig 4k). Collectively, these data indicate that gpASNase1 expression can supply aspartate in physiological contexts and that obtaining aspartate can limit the growth of some tumours. The expression of asparaginases from other organisms can have differential effects on tumour growth, potentially indicating that the mammalian asparaginase used here has enzyme properties that preclude intracellular asparaginase depletion to deleterious levels. Regardless, the observation that SLC1A3 expression can also increase tumour growth supports aspartate production being limited in some cancers.

Different tumours rely on distinct metabolic pathways to support growth, which can be influenced by the cancer genotype, tissue environment and cell lineage. The finding that cancer cells of disparate lineage and genotype can be engineered to grow tumours faster by expressing gpASNase1 suggests that acquiring aspartate may be a metabolic limitation for a subset of cancers. Different pathways can support aspartate levels in tumours from different tissues. For example, glucose supports aspartate synthesis in some lung cancers, whereas some pancreatic cancers catalyze extracellular protein as a source of aspartate. Importantly, inhibition of these pathways in their respective contexts can suppress tumour growth in vivo. Another potential method to suppress aspartate levels in tumours is to inhibit mitochondrial respiration. Indeed, the anti-diabetes drug metformin can inhibit tumour growth via cancer cell-autonomous inhibition of mitochondrial complex I. The anti-cancer activity of metformin can correspond with decreases in intratumoral aspartate levels, suggesting that metformin may suppress tumour growth through aspartate limitation. To test this, we implanted 143B cells with or without gpASNase1 expression into nu/nu mice and, upon tumours reaching 45 mm3, treated the mice with either metformin or without gpASNase1 expression into nu/nu mice and, upon tumours reaching 45 mm3, treated the mice with either metformin or vehicle. Whereas metformin suppressed the growth of control tumours, metformin had no effect on the growth of gpASNase1-expressing tumours (Fig. 4i). These data support the hypothesis that metformin can suppress tumour growth by exacerbating an endogenous aspartate limitation.

Why some cancer cells are more aspartate limited than others is unknown, but may be related to the method by which they acquire aspartate or their metabolic gene expression. One possible contributor is intratumoral hypoxia, which limits access to the electron acceptors that are important for aspartate synthesis. The finding that gpASNase1 has no effect on AsPC-1 tumour growth argues that aspartate is not necessarily a limiting metabolite for the growth of all tumours or, by extension, all proliferating cells in normal tissues. Thus, these data argue that targeting aspartate production may have particular efficacy to inhibit the growth of some tumours.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41556-018-0125-0.

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Author contributions
L.B.S. performed the metabolite permeability experiments, the metabolite consumption assays, GCMS metabolite quantitation and the statistical analysis. L.B.S., A.L., L.V.D., L.N.B., F.F.D., S.E. and S.M. performed the mouse experiments. L.B.S. and L.N.B. performed the cloning, the proliferation rate measurements and the western blots. A.M.H. performed the recombinant gpASNase1 purification and activity measurements. L.B.S. and A.N.L. performed the metabolite tracing into protein experiments. L.B.S. and C.A.L. performed the LCMS labelling and quantitation experiments. L.B.S. and M.G.V.H. designed the study and wrote the manuscript.

Competing interests
M.G.V.H. is a consultant and scientific advisory board member for Agios Pharmaceuticals and Aeglea Biotherapeutics.

Additional information
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Methods

Cell culture and the generation of cell lines. All cell lines were passed in DMEM with pyruvate (50-003, Corning) supplemented with 10% FBS (Sigma) and penicillin–streptomycin (Corning). The identity of 143B, 668-6, A172, AsPC-1, HCT116, HT1080, and TT cells were confirmed by satellite tandem repeat testing and referenced to the American Type Culture Collection (ATCC) values. AL1376 cells were generated in-house from a pancreatic tumour from the K/P–C (Kraut et al., 2003; Piel et al.) mouse model of pancreatic adenocarcinoma. All cell lines were negative for mycoplasma (MycoAlert, Lonza). For experiments, media were changed to DMEM without pyruvate (50-013, Corning) supplemented with 10% dialysed FBS (Sigma), penicillin–streptomycin and treatments as indicated. 143B CytB cells were also supplemented with 0.1 mg/ml uridine.

The protein sequence of gpASNase 1 was reverse translated into a cDNA sequence, which was synthesized and inserted into a pcDNA3 plasmid (Biomatic). Primers were designed to amplify the gpASNase 1 sequence, encode a carboxyl-terminal FLAG tag and use restriction cloning to insert into pcDNA3 into the plasmid pHLX. The primers used were: AAGATTATAACGTGACCGGCAAGGTGTTAG and CACGAGTTCGGGACCT GGCATGTGCTTGTGCTAACAAGACGGTGAAGCTGATG. A retrovirus was generated in HEK293 T cells by transfection using standard techniques. Cells were infected with virus containing either ev pLHCX or gpASNase 1 pHLX and were selected with 200 μg/ml (143B, 143B CytB, UOK262 and AsPC-1) or 500 μg/ml (AL1376 and HCT116) hygromycin B (Invitrogen). Cells were maintained in hygromycin B-containing media until all uninfected control cells had died.

The expression of SLC1A3 was accomplished in 143B cells using virus generated from PMX-SLC1A3, a gift from D. Sabatini (Addgene plasmid 72873). Standard techniques were used and cells were selected in 5 μg/ml blasticidin (Invitrogen).

Knockdown of ASNS was accomplished in 143B cells using standard techniques to generate virus containing pLKO.1 ARNA targeting either human ASNS (TRCN000290103 (ASNS1) and TRCN000290105 (ASNS2), Sigma) or GFP (Addgene plasmid 30323). Cells were maintained in 2 μg/ml puromycin containing media until all uninfected control cells had died.

Metabolite permeability assays. Cells were plated in 6-well dishes at 2.5 × 10^5 cells per well and allowed to incubate overnight. The next day, cells were washed twice with PBS, 4 ml media containing the indicated concentrations of U-13C-labelled asparagine and allowed to incubate overnight. The next day, cells were washed two times with PBS, 4 ml media containing 1 mM U-13C-labelled asparagine was added and cells were incubated for the indicated time. At the end point, cells were washed three times with ice-cold saline and extracted with 300 μl 80% methanol containing norvaline standard. Extracts were centrifuged at 21,000 × g for 10 min at 4°C, 200 μl supernatant was moved to a fresh microcentrifuge tube and extracts were dried under N2 gas. Dried extracts were then frozen at −80°C before subsequent derivatization and measurement by GCMS.

Purification of recombinant asparaginase. gpASNase 1 was cloned into pET28a* by Gibson Assembly (New England Biolabs) according to the manufacturer’s protocol. The resulting vector encodes gpASNase 1 with a C-terminal 6×His tag following a Ser-Gly-Ser-Gly linker. The vector was transformed into BL21(DE3) Escherichia coli, which were grown at 37°C to an optical density of 0.7 at 600 nm before asparaginase expression (final concentration 2 μg/ml). The culture was induced with 0.1 mM isopropyl-β-d-1-thiogalactopyranoside for 6 h at room temperature. Bacteria were flash frozen, resuspended in lysis buffer (50 mM Tris-Cl (pH 8.5), 300 mM NaCl, 10% (v/v) glycerol and 5 mM imidazole) and lysoned by sonication. The clarified lysate was incubated with Ni-NTA agarose beads (Qiagen), and the bound material was washed extensively with lysis buffer (lysate buffer containing 30 mM imidazole) before elution into elution buffer (50 mM Tris-Cl (pH 8.5), 250 mM NaCl, 10% (v/v) glycerol and 250 mM imidazole). The eluted enzyme was dialysed against dialysis buffer (50 mM Tris-Cl (pH 7.5), 25 mM NaCl and 20% (v/v) glycerol) overnight before being aliquoted and frozen.

Recombinant asparaginase activity. Asparaginase catalytic activity was monitored by a linked assay described previously. In this assay, aspartate produced by the hydrolysis of asparagine is transaminated to oxaloacetate that is reduced to malate, changes over time cannot be used to interpret its relative concentration in cells or media. However, a change in the metabolite levels after asparagine pore formation relative to untreated saline indicates that the metabolite is not released from cells in saline as readily as when cells were permeabilized.

Cellular asparaginase activity. Cells were plated into 6-well dishes at 1.5 × 10^5 cells per well and allowed to incubate overnight. The next day, cells were washed twice with PBS, 4 ml media containing 1 mM U-13C-labelled asparagine was added and cells were incubated for the indicated time. At the end point, cells were washed three times with ice-cold saline and extracted with 300 μl 80% methanol containing norvaline standard. Extracts were centrifuged at 21,000 × g for 10 min at 4°C, 200 μl supernatant was moved to a fresh microcentrifuge tube and extracts were dried under N2 gas. Dried extracts were then frozen at −80°C before subsequent derivatization and measurement of aspartate isoform abundance by GCMS.

Proliferation assays. Cells were plated in replicate in 6-well dishes (Corning), with an initial seeding density of 20,000 cells per well for 143B cells and 30,000 cells per well for 143B CytB and UOK262 cells. After seeding, cells were allowed to equilibrate overnight and one six-well dish was counted to quantify the starting cell number prior to treatment. The wells in the remaining dishes were washed twice with PBS and 4 ml of media containing the indicated treatments was added. Final cell counts were determined 4 days after the initial treatment using a Cellerometer Auto T4 Plus Cell Counter (Nexcelom Biosciences). The proliferation rate was determined based on the following formula:

\[
\text{Proliferation rate (doublings per day)} = \log_2 \left( \frac{\text{final cell count (day5)}}{\text{initial cell count (day1)}} \right) \times \frac{4}{\text{days}}
\]

Western blot analysis. Cells expressing either ev or C-terminal FLAG-tagged gpASNase 1 were washed with cold PBS and lysed with cold RIPA buffer containing Complete Mini protease inhibitors (Roche). The protein concentration was determined based on the following formula:

\[
\text{Protein concentration} = \frac{\text{Absorbance at 595 nm}}{\text{Concentration of BSA used for standards}} \times \text{Absorbance at 620 nm of sample}
\]
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determined by BCA Protein Assay (Pierce) using BSA as a standard. Samples were resolved by SDS–PAGE using standard techniques, and protein was detected with the following antibodies: primary antibodies: FLAG (1:1,000; 2368, Cell Signaling), vinculin (1:10,000; ab18058, Abcam), ASNS (1:250; HPA029318, Sigma) and HIF1-α (1:250; 610958, BD Transduction Laboratories); secondary antibodies: IR680LT dye-conjugated anti-rabbit IgG (1:10,000; 925-68021, Licor Biosciences), IR800 dye-conjugated anti-mouse IgG (1:10,000; 925-32210, Licor Biosciences) and horseradish peroxidase-linked anti-mouse IgG (1:10,000; 70765, Cell Signaling). FLAG and ASNS blots were imaged by an Odyssey infrared scanner (Licor Biosciences) and analysed using Image Studio Lite. HIF1-α blots were imaged with film (Denville Scientific).

Metabolite consumption or production. Metabolite consumption or production was determined by measuring the change in the metabolite levels in the medium collected from the culture of proliferating cells. The cell numbers were measured from duplicate treatment plates to determine the proliferation rate, and the metabolite flux was determined with the following formula:

\[ \text{Consumption rate} = \text{change in nutrient(mol)/area under growth curve (cell hours)} \]

\[ \text{Area under growth curve} = \int_0^T N(t) \, dt = \frac{N_0}{k \log_2} (2^T - 1) \]

Where \( N_0 \) is the initial cell count (cells), \( N(t) \) is cell hours at time point \( t \), \( T \) is final time point (hours) and \( k \) is the proliferation rate (doublings per hour).

Asparagine levels in the medium were quantified by LCMS and compared to a standard curve of U-13C asparagine in the medium. Measurements of glucose, lactate, glutamine and glutamate in the medium were measured on a YSI-2950 Biochemistry Analyzer (Yellow Springs Instruments).

Mouse studies. All experiments performed in this study were approved and compliant with ethical regulations regarding animal research provided by the MIT Committee on Animal care (IACUC). Nu/nu mice were purchased from Charles River (888) with ad libitum access to food and water.

Mouse plasma metabolite measurement. To collect mouse plasma samples, animals were anaesthetized using a mixture of isofluorane and oxygen and blood was collected into EDTA-containing tubes via retro-orbital bleeding. Samples were centrifuged at 3,000 r.p.m. for 10 min at 4 °C, and the supernatant was collected and re-centrifuged. Plasma samples were collected and stored at −80 °C until further analysis.

Xenograft tumour growth. Cancer cells were suspended in 100 μl PBS and injected into the flanks of 6–8-week-old, male nu/nu mice at 1 × 10^6 cells per injection (143B and AsPC-1), 2 × 10^6 cells per injection (AL1376) or 3 × 10^6 cells per injection (HCT116). When tumours became palpable, the tumour volume was measured by calipers in two dimensions and the volumes were estimated using the equation \( \text{Volume} = \left(\frac{4}{3}\right)\pi r^3 \), where \( r \) is the radius of the tumour.

Tumour protein extraction. Pieces of frozen tumour tissue (5–20 mg) were cryogenically homogenized (Retsch Cryomill) and extracted with 1.3 ml 6:3:4 methanol:H2O:chloroform. Tumour extractions were vortexed at 4 °C for 10 min, centrifuged at 21,000 g for 10 min at 4 °C, and 500 μl of the upper, aqueous layer was moved to a fresh microcentrifuge tube and dried under N2. Dried extracts were resuspended in 50 μl acetonitrile:H2O and metabolites were measured by LCMS.

Tumour protein extraction. Pieces of frozen tumour tissue (5–20 mg) were homogenized in 300 μl of ice-cold RIPA buffer containing cOmplete Mini protease inhibitors (Roche) and 1% Antifoam Y-30 (Sigma) using gentleMACS dissociators (Miltenya Biotec). Tissue homogenate protein was quantified and analysed as normal for western blot analysis.

Asparaginase tracing in tumours. Following xenograft growth assay of AL1376 cells expressing ev or gpASNSae1, mice were given a retro-orbital bolus of either vehicle (saline) or 30 mg ml⁻¹ U-13C asparagine at time 0 and 30 min. One hour after the initial bolus, mice were killed and tumours were extracted for metabolite analysis by LCMS, as detailed above. Owing to small contaminating amounts of U-13C aspartate in the U-13C asparagine stock, the measurement of M+4 asparate in tumours was confounded by labelled aspartate in the blood. As this blood level of aspartate was far below what is required to enter cells, we rationalized that we could use a downstream metabolite of intracellular aspartate metabolism, malate, which is not detectable in the blood, as a surrogate for intracellular asparaginase activity.

Statistics and reproducibility. Data are presented as the mean ± s.e.m. The sample size (n) indicates the experimental replicates from a single representative experiment; the results of the experiments were validated by independent repetitions. For all experiments, each data point analysed was from an independent biological sample. The experiments in Fig. 4b and Supplementary Figs. 2c, 3d and 4e were repeated once. Statistical significance was typically determined using an unpaired, one-tailed t-test, in which significance was determined as \( P \leq 0.05 \). Determination of the statistical significance of tumour formation efficiency was determined by a two-sided Fisher's exact test.

Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Source data for Figs. 1f,g, 2c,e and 4b,d,i and Supplementary Figs. 1g, 2f–h, 3b and 4a,c,d,f,g,i,j have been provided in Supplementary Table 1. Metabolomics data for Supplementary Figs. 2h and 4j have also been deposited at 10.6084/m9.figshare.6169409. All other data generated that support the findings of this study are available from the corresponding author upon reasonable request.
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
- Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated
- Clearly defined error bars
- State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection  
No software was used to collect data.

Data analysis  
Graphpad Prism 7.0 was used for statistical tests. Image Studio Lite was used to visualize Western blots that were developed using an Odyssey scanner (Licor). Mass spectrometry data was analyzed with XCalibur QuanBrowser 2.2.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All data generated that support the findings of this study are available from the corresponding author upon reasonable request. Individual data points for figures that cannot be presented with a dot plot overlay (Figures 1f, 1g, 2c, 2e, 4b, 4d, 4i and Supplementary Figures 1g, 2f, 2h, 3b, 4a, 4c, 4d, 4f, 4g, 4i, 4j) have been
provided in Supplementary Table 1. Metabolomics data for Supplementary Figures 2h and 4j have also been deposited at https://doi.org/10.6084/m9.figshare.6169409.

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- [ ] Life sciences
- [ ] Behavioural & social sciences
- [ ] Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Experiments were performed using sample sizes based on standard protocols in the field. No statistical test was performed to predetermine sample size. |
|-------------|-----------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data was excluded.                                                                                                           |
| Replication | All attempts to replicate the experiments performed here were successful.                                                        |
| Randomization | Allocation of groups was random.                                                                                             |
| Blinding | The investigator was not blinded to group allocation, although results were qualitatively verified by blinded investigators. For the metformin xenograft experiment, quantitation of tumor volumes was done by a blinded investigator as discussed in methods. |

Reporting for specific materials, systems and methods

Materials & experimental systems

- [ ] Involved in the study
- [ ] Unique biological materials
- [ ] Antibodies
- [ ] Eukaryotic cell lines
- [ ] Palaeontology
- [ ] Animals and other organisms
- [ ] Human research participants

Methods

- [ ] Involved in the study
- [ ] ChIP-seq
- [ ] Flow cytometry
- [ ] MRI-based neuroimaging

Unique biological materials

Policy information about availability of materials

Obtaining unique materials | No restrictions on materials.

Antibodies

Antibodies used

Primary antibodies: FLAG (Cell Signaling, 2368, 1:1000), Vinculin (Abcam, ab18058, 1:10,000), ASNS (Sigma, HPA029318, 1:250), HIF1alpha (BD Transduction Laboratories, 610958, 1:250). Secondary antibodies: IR680LT dye conjugated anti-rabbit IgG (Licor Biosciences, 925-68021, 1:10,000), IR800 dye conjugated anti-mouse IgG (Licor Biosciences, 925-32210, 1:1,000), HRP-linked Anti-mouse IgG (Cell Signaling, 70765, 1:10,000).

Validation

Antibodies were validated by the suppliers and by the appearance of a band at the predicted size and using biologically relevant conditions (hypoxia increasing HIF1alpha). Otherwise the antibodies were not validated before use, but are standard antibodies in the field.
### Eukaryotic cell lines

**Policy information about cell lines**

| Cell line source(s) | All cell lines were obtained from ATCC, aside from AL1376 cells which were derived from a pancreatic tumour from the KP-/- C (KrasG12D/+; Trp53fl/fl; Pdx1-cre) mouse model of pancreatic adenocarcinoma. |
|---------------------|------------------------------------------------------------------------------------------------------------------|
| Authentication      | The identity of 143B, 468, 786-o, A172, AsPC-1, HCT116, HeLa, HT1080, and TT cells were authenticated by satellite tandem repeat testing and referenced to ATCC values. AL1376 cells were generated in house. |
| Mycoplasma contamination | Mycoalert testing was done to test for mycoplasma contamination, as described in methods. |
| Commonly misidentified lines | None of our cell lines are listed in ICLAC as commonly misidentified cell lines. |

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### Animals and other organisms

**Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research**

| Laboratory animals | 6-8 week old male nu/nu mice were purchased from Charles river (088) and used for xenograft studies. |
|--------------------|--------------------------------------------------------------------------------------------------|
| Wild animals       | Study did not involve wild animals.                                                               |
| Field-collected samples | Study did not involve samples collected from the field.                                             |