As oxygen is essential for many metabolic pathways, tumour hypoxia may impair cancer cell proliferation\(^{1,2}\). However, the limiting metabolites for proliferation under hypoxia and in tumours are unknown. Here, we assessed proliferation of a collection of cancer cells following inhibition of the mitochondrial electron transport chain (ETC), a major metabolic pathway requiring molecular oxygen\(^3\). Sensitivity to ETC inhibition varied across cell lines, and subsequent metabolomic analysis uncovered aspartate availability as a major determinant of sensitivity. Cell lines least sensitive to ETC inhibition maintain aspartate levels by importing it through an aspartate/glutamate transporter, SLC1A3. Genetic or pharmacologic modulation of SLC1A3 activity markedly altered cancer cell sensitivity to ETC inhibitors. Interestingly, aspartate levels also decrease under low oxygen, and increasing aspartate import by SLC1A3 provides a competitive advantage to cancer cells at low oxygen levels and in tumour xenografts. Finally, aspartate levels in primary human tumours negatively correlate with the expression of hypoxia markers, suggesting that tumour hypoxia is sufficient to inhibit ETC and, consequently, aspartate synthesis in vivo. Therefore, aspartate may be a limiting metabolite for tumour growth, and aspartate availability could be targeted for cancer therapy.

As solid tumours frequently outgrow their blood supply, cancer cells reside in nutrient- and oxygen-poor environments\(^5\). To sustain proliferation, cancer cells rewire their metabolic pathways and adapt to the tumour nutrient environment. In particular, low oxygen activates a transcriptional program that induces glucose uptake and glycolysis, while suppressing electron transport chain (ETC) activity\(^6\). However, the cellular effects of low oxygen extend beyond central glucose metabolism, as there are more than 145 metabolic reactions that employ molecular oxygen as an electron acceptor\(^7,8\). These oxygen-requiring reactions generate energy and provide critical building blocks such as fatty acids, amino acids, cholesterol and nucleotides. Nonetheless, which of these cellular metabolites are limiting for cancer cell proliferation under hypoxia and in tumours remains poorly understood.

Among the oxygen-requiring metabolic pathways, ETC activity provides a highly efficient route for eukaryotic cells to generate ATP\(^9,10\). ETC inhibition suppresses cancer cell proliferation in vitro and in vivo\(^11,12\), but whether all cancer cells have similar sensitivity to ETC inhibition, and the precise metabolic determinants of this sensitivity, are not clear. To address this, we assessed proliferation of a collection of 28 patient-derived cancer cell lines derived from blood, stomach, breast, colon and lung tumours, and measured the effect of ETC inhibition on cell proliferation (Fig. 1a). Given that inhibition of different complexes of the ETC may have pleiotropic effects on metabolism, we used inhibitors of complex I (piericidin), complex III (antimycin A) and complex V (oligomycin) as well as phenformin, an anti-diabetic drug that inhibits the ETC. Interestingly, cancer cell lines display diverse growth responses to ETC inhibition (Fig. 1a). While proliferation of many lines was strongly affected by ETC inhibitors, a subset was less sensitive or some were completely resistant to ETC inhibition. The sensitivity to inhibition of each ETC complex significantly correlated with the others, suggesting that the effect of ETC inhibition on proliferation is largely independent of the complex inhibited (Fig. 1a and Supplementary Fig. 1a). However, a subset of cancer cell lines exhibited sensitivity to ETC inhibition that was partially complex dependent. For example, the sensitivity profiles of complexes I and III inhibition were more highly correlated with each other than with that of complex V inhibition, reflecting the distinct functions of complexes I/III and V in the ETC. Similarly, the sensitivity profile of complex I inhibitor piericidin most strongly correlated with that of phenformin \((r = 0.90, P = 1.7 \times 10^{-14})\) (Fig. 1b and Supplementary Fig. 1a), consistent with the previous findings that the major cellular target of anti-diabetic biguanides such as metformin and phenformin is complex I\(^13,14\).

Given these results, we hypothesized that fundamental differences in metabolic function might exist between the cancer cell lines most sensitive (P12-ICHIIKAWA, ALL-SIL, HPB-ALL, MV-4-11, A549 and SU-DHL-8) and least sensitive (COLO-704, MDA-MB-157, NOMO-1, RPMI-8226, COLO-320DM and SNU-1) to ETC inhibition. To understand why a subset of cancer cell lines is less sensitive to ETC inhibition, we compared the effects of piericidin treatment on levels of key metabolites in the two groups (Fig. 1c). Even though ETC inhibition caused profound changes in the metabolism of all cancer cell lines, we were only able to detect significant differences in 19 metabolites between the two groups (Fig. 1c and Supplementary Fig. 1b). Among these were TCA cycle intermediates (malate, citrate and fumarate), nucleotides (UMP, ADP, UDP and CDP) and the amino acids aspartate and argininosuccinate. Synthesis of aspartate is limiting for cancer cell proliferation under hypoxia and in tumours remains poorly understood.
These results suggest that maintaining aspartate levels by import aspartate import to ETC inhibitors (Fig. 2b and Supplementary Fig. 2a), indicating that increased aspartate transport is a close homologue of SLC1A3, which is also capable of importing aspartate from the extracellular environment. Remarkably, unlike ETC-inhibition-sensitive cancer cell lines, while its levels are mostly maintained in the resistant counterparts (Fig. 1d). Therefore, our results strongly point to the change in aspartate levels as an important determinant of sensitivity to ETC inhibition across different cancer cell lines.

We next sought to understand how a subset of cancer cells could maintain aspartate levels under ETC inhibition and whether maintenance of aspartate levels decreases sensitivity to ETC inhibition. Cells can increase aspartate availability by (1) increasing de novo synthesis from oxaloacetate or (2) importing aspartate from the extracellular environment. Remarkably, unlike ETC-inhibition-sensitive cancer cell lines, while its levels are mostly maintained in the resistant counterparts (Fig. 1d). Therefore, our results strongly point to the change in aspartate levels as an important determinant of sensitivity to ETC inhibition across different cancer cell lines.

Aspartate is a charged amino acid at physiological pH and requires the presence of specific transporters for cellular import (Supplementary Fig. 2c), as most mammalian cells cannot uptake aspartate from their environment. Expression of these known transporters is normally restricted to neuronal tissues. To identify the transporter(s) that import aspartate in ETC inhibition resistant cancer cell lines, we asked whether the expression of any particular transporter is most predictive of the response to ETC inhibition. Correlation of antimycin’s effect on cell viability with transcriptome-wide mRNA expression data from the Cancer Cell Line Encyclopedia (CCLE) revealed SLC1A3 as a top scoring gene (Fig. 2c). Indeed, many of the cancer cell lines with detectable aspartate import activity express SLC1A3 protein (Fig. 2d). One exception to this correlation, COLO-704, expresses SLC1A2, a close homologue of SLC1A3, which is also capable of importing aspartate (Supplementary Fig. 2d,e). Two additional resistant cell lines (RPMI 8226 and DU4475) do not import aspartate, suggesting the existence of alternative mechanisms to overcome ETC inhibition.

SLC1A3 is a glutamate-aspartate transporter localized on the plasma membrane (Supplementary Fig. 2f) and normally expressed aspartate is the only one that drops substantially (~3-fold) in ETC-inhibition-sensitive cancer cell lines, while its levels are mostly maintained in the resistant counterparts (Fig. 1d). Therefore, our results strongly point to the change in aspartate levels as an important determinant of sensitivity to ETC inhibition across different cancer cell lines.
Aspartate import underlies the resistance of cancer cells to ETC inhibition. a. 14C-Aspartate uptake in ETC-inhibition-resistant and -sensitive cancer cell lines (mean ± S.E.M., n = 3 biologically independent samples). b. Aspartate depletion sensitizes aspartate-importing cancer cell lines to ETC inhibitors. L-aspartate (150 μM), antimycin (AntiA, 30 nM) and piericidin (Pier., 10 nM) were added as indicated (mean ± S.E.M., 5 cell lines in each group, n = 3 biologically independent samples). c. Correlation of the sensitivities to ETC inhibitors with transcriptome-wide mRNA expression data from the Cancer Cell Line Encyclopedia (CCLE). The resulting Pearson correlation coefficients were sorted and plotted. SLC1A3 is indicated. n = 28 cell lines from Fig. 1a, d. Immunoblot analysis of SLC1A3 in ETC-inhibition-resistant and -sensitive cell lines. Actin was used as a loading control. e. Immunoblot analysis of SLC1A3 in wild-type, SLC1A3-null and rescued null SNU-1 cells. Actin was used as a loading control. f. 14C-aspartate uptake in wild-type, SLC1A3-null and SNU-1 cells treated with an SLC1A3 inhibitor (TFB-TBOA, 20 μM) (mean ± S.E.M., n = 3 biologically independent samples). g. Loss of SLC1A3 sensitizes cancer cells to antimycin and piericidin in RPMI. Relative cell number of wild-type (black), SLC1A3-null (blue) and rescued SLC1A3-null (grey) SNU-1 cells in the absence and presence of pyruvate (1 mM) or TFB-TBOA (20 μM) after treatment with antimycin (10 nM) and antimycin (30 nM) for 5 days (mean ± S.E.M., n = 3 biologically independent samples). h. Pharmacologic inhibition of SLC1A3 sensitizes aspartate importing cancer cell lines to antimycin (30 nM) and piericidin (10 nM). Cells were grown for 5 days in the absence or presence of TFB-TBOA (20 μM) (mean ± S.D., 5 cell lines in each group, n = 3 biologically independent samples). i. Expression of SLC1A3 rescues A549 from the anti-proliferative effects of antimycin and piericidin in standard RPMI media, which contains 150 μM aspartate. Relative cell number of control (black), and SLC1A3-overexpressing (grey) A549 cells in the absence and presence of pyruvate (1 mM) or TFB-TBOA (20 μM) after treatment with antimycin (10 nM) and antimycin (30 nM) for 5 days. (mean ± S.E.M, n = 3 biologically independent samples). Statistics: two-tailed unpaired t-test. **P < 0.001, ***P < 0.0001, ****P < 0.0001. For individual P-values, see Supplementary Table 1. Statistics source data are provided in Supplementary Table 1.

Fig. 2 | Aspartate import underlies the resistance of cancer cells to ETC inhibition. a. 14C-Aspartate uptake in ETC-inhibition-resistant and -sensitive cancer cell lines (mean ± S.E.M., n = 3 biologically independent samples). b. Aspartate depletion sensitizes aspartate-importing cancer cell lines to ETC inhibitors. L-aspartate (150 μM), antimycin (AntiA, 30 nM) and piericidin (Pier., 10 nM) were added as indicated (mean ± S.E.M., 5 cell lines in each group, n = 3 biologically independent samples). c. Correlation of the sensitivities to ETC inhibitors with transcriptome-wide mRNA expression data from the Cancer Cell Line Encyclopedia (CCLE). The resulting Pearson correlation coefficients were sorted and plotted. SLC1A3 is indicated. n = 28 cell lines from Fig. 1a, d. Immunoblot analysis of SLC1A3 in ETC-inhibition-resistant and -sensitive cell lines. Actin was used as a loading control. e. Immunoblot analysis of SLC1A3 in wild-type, SLC1A3-null and rescued null SNU-1 cells. Actin was used as a loading control. f. 14C-aspartate uptake in wild-type, SLC1A3-null and SNU-1 cells treated with an SLC1A3 inhibitor (TFB-TBOA, 20 μM) (mean ± S.E.M., n = 3 biologically independent samples). g. Loss of SLC1A3 sensitizes cancer cells to antimycin and piericidin in RPMI. Relative cell number of wild-type (black), SLC1A3-null (blue) and rescued SLC1A3-null (grey) SNU-1 cells in the absence and presence of pyruvate (1 mM) or TFB-TBOA (20 μM) after treatment with antimycin (10 nM) and antimycin (30 nM) for 5 days (mean ± S.E.M., n = 3 biologically independent samples). h. Pharmacologic inhibition of SLC1A3 sensitizes aspartate importing cancer cell lines to antimycin (30 nM) and piericidin (10 nM). Cells were grown for 5 days in the absence or presence of TFB-TBOA (20 μM) (mean ± S.D., 5 cell lines in each group, n = 3 biologically independent samples). i. Expression of SLC1A3 rescues A549 from the anti-proliferative effects of antimycin and piericidin in standard RPMI media, which contains 150 μM aspartate. Relative cell number of control (black), and SLC1A3-overexpressing (grey) A549 cells in the absence and presence of pyruvate (1 mM) or TFB-TBOA (20 μM) after treatment with antimycin (10 nM) and antimycin (30 nM) for 5 days. (mean ± S.E.M, n = 3 biologically independent samples). Statistics: two-tailed unpaired t-test. **P < 0.001, ***P < 0.0001, ****P < 0.0001. For individual P-values, see Supplementary Table 1. Statistics source data are provided in Supplementary Table 1.

in glioblastomas29. However, SLC1A3 is also highly expressed in a subset of non-glioblastoma tumours and its genomic locus is significantly amplified (Supplementary Fig. 2g). To determine whether SLC1A3 expression is necessary for cancer cell proliferation under ETC inhibition, we used the CRISPR/Cas9 system and generated a clonal SLC1A3 knockout of the SNU-1 cell line (SLC1A3_KO), in which SLC1A3 is normally highly expressed (Fig. 2e). SLC1A3_KO cells take up 10-fold less aspartate and are more sensitive to piericidin and antimycin than wild-type counterparts (Fig. 2f,g). Importantly, expression of an sgSLC1A3-resistant human SLC1A3 cDNA in the
null cells or addition of pyruvate, which facilitates aspartate synthesis despite ETC inhibition, completely restored resistance to ETC inhibitors (Fig. 2g). Small molecule inhibitors targeting SLC1A3 (TFB-TBOA and UCPH-101) effectively block aspartate/glutamate uptake (Fig. 2f)²⁻⁵. These pharmacologic inhibitors of SLC1A3 sensitize SNU-1 cells and other ETC-inhibition-resistant cell lines with high SLC1A3 expression, including those with SLC1A3 amplification (NCI-H596, SNU-182 and Detroit-562), to ETC inhibitors (Fig. 2h and Supplementary Fig. 2hi). Additionally, we found that overexpression of SLC1A3 in A549, a cell line with undetectable endogenous SLC1A3 expression, is sufficient to enable the aspartate uptake and the proliferation of these cells under ETC inhibition (Fig. 2i and Supplementary Fig. 2j). Taken together, these findings reveal that SLC1A3 expression permits cancer cell proliferation under ETC inhibition, and that expression of the aspartate transporter SLC1A3 is a predictor of the proliferative response to ETC inhibition.

Aspartate levels can be limiting for cell proliferation under pharmacologic inhibition of mitochondrial respiration⁶⁻¹⁰, but whether aspartate is a limiting metabolite under conditions relevant to tumour growth is not known. As cancer cells in tumours are often starved for oxygen due to dysfunctional vasculature, we hypothesized that insufficiency of oxygen probably restricts aspartate biosynthesis via impairing ETC function and redox balance (Supplementary Fig. 3a). To first test whether aspartate is depleted in hypoxia, we measured amino acid levels of A549 and PANc-1 cancer cells, which have no detectable SLC1A3 expression, in standard atmospheric oxygen conditions (21%) or in reduced oxygen similar to that found in hypovascularized tumours (0.5%)²⁻⁷. Hypoxia causes profound metabolic changes, particularly a 4-fold decrease in the levels of aspartate and asparagine, which is synthesized from aspartate (Fig. 3a and Supplementary Fig. 3b). These data raise the possibility that low aspartate levels can limit proliferation under low oxygen, similar to what we observed for ETC inhibition, and that increasing aspartate levels may also promote proliferation in hypoxia. To test this possibility, we expressed SLC1A3 in A549 and PANc-1 cell lines as well as counterparts derived from KrasG12D/p53−/−-driven lung and pancreas cancer mouse models (KP lung and KP pancreas). Expression of SLC1A3 in these four cancer cell lines raised aspartate levels and increased their proliferation rates in hypoxia (Fig. 3b,c). Improved proliferative capacity under hypoxia depends on the aspartate transport activity of SLC1A3, as aspartate depletion or inhibition of SLC1A3 with a small molecule inhibitor completely eliminates the rescue phenotype (Fig. 3c). While aspartate is present in standard RPMI media at a concentration of 150 μM, its concentration in human and mouse serum is much lower (10–30 μM) (Supplementary Fig. 3c)²¹. However, culturing SLC1A3-expressing cancer cells in physiological aspartate concentrations²² is still sufficient to enable proliferation under ETC inhibition or hypoxia (Supplementary Fig. 3e,f), validating that SLC1A3 is a high-affinity transporter and can import aspartate at 20 μM, a concentration on par with the levels of aspartate found in circulation. Therefore, our data indicate that aspartate is a major limiting metabolite for optimal cell proliferation under hypoxia.

We next asked whether low aspartate is also a metabolic limitation for tumour growth in vivo. To test the role of aspartate availability in tumour growth, we performed a competitive proliferation assay between vector and SLC1A3-infected A549 and KP lung cancer cells under hypoxia, pharmacologic ETC inhibition and as tumour xenografts. Indeed, exogenous expression of SLC1A3 provides a growth advantage compared to vector infected controls in hypoxia or upon ETC inhibition in culture, and in tumour xenografts (Fig. 3d and Supplementary Fig. 3d). Of note, SLC1A3-expressing tumours have 2-fold higher aspartate levels compared to wild-type counterparts (Supplementary Fig. 3g). Aspartate limitation is not necessarily common to all cancers, as the growth advantage conferred by SLC1A3 expression was not observed in Kras mutant mouse pancreas tumours (Supplementary Fig. 3h). This suggests that some cancer types might meet their aspartate supply through alternative routes such as macropinocytosis in vivo²³⁻²⁵. Collectively, our results provide evidence that aspartate can be a growth-limiting metabolite in tumours.

Aspartate is a proteinogenic amino acid but can also contribute to nucleotide synthesis and TCA cycle anaplerosis. To understand why aspartate is limiting for cancer cell proliferation under hypoxia, we measured the generation of key metabolites derived from isotope labelled aspartate in A549 and PANc-1 cells. Aspartate can be directly used as a substrate to replenish aspartate, glutamate, and asparagine levels in hypoxic cells. Aspartate transaminases, generating citrate with four 13C atoms (m+4) and malate with two 13C atoms (m+2). However, we observe only modest labelling of malate/fumarate (m+2) and citrate (m+4) from 13C-aspartate in these cell lines (Supplementary Fig. 4a). Consistent with this observation, SLC1A3 expression does not increase oxygen consumption, which would be expected if aspartate supplementation substantially drove TCA flux (Supplementary Fig. 4b). We then

Fig. 3 | Aspartate is a limiting metabolite for cancer cell proliferation under hypoxia and in tumours. a, Differential intracellular amino acid abundances (log2) of A549 and PANc-1 cells with pericidin (10 nM) treatment or under 0.5% oxygen, relative to untreated control cells under 21% oxygen. The cells were cultured in RPMI media without glutamate and asparagine. b, Aspartate abundance in control (vector, black) and SLC1A3-expressing (grey) cell lines under hypoxia, relative to controls under 21% oxygen (mean ± S.E.M., n = 3 biologically independent samples). c, Relative cell number of control (vector) or SLC1A3-overexpressing cell lines under 0.5% oxygen with or without supplementation of aspartate (150 μM) and TFB-TBOA (20 μM) to untreated cells under 21% oxygen. The cells were cultured in RPMI media without aspartate, glutamate and asparagine. KP lung and pancreas indicates KrasG12D/p53−/− mouse cancer cell lines (mean ± S.E.M., n = 4 biologically independent samples for PANc-1 and A549, 3 biologically independent samples for KP lines). d, Left: the general scheme of the cancer cell competition experiment. Right: the relative abundance of A549 (top) and Kras/p53 (KP) lung (bottom) cancer cells transduced with a control vector or with SLC1A3 cDNA grown in vivo as xenografts or in vitro under antimycin (30 nM) treatment or 0.5% oxygen. Results are plotted relative to untreated cells under 21% oxygen (mean ± S.E.M., n = 6 biologically independent samples for in vitro conditions, n = 8 biologically independent samples for A549 tumours, n = 14 biologically independent samples for KP lung tumours). e, Left: a schematic depicting the metabolic routes of aspartate in pyrimidine and purine synthesis. Filled circles represent 13C or 15N atoms derived from [U-13C]-L-aspartate or [15N]-L-aspartate. Right: the fractions of labelled nucleotide precursors derived from labelled aspartate in control and SLC1A3-overexpressing A549 cells cultured for 24 h with [U-13C]-L-aspartate (150 μM) or [15N]-L-aspartate (150 μM) with pericidin treatment (10 nM) or under 0.5% oxygen. Colours indicate mass isotopomers (mean ± S.D., n = 3 biologically independent samples). f, Relative cell number of control (vector) or SLC1A3-overexpressing cell lines under 0.5% oxygen with or without supplementation of aspartate (150 μM) or nucleosides (thymidine, uridine, adenosine, cytidine and inosine; 100 μM). The cells were cultured in RPMI media without aspartate, glutamate and asparagine. KP lung and pancreas indicates KrasG12D/p53−/− mouse cancer cell lines. Results are plotted relative to cells cultured under 0.5% oxygen without aspartate or nucleoside supplementation. (mean ± S.E.M., n = 3 biologically independent samples, *P < 0.01, **P < 0.001, ***P < 0.0001). Statistics: two-tailed unpaired t-test. For individual exact P-values, see Supplementary Table 1. Statistics source data are provided in Supplementary Table 1.
focused our attention on the contribution of imported aspartate to nucleotide synthesis. In purine biosynthesis, aspartate acts as a nitrogen donor for both the purine ring and for the production of adenine from hypoxanthine. In pyrimidine biosynthesis, aspartate contributes three carbon atoms during the production of UMP and TMP via the intermediate orotate. Interestingly, 50 to 80% of orotate, dTMP and UMP in SLC1A3-expressing cells are derived from imported aspartate specifically under hypoxia and ETC inhibition, and only minimally under normoxic conditions (Fig. 3e and Supplementary Fig. 4a). Additionally, a significant portion of aspartate can contribute to purine synthesis as measured by $^{15}$N-aspartate incorporation into AMP in SLC1A3-expressing cells (Fig. 3e and Supplementary Fig. 3c). We observed similar results even under physiological aspartate concentrations (Supplementary Fig. 4d). These observations raise the possibility that low aspartate levels might limit nucleotide production.
necessary for cell proliferation under hypoxia and ETC inhibition, conditions where de novo aspartate synthesis is inhibited. Remarkably, in 3 out of the 4 cell lines, nucleoside supplementation alone substantially rescued growth inhibition by hypoxia even in the absence of aspartate, consistent with the limiting role of nucleotide biosynthesis (Fig. 3f). Thus, our data argue that imported aspartate is a major source for nucleotide biosynthesis and that aspartate-derived nucleotide synthesis is limiting for proliferation of a subset of cancer cells under hypoxia.

Given our results demonstrating that hypoxic cells are deficient in aspartate production, we asked whether aspartate could be a metabolic marker predicting the degree of hypoxia in primary human tumours. Hypoxia activates a specific transcriptional program through hypoxia-inducible factors, which strongly induces transcription of genes related to metabolism and angiogenesis, most notably VEGFA, CA9 and HK2. To test whether tumour hypoxia impacts aspartate levels in glioblastomas, we collected 24 snap-frozen glioblastomas for metabolomics and transcriptomic analyses, and correlated mRNA expression of hypoxia markers with the abundance of individual metabolites. Remarkably, among 128 metabolites detected, steady-state aspartate levels correlate significantly ($P<0.01$) with these established markers of tumour hypoxia (Fig. 4a and Supplementary Fig. 4e,f). Interestingly, steady-state aspartate levels correlated with the hypoxia markers even more strongly than lactate and acylcarnitines, metabolites commonly viewed as being increased in hypoxic tumour regions (Fig. 4b). These results suggest that the degree of hypoxia experienced by human tumours may inhibit aspartate synthesis through ETC inhibition, making aspartate a potential metabolic marker for tumour hypoxia in glioblastomas.

Our data are consistent with a model whereby tumour hypoxia limits ETC function, blocking aspartate availability and nucleotide biosynthesis (Fig. 4c). Similar to our results, heterologous expression of a guinea pig asparaginase also increases aspartate availability and tumour growth. Consistent with this observation, the degree of ETC inhibition present in primary human tumours is sufficient to limit aspartate production, as hypoxic markers correlate with aspartate levels. While low aspartate levels may restrict tumour growth by impacting protein and nucleotide synthesis, nucleoside supplementation alone can restore proliferation of a subset of aspartate-limited cancer cells, implying differential metabolic needs of different cancer types under hypoxia. Finally, acquisition of the ability to import aspartate through upregulation of gene expression or genomic amplification of aspartate transporters may be an important metabolic adaptation for tumours that develop in or are selected by transient rounds of hypoxia. Our findings indicate that low oxygen levels may constrain in vivo tumour growth through aspartate limitation, and pathways relevant to aspartate availability could thus be targeted for therapy in a subset of tumours.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41556-018-0118-z.

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Author contributions

K.B. and J.G.-B. conceived the project and designed the experiments. J.G.-B. and L.B. performed most of the experiments. K.L. performed computational analysis. X.G.Z assisted with the mouse xenograft experiments. T.P. assisted with the mouse xenograft experiments. K.B. and J.G.-B. conceived the project and designed the experiments. J.G.-B. and L.B. performed most of the experiments. K.L. performed computational analysis. X.G.Z assisted with the immunofluorescence experiments. C.A.L., J.F. and H.M. performed metabolite profiling experiments. T.P. assisted with the mouse xenograft experiments. R.L.P., M.S. and V.O.S. provided human tumour data and analysis. K.B. and J.G.-B. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Methods

Cell lines, compounds and constructs. Antibodies to SLC1A3 (GT2X0262, 1:1,000 for western blot; 1:500 for immunofluorescence) and β-actin (GTX109639, 1:10,000) were obtained from GeneTex; HRP-conjugated anti-rabbit antibody from Santa Cruz (sc-2357, 1:5,000); Alexa Fluor 488 donkey anti-rabbit antibody from Invitrogen (A-21206, 1:250); DAPI from Vector Laboratories; sodium pyruvate, polybrene, puromycin, thymidine, uridine, adenosine, cytidine and inosine from Sigma; aspartic acid from Acros; glutamic acid from Fisher Scientific; blasticidin from Invivogen; anticytin A and piericidin from Enzo Life Sciences; oligomycin from EMD Millipore; phenformin from Fluka; TFB-TBOA and UCPH 101 from Tocris; and Matrigel from Corning.

The identities of all the cell lines used in this study were authenticated by single tandem repeat (STR) profiling. All the cell lines were routinely tested for mycoplasma contamination every two months. Among all the cell lines, three of them were in ICLAC as misidentified cell lines but included in our analysis for diversity due to their oncogene status and metabolic phenotype: RPMI-8402 is an asparaginase auxotrophic leukemia cell line, U-937 is a rare histiocytic lymphoma cell line, and HPB-ALL is a cell line with Notch-activating mutations. Identities of these cell lines were authenticated by STR profiling (Supplementary Fig. 1c). Cell lines were cultured in RPMI medium containing 1 mM glucose, 10% fetal bovine serum, penicillin and streptomycin. For proliferation assays under aspartate and glutamate depletion and for tracing experiments, RPMI without amino acids (US Biologicals-R8999) was used, supplemented with individual amino acids at RPMI concentrations. For oxygen consumption experiments, assay media composed of RPMI media with L-glutamine and without sodium bicarbonate (Corning, 50-020-PC). Drugs used were used in aspartate and glutamate depletion experiment was prepared using Dialysis Tubing (Fisher Scientific) and fetal bovine serum (Sigma). For tracing experiments, ([U-13C]L-aspartic acid (CIL, CLM-1801-H) and L-aspartic acid-15N (CIL, NLM-718-05) were used. ([14C]U)-L-aspartic acid (Moravek, MC139) was used for aspartate uptake assays. Generation of the lentiviral sgSLC1A3 was achieved via ligation of hybridized oligos (below) into lenticRISPR-v1 vector linearized with BsmBI by ligation (NEB). Lentiviral vector expressing the sgRNA was transfected into HEK293T cells with lentiviral packaging vectors VSVG and Delta-VPR using XtremeGene transfection reagent (Roche). For overexpression of aspartate transporters, retroviral vectors with indicated cDNAs were transfected along with retroviral packaging plasmids (Gag-pol and VSVG-G) to HEK293T cells. 48 h after transfection, virus particles were collected and filtered using a 0.45 µm filter. The cell lines were transfected with 2 µl of viral particles and infected in media containing the virus and 8 µg/ml of polybrene. To increase transduction efficiency, a spin infection was performed by centrifugation at 1,200 g for 1.5 h. 48 h after the infection, the selection of transduced cells was performed by addition of puromycin (for sgRNA lentiviral vector) or blasticidin (for overexpression retroviral vector). sgRNA-ko single-cell isolated by serial dilution into a 96-well plate containing 0.1 ml of media. Single-cell clones were grown for three weeks, and the resultant sgSLC1A3−/− clones were validated by western blot and expanded.

Metabolite profiling and isotope tracing. For the initial metabolite profiling experiment in resistant and sensitive lines, each indicated cell line (1.5 million cells per replicate) was cultured as triplicates in 6-well plates and treated for 8 h with piericidin (10 nM). For aspartate tracing experiments in A549 and PANC-1 cell lines, 150,000 cells per well were pretreated with 10 nM piericidin or pre-incubated at 0.5% oxygen, as well as in RPMI without piericidin and at 21% oxygen. After 12 h, cells were harvested and centrifuged with 2 µl of 20% cold RP1-BSA, mixing aspartate, glutamate and asparagine and supplemented with [1-15C]-L-aspartate (150 µM or 20 µM) or [1-15N]-L-aspartate (150 µM or 20 µM) under indicated treatments for a initial 24 h. Cells were washed three times with 1 ml of cold 0.9% NaCl, and polar metabolites were extracted in 1 ml of cold 80% methanol containing internal standards (MSK-A2-1.2, Cambridge Isotope Laboratories). After extraction, samples were nitrogen-dried and stored at −80°C until analysis by liquid chromatography-mass spectrometry (LC-MS). Analysis was conducted on a QExactive benchtop orbitrap mass spectrometer equipped with an Ion Max source and a HESI II probe, which was coupled to a Dionex UltiMate 3000 UPLC system (Thermo Fisher Scientific, San Jose, CA). External mass calibration was performed using the standard calibration mixture every 7 days. Dried polar samples were resuspended in 100 µl water, and 2 µl wasinjected into a ZIC-HILIC 150 × 2.1 mm (5 µm particle size) column (EMD Millipore). Chromatographic separation was achieved using the following conditions: buffer A was 20 mM ammonium carbonate, 0.1% ammonium hydroxide, buffer B was acetonitrile. The column oven was held at 2°C and 4°C, respectively. The chromatographic gradient was run at a flow rate of 0.150 ml/min as follows: 0–20 min: linear gradient from 80% to 20% B; 20–20.5 min: linear gradient from 20% to 80% B; 20.5–28 min: hold at 80% B. The mass spectrometer was operated in full-scan, polarity switching mode with the spray voltage set to 3.0 kV, the heated capillary held at 275 °C, and the HESI probe held at 350 °C. The sheath gas and auxiliary gas was set to 40 units, the auxiliary gas flow was set to 15 units, and the sweep gas flow was set to 1 unit. The MS data acquisition was performed in a range of 70–1,000 m/z, with the resolution set at 70,000, the AGC target at 1 × 106, and the maximum injection time at 20 msec. Relative quantitation of polar metabolites was performed with XCalibur QuanBrowser 2.2 (Thermo Fisher Scientific) using a 5ppm mass tolerance and referencing an in-house library of chemical standards. For stable isotope tracing studies, fractional labelling was corrected for natural abundance using an in-house algorithm, based on that discussed previously. Metabolite levels were normalized to the total protein amount for each condition.

Oxygen consumption measurements. Oxygen consumption of intact cells was measured using an XpP69 Extracellular Flux Analyzer (Seahorse Bioscience). For adherent cell lines (A549, KP lung and KP pancreas), 15,000 cells were plated 12 h before the assay using RPMI 8226 (Corning no. 50-020-PC) assay media as previously described. Basal oxygen consumption measurements were normalized by protein levels.

Determination of NAD+/NADH ratios. For measurement of NAD+/NADH, manufacturer instructions for NAD/NADH Glo Assay were followed (Promega) with addition of previously reported modifications to the protocol. Cells were incubated at 21% and 0.5% O2, 24 h before being trypsinized and plated at a confluence of 400,000 cells per well in triplicates. After 24 h incubation, cells were lysed in cold D2O lysis buffer (1% dodecyltrimethylammonium bromide in 0.2 N NaOH diluted 1:1 in PBS). Briefly, to measure NADH, a portion of the extracts was heated to 75°C for 30 min in a basic lysis buffer. For NAD+ measurement, samples were further diluted 1:1 with 0.4 N HCl and heated to 60°C for 15 min, with the acidic conditions of this incubation degrading NADH. After quenching
both reactions by addition of 0.25 M Tris, 0.2 N HCl (NADH reaction) or 0.5 M Tris base (NAD+ reaction), manufacturer instructions to measure NAD+/NADH were followed.

**Immunoblotting.** Cell pellets were washed twice with ice-cold PBS and lysed in a standard lysis buffer (10 mM Tris Cl pH 7.5, 150 NaCl, 1 mM EDTA, 1% Triton X-100, 2% SDS, 0.1% CHAPS) supplemented with protease inhibitors (Roche). After sonication of each cell lysate and centrifugation for 10 min at 20,000 g, supernatants were collected and protein concentrations were determined by using Pierce BCA Protein Assay Kit (Thermo Scientific) with bovine serum albumin as a protein standard. Samples were then resolved on a 12% SDS-PAGE gel and analysed as previously described.

**Cell mixing competition assays.** Each indicated cell line transduced with control vector and expressing SLC1A3 cDNA was trypsinized and mixed in equal proportion. An initial sample of this mix was collected for further normalization. The mixed population of both lines was then cultured in RPMI media or custom media with varying concentrations of aspartate, in the presence of 30 nM antimycin A, 10 nM piericidin or subjected to growth at 0.5% O2 in a hypoxic proportion. An initial sample of this mix was collected for further normalization. An initial sample of this mix was collected for further normalization. The mixed population of both lines was then cultured in RPMI media or custom media with varying concentrations of aspartate, in the presence of 30 nM antimycin A, 10 nM piericidin or subjected to growth at 0.5% O2 in a hypoxic chamber for 14 days, by splitting every 3 days. The same cell mix was injected subcutaneously into NOD/SCID-γ mice and tumours were grown for the same period of time. Genomic DNA was isolated from initial samples, cells cultured under different conditions in vitro, and the tumours collected from mice using the Blood and Tissue Kit (Qiagen) following the manufacturer’s protocol. qPCR reactions were assayed on an ABI Real Time PCR System (Applied Biosystems) using SYBR Green Mastermix (Applied Biosystems) and the following primers: a common forward primer targeting the vector (GGTGGACCATCCTCTAGACT) and reverse primers either targeting the vector (GCAGGAAATTCCGTACCAC) or SLC1A3 cDNA (CATCTTGGGCTCTTCTCATT).

**Immunofluorescence.** For immunofluorescence assays, cells were seeded on coverslips previously coated with fibronectin. Cells were then fixed for 15 min with 4% paraformaldehyde diluted in PBS at room temperature. After three washes with PBS, cells were permeabilized and treated with 0.01% Triton X-100 in PBS for 5 min before three extra PBS washes. After incubation with a blocking solution (5% normal donkey serum (NDS) in PBS) for 1 h, coverslips were incubated with SLC1A3 primary antibody (GT2X0262, 1:500 in blocking solution) for an additional hour, and washed 3 times with PBS. Secondary Alexa Fluor 488 donkey anti-rabbit antibody was diluted in blocking solution (1:250) and added to the coverslips for 45 min at room temperature, prior to three washes with PBS. Coverslips were then incubated with a 200 nM solution of DAPI in PBS for 5 min and mounted onto slides with Prolong Gold antifade mounting media (Invitrogen). Images were taken on a Delta Vision bright-field fluorescence Inverted Olympus IX-71 microscope (GE Healthcare).

**Mouse studies.** All animal studies and procedures were conducted according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the Rockefeller University. All mice were maintained on a standard light–dark cycle with food and water ad libitum. Xenograft tumours were initiated by injecting 1.5 million cells per 100 μl of 30% Matrigel subcutaneously. After injections in the left and right flanks of male and female 6–9-week-old NOD/SCID-γ (NSG) mice (Jackson labs), tumours were grown for 14 days. For in vivo metabolite profiling experiments, 1.5 million cells A549 cells expressing SLC1A3 or a control vector were injected subcutaneously in opposite flanks of NSG mice and grown for 14 days before the experiment. Animals were euthanized and the tumours were extracted and immediately lysed in cold 80% methanol containing internal standards by using a Bead Ruptor 24 (Omni International) by 6 cycles of 20 s at 6 m s⁻¹. Supernatants were collected after 10 min centrifugation at 10,000g, nitrogen dried and analysed.

**Primary tumour samples.** Fresh frozen glioblastoma human primary tumour samples (24 IDH WT glioblastomas, age: min. = 29, max. = 84, median = 65; 12 male, 12 female) were obtained for metabolomics and RNA-seq using standard protocols. The tissues were homogenized in a methanol/chloroform solution and separated into non-polar and polar fractions. Dried polar fractions were then analysed for metabolomics analysis and normalized by total amino acid signal as described above. Tumour tissues were obtained from the archives of the Department of Neuropathology New York University (NYU) Langone Health after Institutional Review Board (IRB no. 114-00948) approval and the study is compliant with relevant ethical regulations. Informed consent was obtained from all participants. All specimens were reviewed and classified according to the World Health Organization (WHO) classification by a board certified neuropathologist to confirm histological diagnosis and select blocks for molecular analysis.

**Statistics and reproducibility.** GraphPad PRISM 7 and Microsoft Excel 15.21.1 software were used for statistical analysis. Error bars, P-values and statistical tests are reported in the figure captions. All experiments (except metabolite profiling and RNA-seq data of primary tumour samples in Fig. 4 and Supplementary Fig. 4e,f, which were done once) were performed at least two times with similar results. Both technical and biological replicates were reliably reproduced.

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

**Data availability.** Deep-sequencing (RNA-seq) data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) under accession code GSE113474. Metabolomics data of primary tumour samples (Fig. 4 and Supplementary Fig. 4e,f) have been deposited in Figshare (10.6084/m9.figshare.6167711). Source data for metabolite profiling experiments shown in Figs. 1 and 3 and Supplementary Figs. 1 and 4 have been provided as Supplementary Table 1. Correlation analysis for Fig. 4 is provided as Supplementary Table 2. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

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Reporting Summary

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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).

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| ☑   | 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 specific software was used for data collection. |
| Data analysis   | Microsoft Excel 15.21.1, GraphPad PRISM 7.0 and XCalibur QuanBrowser 2.2 were used for data analysis. |

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

Deep-sequencing (RNA–seq) data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) under accession code GSE113474. Metabolomics data included in Fig. 4 and Supplementary Fig. 4e–f have been deposited in Figshare with the accession number 10.6084/m9.figshare.6167711. Source data for metabolite profiling experiments shown in Fig. 1, 3, 4 and Supplementary Fig. 1, 4 have been provided as Supplementary
Table 1. Correlation analysis for Figure 4 is provided as Supplementary Table 2. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

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Life sciences study design

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

| Sample size | All the experiments were performed using sample sizes based on standard protocols in the field. No statistical methods were used to predetermine sample sizes. |
|-------------|-------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data points were excluded in any case. |
| Replication | All experiments (except metabolite profiling and RNA-seq data of primary tumor samples in Fig. 4 and Supp. Fig. 4e-f, which were done once) were done either 2 (cell competition experiments, oxygen consumption assays, metabolite profiling in cell lines) or 3 times with similar results. Both technical and biological replicates were reliably reproduced. |
| Randomization | Sample groups were allocated randomly. |
| Blinding | Studies were not conducted blinded. In metabolite profiling experiments bias is not possible. In vivo studies did not need any blinding due to its nature as cell competition assays. |

Reporting for specific materials, systems and methods

Materials & experimental systems

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

Methods

- n/a
- Involved in the study
- CHIP-seq
- Flow cytometry
- MRI-based neuroimaging

Antibodies

- Antibodies used
  - Primary Antibodies: EAAT1/SLC1A3 (GeneTex, GTX20262, Lot. No 821605369, 1 : 1,000 for Western blot, 1 : 500 for Immunofluorescence).
  - Beta-actin (GeneTex, GTX109639, Lot. No. 42810, 1 : 10,000).
  - Secondary Antibodies: HPR-conjugated Anti-Rabbit IgG (Santa Cruz, sc-2357, Lot. No. L0617, 1 : 5,000).
  - Alexa Fluor 488 donkey anti-rabbit antibody from Invitrogen (# A-21206, Lot. No. 1796375, 1:250).

- Validation
  - Each antibody used in this work was validated prior to its use following manufacturer’s instructions.

Eukaryotic cell lines

- Policy information about cell lines
- Cell line source(s)
  - All the cell lines used were obtained from Sabatini and Weinberg labs (Whitehead Institute) and originally purchased from ATCC or from DSMZ. We have STR analyzed all of our cell lines except for Kras/p53 mutant mouse cell lines, which were derived from Kras/p53 pancreas and lung mouse cancer models by Tyler Jacks/Thales Papagiannakopoulos and Nabeel Bardeesy labs, respectively. These mouse cancer cell lines are not commercially available and were directly obtained from the corresponding labs.
Authentication

Short Tandem Repeat (STR) profiling of all the cell lines used in this work was performed in collaboration with the Integrated Genomics Operations Center in Memorial Sloan Kettering Cancer Center. The KRAS/p53 mouse cancer cell lines are not commercially available and were directly obtained from the corresponding labs (Bardeesy and Papagiannakopoulos).

Mycoplasma contamination

PCR analysis confirming the absence of mycoplasma contamination was performed routinely every 6 months.

Commonly misidentified lines

Among all the cell lines used in this study, 3 of them were in ICLAC as misidentified cell lines but included in our analysis for diversity due to their oncogene status and metabolic phenotypes: RPMI-8402 is an asparagine auxotroph leukemia cell line, U-937 a rare histiocytic lymphoma cell line that is cholesterol auxotrophic, and HPB-ALL is a cell line with Notch activating mutations. Identities of these cell lines were authenticated by STR profiling.

Palaeontology

Specimen provenance

Provide provenance information for specimens and describe permits that were obtained for the work (including the name of the issuing authority, the date of issue, and any identifying information).

Specimen deposition

Indicate where the specimens have been deposited to permit free access by other researchers.

Dating methods

If new dates are provided, describe how they were obtained (e.g. collection, storage, sample pretreatment and measurement), where they were obtained (i.e. lab name), the calibration program and the protocol for quality assurance OR state that no new dates are provided.

Animals and other organisms

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

Laboratory animals

All animal studies and procedures were conducted according to a protocol approved by the Institutional Animal Care and Use Commitee (IACUC) at the Rockefeller University. Male and female 6-14 weeks old and 20-30 grams of weight NOD scid gamma (NSG) mice (Taconic) were used.

Wild animals

Provide details on animals observed in or captured in the field; report species, sex and age where possible. Describe how animals were caught and transported and what happened to captive animals after the study (if killed, explain why and describe method; if released, say where and when) OR state that the study did not involve wild animals.

Field-collected samples

For laboratory work with field-collected samples, describe all relevant parameters such as housing, maintenance, temperature, photoperiod and end-of-experiment protocol OR state that the study did not involve samples collected from the field.

Human research participants

Policy information about studies involving human research participants

Population characteristics

Fresh frozen glioblastoma human primary tumor samples (24 IDH WT glioblastomas, Age: Min = 29, Max = 84, median = 65; 12 Male, 12 Female) were obtained for metabolomics and RNAseq using standard protocols. Tumor tissues were obtained from the archives of the Department of Neuropathology New York University (NYU) Langone Health after Institutional Review Board (IRB# i14-00948) approval. All specimens were reviewed and classified according to the World Health Organization (WHO) classification by a board certified neuropathologist to confirm histological diagnosis and select blocks for molecular analysis.

Recruitment

Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.