Glutamine synthetase activity fuels nucleotide biosynthesis and supports growth of glutamine-restricted glioblastoma

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L-Glutamine (Gln) functions physiologically to balance the carbon and nitrogen requirements of tissues. It has been proposed that in cancer cells undergoing aerobic glycolysis, accelerated anabolism is sustained by Gln-derived carbons, which replenish the tricarboxylic acid (TCA) cycle (anaplerosis). However, it is shown here that in glioblastoma (GBM) cells, almost half of the Gln-derived glutamate (Glu) is secreted and does not enter the TCA cycle, and that inhibiting glutaminolysis does not affect cell proliferation. Moreover, Gln-starved cells are not rescued by TCA cycle replenishment. Instead, the conversion of Glu to Gln by glutaminolysis does not affect cell proliferation. Additionally, the conversion of Glu to Gln by glutamine synthetase (GS; cataplerosis) confers Gln prototrophy, and fuels proliferation. Moreover, Gln-starved cells are not rescued by TCA cycle replenishment. Instead, the conversion of Glu to Gln by glutaminolysis does not affect cell proliferation. However, it is shown here that in glioblastoma (GBM) cells, almost half of the glutamate (Glu) is secreted and does not enter the TCA cycle, and that inhibiting glutaminolysis does not affect cell proliferation. Moreover, Gln-starved cells are not rescued by TCA cycle replenishment. Instead, the conversion of Glu to Gln by glutamine synthetase (GS; cataplerosis) confers Gln prototrophy, and fuels de novo purine biosynthesis. In both orthotopic GBM models and in patients, 13C-glucose tracing showed that Gln produces Glu from TCA-cycle-derived carbons. Finally, the Gln required for the growth of GBM tumours is contributed only marginally by the circulation, and is mainly either autonomously synthesized by GS-positive glioma cells, or supplied by astrocytes.

Gln and Glu constitute a metabolic hub in cellular physiology. An increased demand for Gln by transformed cells has been recognized by biochemists for almost a century, and it has been linked to its role as an abundant circulating respiratory fuel. Notably, Gln carbons can support anabolism through entering the TCA cycle through glutaminolysis. Only specific tumour types exhibit Gln dependency, and its genetic and metabolic basis remains debatable. In certain cancer models, the inhibition of glutaminase (GLS), which deaminates Gln to Glu, reduces proliferation and tumorigenicity.

Conversely, GLS2 can be induced by the tumour suppressor p53 (ref. 11), and in human hepatocellular carcinoma, β-catenin increases the expression of GS, which catalyses the reversed GLS reaction. Originally, tuning of the Gln–Glu cycle was observed in the central nervous system where Glu is the most abundant neurotransmitter. Unlike astrocytes, glioma cells can release neuro-excitotoxic amounts of Glu, potentially promoting tumorigenesis. Gln addiction has been proposed as a mark of GBM, the most aggressive glioma. Here, we dissected the differential metabolic roles of Gln-derived carbon and nitrogen atoms in sustaining anabolism and growth in six human established GBM cell lines, in primary GBM stem-like cells, and in normal astrocytes. Additionally, Gln-related metabolism was investigated in both primary orthotopic murine xenografts and in GBM patients, leading to the identification of a GBM–astrocyte metabolic crosstalk.

RESULTS

Gln starvation reduces GBM cell proliferation unsystematically

To explore their growth response to different nutrient supplies, GBM cells were incubated either in DMEM containing supra-physiological concentrations of glucose and lacking some of the non-essential amino acids, or in a newly formulated SMEM, containing nutrient concentrations comparable to human serum (Supplementary Table 1).

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Both media were supplemented with various concentrations of Gln (Fig. 1a). In serum-like medium, all cell lines grew comparably to or faster than cells cultured in DMEM. In both media, the minimal Gln concentration required for maximal growth was below 0.65 mM, hereafter used as the control concentration. In the absence of Gln, cells grew faster in SMEM, demonstrating that medium formulation affects the response to Gln deprivation. Gln starvation hindered proliferation to different extents (Fig. 1b and Supplementary Fig. 1a) without inducing cell death, contrary to previous reports3,4, and to their response to glucose withdrawal (Fig. 1c). DNA flow-cytometry analysis showed that Gln starvation did not cause cell cycle arrest at any particular phase (Fig. 1d and Supplementary Fig. 1b). Overall, Gln withdrawal resulted in cell line-specific growth inhibition, ranging from 20% for U251 and SF188, to 80% for LN18 cells (Fig. 1e), independently of the initial proliferation rate (Fig. 1f).

Gln-based anaplerosis is not essential for the proliferation of GBM cell lines

To investigate cellular metabolic alterations on Gln starvation, the exchange rate of metabolites between cells and medium was analysed by high-performance liquid chromatography–mass spectrometry (HPLC–MS). Gln was the second most consumed nutrient by all cell lines (Supplementary Fig. 2 and Supplementary Table 2). However, no clear relationship emerged between Gln consumption and Gln dependency (Fig. 2a and Supplementary Fig. 3a). In contrast, all cell lines showed a net secretion of Glu despite its presence in the cell culture medium. The minimal concentration of Glu in serum-like medium was 0.65 mM, hereafter used as the control concentration. In the absence of Gln, cells grew faster in SMEM, demonstrating that medium formulation affects the response to Gln deprivation. Gln starvation hindered proliferation to different extents (Fig. 2b) without inducing cell death, contrary to previous reports3,4, and to their response to glucose withdrawal (Fig. 2c). DNA flow-cytometry analysis showed that Gln starvation did not cause cell cycle arrest at any particular phase (Fig. 2d and Supplementary Fig. 2b). Overall, Gln withdrawal resulted in cell line-specific growth inhibition, ranging from 20% for U251 and SF188, to 80% for LN18 cells (Fig. 2e), independently of the initial proliferation rate (Fig. 2f).
Figure 2 The effects of Glu secretion and Gln inhibition on GBM cell growth and metabolism. (a, b) Cells were incubated for 24 h with/without $^{13}$C$_2$-Gln. Secretion (positive bars) and consumption (negative bars) rates of Gln and Glu isotopologues are shown. Mean ± s.e.m. $n=3$ independent experiments. (c-f) Cells were incubated as in (a, b) and the levels of intracellular Gln, Glu, acetyl-CoA and oleate isotopologues are shown. Mean ± s.e.m. $n=3$ independent experiments. (g, h) LN18 and SF188 cells were incubated for 24 h with/without Gln in media where glucose (g) or alanine (h) was fully replaced by $^{13}$C$_2$-glucose or $^{13}$N$_2$-alanine, respectively. The isotopologue distributions of Glu released in the medium are shown. Mean ± s.e.m. $n=3$ independent experiments. (i) Scatter plot of Glu secretion observed in the absence of Glu, in relation to the growth inhibition caused by Glu starvation. Mean ± s.e.m. $n=3$ independent experiments. (j) A schematic representation of the XCL activity in the context of Glu metabolism. (k) LN18 cells were incubated for 24 h with/without Gln in media supplemented or not with Glu, α-ketoglutarate dimethylester (dm-αKG), sulphasalazine (SSZ) or cystine, at the indicated concentrations, and the secretion and consumption rates of Glu are shown. (l-o) LN18 cells were incubated as in (k) and the intracellular levels of Glu (l), aspartate (m), citrate (n) and the reduced form of glutathione (o) are shown as a percentage of the untreated control. (p) LN18 cells were incubated for 72 h as described for (k). Cell number is shown as a percentage of the untreated control. Mean ± s.e.m. $n=4$ independent experiments. $P$ values refer to a two-tailed t-test for unpaired samples. (q, r) Cells were pre-incubated in medium with 0, 2.5, 5, 10, 15, 30 μM BPTES for 3 h. At t=0, the medium was replaced with one containing $^{13}$C$_1$-Glu (q) or $^{13}$C$_2$-Gln (r) in the medium was monitored over time. In all conditions, cells were exposed to 0.3% DMSO. (s) Cells were incubated in medium with/without 2.5 μM BPTES for 72 h, and counted. DMSO was 0.3% in all conditions. Mean ± s.e.m. $n=3$ independent experiments. In (k-o) or twice (q, r). Raw data from independent repeats are provided in Supplementary Table 5.
Nor oleate (Fig. 2f) levels were reduced, re-affirming that Gln does not sustain fatty acids biosynthesis under normoxic condition\textsuperscript{16–20}. Consistently, in all cell lines, less than 15% of the citrate was derived from reductive carboxylation (\textsuperscript{13}C\textsubscript{5}-citrate; Supplementary Fig. 3b), and labelled acetyl-CoA and oleate were barely detectable (Fig. 2c).

\textbf{Figure 3} GS sustains cell growth during Gln starvation. (a) GS- and GLS-catalysed reactions. (b) Cells were incubated for 24 h with/without Gln and protein expression was assessed. (c) Scatter plot of GS protein expression observed in the Gln-fed condition (arbitrary units, a.u.) in relation to the growth inhibition caused by Gln starvation. Mean ± s.e.m. \( n=3 \) independent experiments. (d) LN18 and SF188 cells were incubated for 24 h with/without Gln in the presence of 0.8 mM NH\textsubscript{4}\textsuperscript{+}. The intracellular levels of Gln isotopologues are shown as a percentage of \( ^{15}\text{N}_0-\text{Gln} \) in LN18 cells. Data derive from one experiment performed twice. Raw data from independent repeats are provided in Supplementary Table 5. (e) SF188 and U251 cells were incubated for 72 h with/without Gln in medium supplemented with 4 mM Glu and 0.8 mM NH\textsubscript{4}\textsuperscript{+}, as indicated. Cell number is shown as a percentage of the respective Gln-fed control. Mean ± s.e.m. \( n=3 \) independent experiments. (f) Cells were incubated for 12–17 days with/without Gln in medium supplemented with 4 mM Glu and 0.8 mM NH\textsubscript{4}\textsuperscript{+} as indicated. Colonies obtained in representative wells are shown. \( n=4 \) independent experiments, quantified as shown in Supplementary Fig. 4b. Unprocessed original scans of western blots are shown in Supplementary Fig. 8.
To identify the carbon source for Glu synthesis under Gln starvation, Gln-deprived LN18 and SF188 cells were incubated with \(^{13}\)C\(_6\)-glucose. In both cases, the contribution of glucose carbons to Glu was marked increasing (Fig. 2g). Accordingly, alanine consumption was increased (Supplementary Fig. 2, inset), providing the nitrogen required for Glu production (Fig. 2h). Moreover, a strong direct correlation between Glu efflux and growth inhibition was observed (Fig. 2i), suggesting that on Gln withdrawal, Glu efflux limits the intracellular Glu available for reactions essential for growth.

To test whether Glu efflux indeed limited both its availability and the proliferation of Gln-starved cells, LN18 cells were incubated with sulphasalazine, an inhibitor of X\(_c\), a Glu/cystine antiporter (Fig. 2j) that is active in glioma cells\(^{15}\), or with 4 mM Glu, largely exceeding the K\(_s\) for X\(_c\) (ref. 21). In both conditions Glu release was largely inhibited (Fig. 2k). Consistently, the activation of X\(_c\) by increasing the extracellular concentrations of cystine, boosted Glu efflux (Fig. 2k), firmly associating X\(_c\) with the escape of Glu from GBM cells. Inhibiting X\(_c\) prevented the drop in intracellular Glu, aspartate and citrate, caused by Gln withdrawal (Fig. 2l–n).

The kinetics of Glu synthesis under Gln starvation, intracellular oleate was unaffected (Fig. 2f), and glucose-dependent Glu production increased (Fig. 2g–h), these results imply that the contribution of Gln to growth is largely independent of anaerolysis.

To evaluate this directly, BPTES, a GLS inhibitor, was employed. The kinetics of Gln-derived Glu secretion from GBM lines (Fig. 2i) that is active in glioma cells\(^{15}\), or with 4 mM Glu, largely exceeding the K\(_s\) for X\(_c\) (ref. 21). In both conditions Glu release was largely inhibited (Fig. 2k).

Glutamine synthetase sustains purine availability and cell growth under Gln starvation

Next, a genome-scale constraint-based metabolic modelling approach was employed\(^{22}\), searching for reactions that become essential when Gln is removed from the SMEM medium. According to the model, GS was the only enzyme essential for sustaining biomass production after Gln starvation (Supplementary Note).

Together, GS and GLS control Gln homeostasis by catalysing opposite reactions (Figs 2 and 3a). The messenger RNA levels of GS and GLS in all cell lines revealed no pattern of Gln dependency (Supplementary Fig. 4a). Although it has been proposed that c-Myc determines Gln addiction by increasing GLS expression\(^{13,23,24}\), MYC-induced lung tumours were shown to increase GS expression\(^2\). In line with this, SF188 cells, harbouring MYC amplification\(^{25}\), and expressing high levels of c-Myc (Fig. 3b and Supplementary Fig. 4a), showed the highest levels of GS mRNA and protein (Fig. 3b and Supplementary Fig. 4a). In most cell lines, GS protein levels rose on Gln deprivation (Fig. 3b). Furthermore, GS protein levels tend to increase in cells with decreased sensitivity to Gln withdrawal (Fig. 3c).

Nevertheless, GS did not match the residual low amount of Gln found in Gln-starved cells (Fig. 2c). To investigate this apparent discrepancy, the metabolic flux through GS was assessed by the incorporation of \(^{15}\)N-labelled ammonia (\(^{15}\)NH\(_4^+\)) into Gln in LN18 and SF188 cells, which exhibit low and high levels of GS, respectively. Significant levels of \(^{15}\)N-labelled Gln were indeed detected in SF188 but not in LN18 cells (Fig. 3d). Next, SF188 and U251 cells, exhibiting high levels of GS and low sensitivity to Gln withdrawal, were incubated with \(^{1}\)methionine sulfoximine (MSO), a selective irreversible inhibitor of GS. MSO sensitized cells to Gln starvation and, further, abolished the protective effect of Glu supplementation (Fig. 3e). To complement this approach, GS expression was stably silenced in these cells by two short hairpin RNA (shRNA) sequences (Fig. 3f). On Gln starvation, cell proliferation (Fig. 3g) as well as colony formation (Fig. 3h and Supplementary Fig. 4b) was lowered by GS silencing. Supplementation with the GS substrates Glu and ammonia rescued Gln-deprived control cells more effectively than GS-silenced cells (Fig. 3g,h).

To corroborate the causal link between Gln biosynthesis and Gln dependency, GS was overexpressed in LN18 cells, which exhibit low GS levels and high sensitivity to Gln deprivation. To this end, LN18-derived clones stably expressing infrared fluorescent protein alone\(^{26,27}\) (iRFP) or iRFP and GS were established. To eliminate intrinsic clonal variability, GS expression and its effect on growth under Gln starvation were evaluated in multiple clones (6 iRFP and 9 iRFP–GS). After five days of starvation, growth of iRFP control cells reached, on average, 16 ± 5% of control Gln-fed cells, and iRFP–GS clones reached, on average, 54 ± 12% (Fig. 4a).

Under Gln supplementation, the iRFP–GS\(_5\) clone proliferated slower than iRFP controls. This was not rectified by GS inhibition with MSO (Fig. 5b), consistent with a reported non-metabolic, anti-proliferative role of GS (ref. 28). Nevertheless, iRFP–GS\(_5\), but not control iRFP\(_K\) cells, proliferated and formed colonies in Gln-free medium, and this growth advantage was blocked by MSO (Fig. 4b,c). These results imply that under Gln starvation the amidation of Glu catalysed by GS sustains cell growth. In line with this, when supplemented with \(^{15}\)N\(_2\)–ammonia, GS-expressing cells exhibited \(^{15}\)N incorporation into ~50% of the total Gln pool, even when Gln fed (Fig. 4d). When incubated with \(^{15}\)N\(_2\)–ammonia without Gln, residual intracellular Gln was higher in iRFP–GS\(_5\) cells compared with control iRFP\(_K\) cells, and produced entirely by GS as judged by \(^{15}\)N incorporation (Fig. 4d).

To explore the essentiality and metabolic fate of de novo synthesized Gln under Gln starvation, we employed flux imbalance analysis\(^{29}\). In silico, nucleotide biosynthesis delimited cell growth, with a higher weighted cost for purine biosynthesis (Supplementary Note). Indeed, regardless of GS status, Gln removal only marginally affected the levels of the pyrimidine nucleotide uridine monophosphate (UMP; Fig. 4e). Moreover, the contribution of GS-derived...
Figure 4 GS activity regulates cell growth and purine availability under Gln starvation. (a) Top: LN18 clones stably expressing iRFP or iRFP–GS were incubated with/without Gln for 5 days and protein expression was assessed. Unprocessed original scans of western blots are shown in Supplementary Fig. 8. Bottom: For each clone, growth was determined from the protein amount and presented as a percentage of the respective control. Dashed lines show the mean percentage values obtained without Gln. (b) iRFP 4 and iRFP–GS 5 cells were incubated for the indicated times in medium with/without Gln and MSO (1 mM), and counted. (c) iRFP 4 and iRFP–GS 5 cells were incubated with/without Gln and MSO (1 mM) for 21 days, and colonies in representative wells are shown. Data derive from one experiment performed once. (d–j) iRFP 4 and iRFP–GS 5 cells were incubated with/without Gln for 24 h in medium supplemented with 0.8 mM 15N1Gln. The intracellular isotopologues of Gln, UMP, AICAR, IMP, AMP, ATP, and GTP are shown as a percentage of the values obtained for the 15N0 metabolites in iRFP 4 cells in the presence of Gln. (k) Cell lines were incubated with/without Gln for 24 h and the relative amount of intracellular IMP is shown. Mean ± s.e.m. n = 3 independent experiments. (l) Scatter plot of the changes in intracellular IMP levels in relation to the growth inhibition caused by Gln starvation. Mean ± s.e.m. n = 3 independent experiments. (m,n) iRFP 4 (m) and iRFP–GS 5 (n) cells were incubated for 72 h with/without Gln in medium containing adenosine (A), guanosine (G), cytidine (C), thymidine (T) and uridine (U), each at 0.2 mM, or in combination (AGCTU) at 0.2 mM each, Gln (4 mM) and MSO (1 mM), as indicated. Cell numbers are shown as percentage of untreated control. Dashed lines show percentage values obtained in the absence of Gln without any further supplementation. Mean ± s.e.m. n = 3 independent experiments. In a,b,d–j, the data derive from one experiment performed once (a,b) or twice (d–j). Raw data from independent repeats are provided in Supplementary Table 5.
\[^{15}N_{-}\text{Gln}\] to \[^{15}N_{-}\text{UMP}\] showed that the low GS activity in iRFP\textsubscript{4} cells could maintain UMP production. Similarly, the biosynthesis of UDP-\textit{N}-acetylglucosamine, an intermediate of hexosamine biosynthesis, which requires Gln-derived nitrogen, was also sustained in iRFP\textsubscript{4} cells during Gln starvation (Supplementary Fig. 4c). In contrast, 5-aminimidazole-4-carboxamide ribotide (AICAR), a purine
precursor for inosine monophosphate (IMP), dropped to undetectable levels in Gln-starved iRFPGS cells (Fig. 4f). The ammonia-derived AICAR ([15N2]-isotopologue) in Gln-starved iRFPGS cells demonstrates that GS contributes the two Gln nitrogen atoms required for its biosynthesis (Fig. 4f). Thus, [15N2]-IMP accumulated in Gln-deprived iRFPGS(GS) cells, but not in iRFPGS(GS) cells (Fig. 4g). The IMP found in iRFPGS(GS) starved cells corroborated the reported inhibitory effect of Gln deprivation on IMP dehydrogenase19, and suggests that under these conditions over-activity of GS exceeds the rate of IMP conversion to AMP and GMP. Indeed, AMP levels were not significantly affected by either Gln presence or GS overexpression (Fig. 4h). Moreover, ATP and GTP levels, indices of the cell’s bioenergetic state, were comparable between iRFPGS(GS) and iRFPGS(GS) starved cells (Fig. 4i,j). Conversely, the fractions of [15N]-labelled AMP, ATP and GTP found in iRFPGS(GS) cells exceeded those of iRFPGS cells, demonstrating that, under Gln starvation, GS sustains de novo biosynthesis of purine nucleotides.

Notably, the reduction in IMP caused by Gln starvation in the six GBM cell lines correlated with Gln dependency (Fig. 4k,l). Accordingly, adenosine, but not guanosine or pyrimidine nucleosides, partially restored Gln-independent growth of iRFPGS(GS) cells (Fig. 4m). Furthermore, the combined addition of adenosine and Gln compensated for the lack of exogenous Gln in iRFPGS(GS) cells (Fig. 4m), and Gln alone completely restored proliferation of iRFPGS(GS) starved cells (Fig. 4n). In both lines, MSO prevented the Gln rescue, confirming that under Gln starvation, Gln availability determines Gln production rather than anaplerosis. The effect of adenosine on Gln-starved cells was MSO-independent (Fig. 4m,n), because it supported proliferation downstream of GS.

Primary human GBM stem-like cells are self-sufficient for Gln requirements

The clinical relevance of studying established glioma cell lines has been questioned owing to their inability to form tumours that recapitulate human pathology. Therefore, we used three primary patient-derived GBM cell lines (E2, R10, R24), generating paired populations of differentiated cells (DIFF) and glioma stem-like cells (GSC). Stem cell markers such as CD133, Olig2 and Sox2 were predominantly expressed in GSC but not DIFF; however, the astrocytic marker glial fibrillary acidic protein (GFAP) was not consistently associated with the DIFF population (Fig. 5a). The expression of GS was markedly higher in all GSC compared with DIFF (Fig. 5a) and whereas DIFF proliferation was attenuated in the absence of Gln, GSC grew independently of Gln supplementation (Fig. 5b). Once more, the growth of Gln-starved DIFF and GSC was abolished by MSO (Fig. 5b).

Next, the exchange rates (Fig. 5c,d) and intracellular composition of metabolites (Fig. 5e–h) were analysed in these primary Gln-starved or control cells, in the presence of [15N1]-ammonia. The net Gln consumption was consistently higher in DIFF compared with GSC, whereby R24-GSC demonstrated no net Gln consumption (Fig. 5c). As the intracellular [15N1]-Gln fraction shows, GSC have higher GS activity compared with DIFF and sustain higher residual Gln levels on starvation (Fig. 5e). The differences in Glu exchange rates were also striking: GSC exhibited a net uptake of Glu, whereas the reverse occurred in DIFF (Fig. 5d). Also, on Gln withdrawal, intracellular levels of citrate (Fig. 5g) decreased in DIFF, but remained unaltered in GSC, showing that Gln-derived anaplerosis was redundant to the stem-like population. Finally, ammonia-derived [15N] incorporation into purine nucleotides ([15N2]-AMP) under Gln starvation was greater in GSC compared with the paired DIFF.

Human GBM tumours rely on in situ de novo Gln synthesis

As above, GS activity, largely determining Gln dependency, varies between established and primary human GBM cells. Similarly, tissue microarray (TMA) analysis showed that GS expression varies between human GBM patients (n = 209), resembling a Gaussian distribution ranging from tumours with low GS levels, comparable to neurons (25% of patients), to high-expression tumours comparable to astrocytes (15%; Fig. 6a,b). However, GS expression did not predict patient median survival (Fig. 6c). Of twenty biopsies, from which core TMA were sampled, five showed substantial intratumoral GS immunostaining heterogeneity (three examples are reported in Supplementary Fig. 5). Most GBMs showed either GS uniformity, or a mosaic infiltration of GS-positive cells, suggesting autonomous intratumoral Gln biosynthetic capacity. To assess this hypothesis in human tumours, seven GBM patients were injected with [13C6]-glucose before surgery, and metabolites were extracted from the resected tumours and their oedematous margins. The metabolic analysis reliably discriminated between tumour and adjacent tissues by using the choline to creatine ratio, a parameter for classifying brain tumours by magnetic resonance spectroscopy22 (Fig. 6d). No significant difference in Gln content was observed between tumour and adjacent tissues (Fig. 6e). At the time of resection, [13C6]-glucose enrichment in sera ranged between 16 and 50% (Fig. 6f, and Supplementary Fig. 6a). Glucose-derived [13C]-Gln was detected in 6/7 tumours and in 7/7 adjacent oedematous tissues with an enrichment ranging between 1 and 12% (Fig. 6g). In 3/5 patients the fraction of glucose-derived Gln in the tumour was higher than in the serum sample, and so not in equilibrium with the circulating Gln, suggesting that the tumour Gln pool is synthesized in situ and/or provided by adjacent normal brain.

To complement the analysis in patients, mice were orthotopically transplanted with a GS-positive human GBM (P3, Fig. 6h) and injected with [3-13C]-labelled glucose or Gln ~20 min before tissue extraction. An enrichment of 44 ± 3% of [13C6]-glucose was found in the blood at the time of tissue sampling. The intracellular hexose phosphate pool derived from [13C6]-glucose was ~10% and 5% in tumour and contralateral brain tissue, respectively (Fig. 6i). Concomitantly, ~10% and 15% of the total Gln was labelled ([13C2]) from [13C6]-glucose in tumours and contralateral brain, respectively (Fig. 6i), consistent with GS activity in those tissues. After [13C3]-Gln injection, the enrichment in circulating [13C3]-Gln at the time of tissue sampling was 17 ± 1%. Isotopologue distribution analysis showed an enrichment in [13C3]-Gln of <5% in both tumour and contralateral brain tissues (Fig. 6j), whereas in the liver, [13C3]-Gln presented 12% of the total (Supplementary Fig. 6e). Products of glutaminolysis, such as Glu and α-ketoglutarate, were labelled below 1% in both tumour and brain tissues but ~5% in liver (Fig. 6j and Supplementary Fig. 6e). These results suggest slow kinetics both for Gln uptake from the blood, and for glutaminolysis in GBM and brain tissues, compared with liver. Similar results were obtained on constant carotid artery [13C6]-Gln infusion: within two hours, circulating [13C6]-Gln levels plateaued at ~20% enrichment, with an overall ~70% increase in steady-state levels of Gln (Supplementary Fig. 6f). Here too, [13C6]-Gln and
Figure 6 Gln metabolism in GBM patients and primary orthotopic xenografts.  
(a) GBM tissue microarray. GS immunostaining of representative tissue cores at low and high magnification (top and bottom, respectively). A, astrocyte; N, neuron. (b) Frequency distribution of GBM patients (n = 209) divided according to their histoscore for GS, and categorized as low, medium, and high. Normal astrocytes were used as a reference for defining maximal immunoreactivity. (c) Kaplan Meier curves for GBM patients divided into low, medium and high GS expression. *P* value refers to a log-rank (Mantel–Cox) test. (d) Creatine, choline, choline to creatine ratio (d) and Gln (e) levels in tumour tissue and adjacent oedematous brain of GBM patients injected with $^{13}$C-glucose before surgical intervention. *n* = 7 patients; *P* values refer to a two-tailed *t*-test for paired samples. (f,i) $^{13}$C-glucose (f), and $^{13}$C-Gln (g) enrichment in serum at time of tumour resection, in tumour tissue, and in adjacent oedematous tissue. Gln isotopologues incorporating one or more $^{13}$C atoms, over the total amount of Gln detected (percentage of total) are shown. NA, not available; ND, not detectable. Values were corrected for the natural abundance of $^{13}$C. (h) Coronal section of a human P3 GBM xenograft grown in the brain of immunocompromised mice, and stained for human nestin and GS. Lower panels are a magnification of the respective framed regions. A, astrocytes; AF, astrocytic end-feet; N, neuron; V, blood vessel. (i,j) Isotopologue distribution of metabolites (hexose phosphates, citrate, $\alpha$KG, Glu, Gln) obtained in mice orthotopically xenografted with human P3 GBM, and injected in the tail vein with a bolus of $^{13}$C-glucose (i) or $^{13}$C-Gln (j). Tissues were sampled 22 min after injection. The values are mean ± s.e.m. *n* = 3 mice for all conditions, except for contralateral brain of mice injected with glucose, where 2 mice were used. For i,j, raw data from independent repeats are provided in Supplementary Table 5.
glutaminolysis products were scarce in tumour and contralateral brain (Supplementary Fig. 6h,i) indicating that physiologically, circulating Gln does not significantly supply Gln to the brain or the tumour within it.

Next, Erwinase, an enzyme that temporarily depletes circulating asparagine (Asn) and significantly reduces Gln (refs 9,33; Fig. 7a), was injected daily 5 times per week into mice bearing GS-negative orthotopic GBM xenografts (T101, Fig. 7b). Erwinase reduced intratumoral and intracerebral Asn but not Gln (Fig. 7c). The diffuse morphology of GS-negative tumours impaired the volumetric quantification of tumours by magnetic resonance imaging (MRI) (Fig. 7d). Histological reconstruction to assess tumour burden (Fig. 7e) revealed no significant differences between control and Erwinase-treated groups (Fig. 7f). Together, these results indicate that Gln is not provided to GBM by the blood, and so the proximity of GS-expressing cells to GS-negative ones (Fig. 7g) revealed a scenario in which Gln, provided by astrocytes, feeds GS-negative cancer cells (Fig. 3g). Indeed, primary astrocytes in culture retain GS expression between patients (Fig. 6b) and within some tumours (Fig. 7j). The stability of Gln levels may explain the heterogeneity in Gln consumption but rather, rapid Glu uptake (Fig. 8d,e), in line with the expression of excitatory amino acid transporters in this cell type. Under Gln starvation, Glu consumption was unaffected and paralleled by an equimolar net Gln efflux (Fig. 8d,e). The absence of Gln in the medium reduced intracellular Gln, but not Glu (Fig. 8f,g). Moreover, $^{13}$C$_6$-glucose tracing showed that only 30–40% of both intracellular Glu and Gln (Fig. 8f,g) was glucose-derived. Astrocytes maintained ~30% of the control level of intracellular Gln under Gln starvation (Fig. 8f,h), fitting with high Gln expression. Gln maintenance depended on GS activity, as seen from both $^{15}$N$_2$-ammonia tracing and GS inhibition by MSO (Fig. 8h). Moreover, GS inhibition markedly elevated the intracellular amounts of its substrate, Glu (Fig. 8i), without changing the steady-state levels of the Gln product, AMP (Fig. 8j). However, combined Gln withdrawal and GS inhibition significantly reduced the labelled fraction of AMP derived from de novo synthesis ($^{15}$N$_2$ and $^{15}$N$_3$; Fig. 8j), and hindered proliferation (Fig. 8k).

Finally, in co-culture, astrocytes enabled the proliferation of GS-negative LN18 iRFP$_3$ cells without Glu supplementation (Fig. 8l). Moreover, Transwell co-culturing of these cells showed that the factor conveying growth was diffusible (Fig. 8m,n). The addition of Erwinase, which depletes both Asn and Gln, prevented the rescue of Gln-deprived cells by astrocytes (Fig. 8m,n). As Asn was present in the media during all Gln-starvation experiments, and because astrocytes consume Asn but produce and secrete Gln (Fig. 8d and Supplementary Fig. 6k), these results designate astrocyte-derived Gln as the growth-supporting factor for Gln-starved GBM cells.

**DISCUSSION**

Gln plays multiple metabolic and non-metabolic roles. Consequently, the dependency of cancer cells on Gln is difficult to discern. Nevertheless, it was demonstrated here that the Gln requirement in GBM goes beyond anaplerosis (Fig. 2q–s). We identified two alternative metabolic determinants for Gln sensitivity: Glu release through the X$^-$ antiporter; and G$^+$-dependent conversion of Glu to Gln. GS and X$^-$ seemingly compete for cytoplasmic Glu, which during Gln starvation becomes limiting (Fig. 2d,l). Nevertheless, the rescuing effect achieved by maintaining intracellular Glu through X$^-$ inhibition depends on G$^+$ activity (Fig. 3e,h,g and Fig. 4m,n). These results demonstrate that on Gln starvation, Glu conversion to Gln constitutes a critically limiting reaction required for growth. This metabolic trait applies to established GBM lines, and to naive primary cells. Indeed, when primary GBM cells were maintained in a stem-like state, GS expression was markedly increased (Fig. 5a) and Glu was taken up rather than released (Fig. 5d). Both responses enable growth of glioma stem-like cells independent of extracellular Gln (Fig. 5b). The fate of newly synthesized Gln in both established and primary GBM cells was followed by $^{15}$N$_1$-ammonia tracing, identifying the AMP biosynthesis pathway as a significant player in Gln dependency (Fig. 4d–j and Fig. 5b).

GS is found in most human GBM (Fig. 6a,b), and its expression is associated with poor prognosis,$^{35}$ although this is not supported by our TMA study. However, we show that GS expression varies greatly between tumours, ranging from negative, comparable to GS expression in neurons, to high, as in normal astrocytes. This variation accords with a Gln-rich tumour microenvironment, which alleviates the need to synthesize Gln. Nevertheless, in agreement with previous reports,$^{36,37}$ it is shown here that most human GBM, as well as GS-proficient orthotopic GBM xenografts, withdraw carbons from the TCA cycle (cataplerosis) to synthesize Gln by means of GS. When compared with the liver, GBM are inclined towards net Gln synthesis, rather than glutaminolysis (Fig. 6i and Supplementary Fig. 6d). Accordingly, the circulation provides minimal amounts of Gln to normal brain$^{38}$ and GBM (Fig. 6i and Supplementary Fig. 6f,h,i). Moreover, a marked decrease in circulating Gln levels did not affect tumour growth (Fig. 7a–h), and GBM expressing low GS had levels of ammonia-derived Gln comparable to contralateral brain (Fig. 7i). The stability of Gln levels may explain the heterogeneity in GS expression between patients (Fig. 6b) and within some tumours (Supplementary Fig. 5): GS-positive astrocytes and/or GBM cells (potentially GSC) excrete Gln that supports the growth of GS-negative GBM cells. Indeed, primary astrocytes in culture retain the metabolic traits of Glu uptake, GS-dependent Gln synthesis and Gln secretion, and support the proliferation of GS-negative, Gln-auxotrophic GBM cells (Fig. 8l–n). Thus, this brain-to-tumour metabolic communication portrays a scenario in which Gln, provided by astrocytes, feeds GS-negative cancer cells (Fig. 3g). Indeed, GS-positive cellular protrusions of astrocytes surround GS-negative GBM cells in orthotopic xenografts and human tumours (Fig. 7b,c).
Figure 7 Glutamine supply for GBM tumours with low GS expression. (a) Gln and Asn levels were measured by HPLC–MS in peripheral blood samples obtained at the indicated time points from immunocompromised mice intraperitoneally injected with Erwinase (5 U g\(^{-1}\) of body weight). Mean ± s.e.m. \(n = 5\) mice. \(P\) values refer to a two-tailed \(t\)-test for paired samples. (b) Coronal section of a human T101 GBM xenograft grown in the brain of immunocompromised mice, and stained for human nestin and GS. Right panels are a magnification of the respective outlined regions. A, astrocytes; N, neuron. (c–f) Immunocompromised mice were orthotopically implanted with T101 GBM tumours and treated with Erwinase for 6 weeks as described in the Methods. (c) Asn and Gln were assessed in the tumour and contralateral brain 6 h after the last Erwinase injection. Mean ± s.e.m. \(n = 3\) mice. (d) MRI-based apparent diffusion coefficient (ADC) maps of T101 GBM tumours. The tumour mask has been manually delineated to highlight the tumour region. IHC staining of brain sections corresponding to the MRI scans is shown. T101 tumours were stained with an anti-human EGFR antibody. (e) Two representative series of coronal sections of T101 brain xenografts were stained for human EGFR. (f) Volumes of T101 orthotopic tumours obtained thorough quantitative imaging of EGFR-stained serial sections of brains. Mean ± s.e.m. \(n = 7\) mice. \(P\) value refers to a two-tailed \(t\)-test for unpaired samples. (g) Coronal section of a human T407 GBM xenograft grown in the brain of immunocompromised mice, and stained for human nestin and GS. Right panels are a magnification of the respective framed regions. V, blood vessel. (h) \(^\text{15}\)N\(_1\)-Gln enrichment in T407 GBM tumours and in contralateral brains, after a 4 h intracarotid infusion with \(^\text{15}\)NH\(_4\)\(^+\). The dashed lines correspond to the natural abundance of \(^\text{15}\)N\(_1\)-Gln. Mean ± s.e.m. \(n = 4\) mice. \(P\) value refers to a two-tailed \(t\)-test for paired samples.
Figure 8 Astrocytes provide GBM cells with Gln. (a,b) Astrocytes were incubated in SMEM for 6 days with 0, 0.1, 0.3, 0.65, 1, 2 and 4 mM Gln (a) or for the indicated times with 0 and 0.65 mM Gln (b). (c) Astrocytes derived from two independent extractions, and cell lines, were incubated for 3 days with/without Gln and protein expression was assessed. Unprocessed scans of western blots are shown in Supplementary Fig. 8. (d–j) Astrocytes were incubated in SMEM for 6 days with 0, 0.1, 0.3, 0.65, 1, 2 and 4 mM Gln and protein expression was assessed. Unprocessed scans of western blots are shown in Supplementary Fig. 8. (k) Astrocytes were incubated for 6 days with/without 0.65 mM Gln, and 1 mM MSO and counted. (l) Astrocytes were grown to confluence in multi-well plates. iRFP4 cells were seeded in wells with/without astrocytes, with/without Gln, and with/without Erwinase (Erw) as indicated. The fluorescence of iRFP4 cells in representative wells is shown. The experiment was performed twice with comparable results. (m) Astrocytes were grown to confluence in multi-well plates. iRFP4 cells seeded in Transwell inserts were co-cultured with/without astrocytes, with/without Gln, and with/without Erwinase (Erw) as indicated. Fluorescence of iRFP4 cells in representative inserts is shown. At day 5 astrocytes were stained with sulforhodamine-B and the fluorescence of representative wells is shown. (n) Quantification of the iRFP4 fluorescence as described for m. In a,b,d–k,n, the data derive from one experiment performed twice. Raw data from independent repeats are provided in Supplementary Table 5.
and Supplementary Fig. 5p). This ‘parasitic’ behaviour of cancer cells could divert the physiological Gln–Glu cycle in the brain and, indeed, an increase in epileptic seizures has been reported for patients with GBM expressing low GS (ref. 39).

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

S.T. conceived the study, designed and performed most experiments, interpreted the data, and wrote the manuscript. A.O. performed the experiments in the orthotopic xenograft models, S.U.A. and A.J.C. provided the differentiated and stem-like primary glioblastoma cells, L.Z. supervised the analysis of LC-MS samples, O.K. performed the MRI analysis, F.F. processed the orthotopic and clinical GBM samples, S.H.M. provided the tissue microarray, A.K.H. designed and provided the iRFP and iRFPGS constructs, A.W. and E.R. generated and employed the IRFP-GS constructs, A.Weinstock, A.Wagner and E.R. generated and employed the metabolic modelling, S.C.B. and S.L.L. provided the primary astrocytes, M.L.-J., S.H.M. and P.O.S. provided the surgical specimens from the patients, S.P.N. and R.B. conceived and supervised the experiments in orthotopic models and human patients, and E.G. conceived and supervised the study, interpreted the data, and revised the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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METHODS

Cell cultures. The human glioblastoma cell lines MOG-G-UVW, LN-18, LN-229, SF-188, U-251 MG and U-87 MG (hereafter referred to as GUVW, LN18, LN229, SF188, U251 and U87, respectively) were obtained from the following sources; University of California, San Francisco, USA; SF188; Brain Tumour Tissue Bank University of California San Francisco, USA; U251 and U87: kindly provided by K. Ryan at the Cancer Research UK—Beatson Institute, UK and A. Chalmers at the University of Glasgow, UK; LN18 and LN229: ATCC; SF188: Brain Tumour Tissue Bank University of California San Francisco, USA; U251 and U87: kindly provided by K. Ryan at the Cancer Research UK—Beatson Institute, UK and A. Chalmers at the University of Glasgow, UK, respectively. The cell lines were authenticated using Promega GenePrint 10 system (STR multiplex assay that amplifies 9 tetranucleotide repeat loci and the Amelogenin gender determining marker). No cell line used in the manuscript has been found in the ICLAC-database of commonly misidentified cell lines. Cortical astrocytes were generated from the cortex of P7 Sprague-Dawley rat pups (unclassified gender). Briefly, both cortices were removed from each pup cleared of meninges and enzymatically dissociated and purified as previously described. Tissue from 2 pups was added to each 75 cm² tissue culture polystyrene-flask coated with laminin at a density of 2 × 10^5 cells per flask. The cells were allowed to attach for 2 h at 37°C in a humidified 5% CO₂ incubator. Cell number was assessed at the specified experimental time as a function of cell viability using a Casy cell counter (Sartorius). The doubling time (DT) was obtained using the exponential growth equation with GraphPad prism 5.0. The percentage of growth inhibition was calculated according to the following equation: 

\[
\frac{\text{Growth inhibition}}{100} = \frac{\text{Surviving cell number}}{\text{Initial cell number}} - 1
\]

The percentage of growth inhibition was calculated according to the following equation: 

\[
\left(1 - \left(\frac{1}{2^{\text{DT}}} \right) \left(\frac{1}{2^{\text{DT}}} \right) \left(\frac{1}{2^{\text{DT}}} \right) \right) \times 100
\]

Cell cycle analysis. Culture media and cells were collected, centrifuged and washed with PBS. Cells were then fixed with cold methanol and stained with a PBS solution containing propidium iodide (100 µg ml⁻¹) and RNase A (250 µg ml⁻¹). DNA nuclear content was measured by using a Beckton Dickinson FACScan flow cytometer and data were analysed with FlowJo software.

Tissue microarray (TMA) and immunohistochemistry (IHC). The collection of human biopsy tissue was approved by the ethical committee at Haukeland University Hospital, Bergen, Norway (REK 2010/130-2). Primary orthotopic human GBM xenografts. Patient-derived GBM xenografts were generated as previously reported [41,42]. Briefly, GBM patient-derived spheroids were stereotactically implanted into the right frontal cortex of 8-12-week-old, male, and female, NOD SCID mice (Charles River) using a Hamilton syringe. After 4–6 weeks a P3 GBM tumour was fully established in the brain, and U-13C6-glucose or U-13C6-glutamine (Cambridge Isotope Laboratories) was injected as a bolus in the tail vein at a dose of 1 mg kg⁻¹ and 0.15 mg kg⁻¹ of body weight respectively. Twenty-two minutes after injection, the animals were euthanized, organs were dissected, and frozen tissues were stored at -80°C for further analysis. The mice were anesthetized by isoflurane and underwent a surgical procedure to expose the right carotid artery. The extracts were spun down at 16,000 g for 10 min and supernatants were stored at -80°C for LC–MS analysis. Human glioblastoma biopsies were obtained from the Neurosurgery Department of the Centre Hospitalier in Luxembourg (CHL). All patients had provided written informed consent, with procedures that were approved by the regional ethical committee at Haukeland University Hospital, Bergen, Norway (REK 2010/130-2).
The following primers were used:

- actin_F 5 primer
- actin_R 3 primer
- MYC_F 5 primer
- MYC_R 3 primer

The secretion/consumption rate for a specific metabolite ($x$) was obtained according to the equation $x = (2 \times (\Delta_{\text{metabolite}})/\mu\text{g prot}_{\text{in}} + \mu\text{g prot}_{\text{out}})$, where $\Delta_{\text{metabolite}} = (x) \text{nmol medium with no cell} - (x) \text{nmol medium with cells}$.

For the IR scanning was performed using a Licor Odyssey scanner and acquired using Image Studio 2.0. All western blots shown are representative of two experiments.

The secretion/consumption rate for a specific metabolite ($x$) was obtained according to the equation $x = (2 \times (\Delta_{\text{metabolite}})/\mu\text{g prot}_{\text{in}} + \mu\text{g prot}_{\text{out}})$, where $\Delta_{\text{metabolite}} = (x) \text{nmol medium with no cell} - (x) \text{nmol medium with cells}$.

For the LC separation of medium samples a ZIC-HILIC (SeaQuant) with a guard column (Hichrom) was used. Mobile phase A was a 0.1% formic acid solution in water and mobile phase B was 0.1% formic acid in acetonitrile. The flow rate of the mobile phase was kept at 0.1 mL min$^{-1}$ and the gradient was as follows: 0 min 10% of B, 12 min 50% of B, 26 min 50% of B, 28 min 20% of B, 36 min 20% of B and 37 to 45 min 80% of B. For the separation, cell extracts were injected on a ZIC-HILIC column with a guard column. (Mobile phase C: 20 mM ammonium carbonate plus 0.1% ammonium hydroxide in water. Mobile phase D: acetonitrile.) The flow rate was kept at 100 $\mu$L min$^{-1}$ and gradient as follows: 0 min 80% of D, 30 min 20% of D, 31 min 80% of D and 45 min 80% of D. The Exact Orbitrap mass spectrometer (Thermo Scientific) was operated in a polarity switching mode.

**Cell transfection and infection.** For stable transfection 10 cm dishes confluent LN18 cells were incubated for 9 h with 10 mL of FBS-free transfection medium containing 1 mg mL$^{-1}$ of DNA (vector containing P2A-IRF5 IRES puro alone, or GS-P2A-IRF5 IRES puro), and 2 $\mu$L mL$^{-1}$ of Lipofectamine2000 (Invitrogen). The transfection mixture was than replaced with 10 mL of complete DMEM. The following day medium was changed and cells were cultured for 3 weeks in selection medium containing 2 mg mL$^{-1}$ of puromycin. Fluorescent colonies were visualized with the Licor Odyssey scanner, picked and amplified in selection medium to obtain stable cultures. For the establishment of cell lines expressing shRNA against glutamine synthetase (GS), four human unique shRNA sequences of 29 nucleotides, and a scrambled negative control non-effective shRNA cassette (NTC) in a lentiviral GFP vector, were employed according to the manufacturer's instructions (TL312740a: 5'-GGTGAGAAAGTCCAGGCCATGTATATCTG-3'; TL312740b: 5'-GAATGGTGCAGGCTGCCATACCAACTTCA-3'; TL312740c: 5'-GGCACAACCTGAAATGACTGGAGAAGGACTGCGCTGCAAGAATGGTGCAGGCTGCCATACCAACTTCA-3'; TL312740d: 5'-GGGCACAACCTGAAATGACTGGAGAAGGACTGCGCTGCAAGAATGGTGCAGGCTGCCATACCAACTTCA-3'). The metabolic modelling methods and results is provided in the Supplementary Note. The values obtained from the macro were normalized to the metabolic model $C$. For infection of recipient cells the supernatants were analysed with trichloroacetic acid, stained with SRB and plates were scanned using a Licor Odyssey scanner. Images were acquired using Image Studio 2.0. SF188 and U251 cells expressing NTC shRNA and GS shRNA sequences were seeded in 6-well plates, at 500 and 350 cells per well for SF188 and U251 cells, respectively. Cells were incubated in complete DMEM and supplemented as indicated in the legend of Fig. 3. Twelve to seventeen days after seeding, colonies were fixed, stained, and representative images obtained as described above. For the quantification of colony surface area an ImageJ macro was designed and run. The values obtained from the macro were normalized for the exact number of days cells were incubated, and presented as percentage of relative control.

**Colony-forming assay.** IRFP and IRFP-GS-expressing cells were plated at 300 cells per well in 6-well plates in complete SMEM, in the absence or presence of 0.65 mM Gln and 1 mM MSO as indicated. After 3 weeks, colonies were fixed with trichloroacetic acid, stained with SRB and plates were scanned using a Licor Odyssey scanner. Images were acquired using Image Studio 2.0. SF188 and U251 cells expressing NTC shRNA and GS shRNA sequences were seeded in 6-well plates, at 500 and 350 cells per well for SF188 and U251 cells, respectively. Cells were incubated in complete DMEM and supplemented as indicated in the legend of Fig. 3.

**Statistical methods.** No statistical method was used to predetermine sample size. For the animal studies, the experiments were not randomized, and the investigators were centrifuged at 16,000g for 10 min at 4 °C and the supernatants were analysed by means of HPLC–MS. The extracted cell monolayer was used for protein determination (μg prot$_{\text{in}}$) with a Lowry assay. Aliquots of freshly prepared SMEM, without FBS, and spiked with known concentrations of lactate were processed in parallel and used as a reference for calibration. The secretion/consumption rate for a specific metabolite ($x$) was obtained according to the equation $x = (2 \times (\Delta_{\text{metabolite}})/\mu\text{g prot}_{\text{in}} + \mu\text{g prot}_{\text{out}})$, where $\Delta_{\text{metabolite}} = (x) \text{nmol medium with no cell} - (x) \text{nmol medium with cells}$.
were not blinded to allocation during experiments. The number of independent experiments performed is reported in the figure legends. For experiments performed once or twice, the raw data of independent repeats are provided in the statistics source data. Error bars represent standard error of the mean (s.e.m.). Two-tailed Student’s t-tests, Pearson correlation tests, and Log-rank (Mantel–Cox) tests were performed with Graph Pad Prism 5.0.

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Supplementary Figure 1 Glutamine starvation reduces GBM cell proliferation.
(a) Representative microscopic fields of cells incubated for 6 days with or without Gln. (b) Cell cycle distribution of cells incubated for 3 days with or without Gln. Mean ± S.E.M. n=3 independent experiments. p value refers to a two-tailed t test for unpaired samples. Raw data of independent repeats are provided in the statistics source data Supplementary Table 5.
Supplementary Figure 2: Exchange rates of metabolites in GBM cell lines. Cells were incubated for 24h +/- U-13C5 Gln, secretion rates (positive bars) and consumption rates (negative bars) of the indicated metabolites are shown. The sum of all isotopologues is reported for clarity. Alanine (Ala) exchange rates are magnified in the inset. Mean ± S.E.M. n=3 independent experiments.
Supplementary Figure 3 Effects of Gln availability and Glutaminase inhibition on GBM cell lines growth and redox state. (a) Correlation between Gln consumption and growth inhibition caused by Gln starvation is shown in six cell lines. Mean ± S.E.M. n=3 independent experiments. (b) Citrate isotopologues distribution of $^{13}$C$_5$-Gln derived atoms. $^{13}$C$_6$-Citrate was not detected. Mean ± S.E.M. n=3 independent experiments. (c) Oxidized (GSSG) to reduced (GSH) glutathione ratio. LN18 cells were incubated for 24h +/- Gln and supplemented with Glu, $\alpha$-ketoglutarate dimethylester (dm-$\alpha$KG), sulfasalazine (SSZ), or cystine, as indicated. GSSG/GSH ratio was assessed by HPLC-MS, and shown as % of untreated control. Data derive from one experiment performed once. Raw data of independent repeats are provided in the statistics source data Supplementary Table 5. (d-i) Cells were incubated with 0, 2.5, 5, 10, 15, 30$\mu$M BPTES for 72h, in the absence (solid line) or presence (dotted line) of 4mM dm-$\alpha$KG and counted. In all conditions cells were exposed to 0.3% DMSO. Mean ± S.E.M. n=3 independent experiments.
Supplementary Figure 4  GS expression and its effects on colony formation capacity, and glucosamine biosynthetic pathway in GBM cells. (a) Cells were incubated for 24h +/- Gln and GLUL (GS), GLS, and MYC mRNA relative expression was assessed by qPCR. Actin expression was used for normalization. Data derive from one experiment performed once. Raw data of independent repeats are provided in the statistics source data Supplementary Table 5. (b) SF188 and U251 cells stably expressing a non-targeting control shRNA (shNTC) and two sequences targeting GS (shGS-1 and shGS-2) were cultured for 12-17 days +/- Gln in medium supplemented with glutamate (4mM), and ammonia (0.8mM) as indicated. The quantification of colonies surface area was obtained as described in the Methods section. Mean ± S.E.M., n=4 independent experiments. (c) iRFP4 and iRFP-GS5 expressing LN18 cells were incubated +/- Gln for 24h in medium supplemented with 0.8 mM 15NH4+. The intracellular isotopologues distribution of UDP N-acetylglucosamine is shown as % of control (value obtained for the metabolites in iRFP4 cells + Gln). Data derive from one experiment performed twice. Raw data of independent repeats are provided in the statistics source data Supplementary Table 5.
Supplementary Figure 5 Heterogeneous GS expression in human GBM biopsies. The whole biotic tissues of three patients included in the tissue microarray were stained for GS, and Hematoxilin and Eosin [H&E], as indicated. Low magnifications of whole biopsies are reported in panels a-b, h-i, n-o. For each biopsy, tissue regions framed in blue and green are magnified in d-g, j-m, q-t. Biopsy 1 shows necrotic tissue [nec] surrounded by a solid tumour core [TC] with uniformly low GS staining (d-f). (d-e) Invasive tumour areas [TI] show infiltrative cells with heterogeneous immunoreactivity for GS. (c) Normal astrocytes [black arrows] in the adjacent normal brain [Br] were positive for GS, while neurons [white arrows] were GS-negative. Biopsy 2 shows distinct areas with low/absent (k) and high (j) immunoreactivity for GS. GS-negative inflammatory tissue [i] surrounding a necrotic region [nec] is evident in panels h-i, k-m. Biopsy 3 shows distinct areas with low/absent (q) and high (r) immunoreactivity for GS. (p) GS-positive reactive astrocytes [arrows] with extensive cellular processes forming a network of GS positive fibers surrounding GS-negative tumour cells.
Supplementary Figure 6 Glucose and glutamine metabolic fates in GBM patients, and orthotopic mouse models. (a) Serum levels of $^{13}$C$_6$ and $^{13}$C$_0$ glucose in three GBM patients injected with $^{13}$C$_6$ glucose before surgical intervention for tumour removal. The dashed line represents the time at which glucose infusion starts. Black arrow points at the time of surgical resection. Values are reported as % of the basal values obtained before infusion. (b) $^{13}$C Lactate and (c) $^{13}$C Glutamate enrichment in serum at time of tumour resection, in tumour tissue, and in adjacent edematous tissue, of seven (1-7) GBM patients injected with $^{13}$C$_6$-glucose. The % of lactate or glutamate incorporating one or more $^{13}$C carbons over the total amount of lactate or glutamate detected is reported; na: not available, nd: not detectable. Values were corrected for the natural abundance of $^{13}$C. (d-e) Isotopologue distribution of metabolites obtained in the liver of mice orthotopically xenografted with human P3 GBM and injected with $^{13}$C$_6$ Glucose (d) or $^{13}$C$_5$-Gln (e). The values are mean ± S.E.M. n=3 mice. (f-g) Isotopologues of Gln (f) and glucose (g) found in the serum of mice orthotopically xenografted with the human GS-positive T16 GBM, and infused for 4h into the carotid artery with $^{13}$C$_5$-Gln. Mean ± S.E.M. n=3 mice. (h-j) Isotopologue distributions of Gln, Glu and α-kg in T16 tumours, contralateral brain, and liver tissues of mice infused as in (f-g). Mean ± S.E.M. n=3 mice. (k) Astrocytes were incubated for 24hours +/- Gln, and asparagine consumption is reported. Data derive from one experiment performed twice. Raw data of independent repeats are provided in the statistics source data Supplementary Table 5.
**Supplementary Figure 7** A toy network used for metabolic modelling. A toy network (a) and its optimal flux distribution (b).
Supplementary Figure 8 Unprocessed scans of western blots accompanied by size markers. Red dotted lines delineate the region presented in the respective Figures as indicated. Images were obtained using a Licor Odyssey scanner and acquired using Image Studio 2.0.
**Supplementary Table 1** Formulation of DMEM and Serum-like Modified Eagles Medium (SMEM).

| Amino Acids          | DMEM μM | SMEM μM |
|----------------------|---------|---------|
| L-Alanine            |         | 510     |
| L-Arginine           | 398     | 64      |
| L-Asparagine         | 41      |         |
| L-Aspartic acid      | 6       |         |
| L-Citrulline         | 55      |         |
| L-Cystine            | 201     | 65      |
| L-Histidine          | 200     | 120     |
| L-Glutamic acid      | 98      |         |
| L-Glutamine          | 2000    | 650     |
| L-Glycine            | 400     | 330     |
| L-Isoleucine         | 802     | 140     |
| L-Leucine            | 802     | 170     |
| L-Lysine             | 798     | 220     |
| L-Methionine         | 201     | 30      |
| L-Ornithine          | 80      |         |
| L-Phenylalanine      | 400     | 68      |
| L-Proline            | 360     |         |
| L-Serine             | 400     | 140     |
| L-Threonine          | 798     | 240     |
| L-Tryptophan         | 78      | 78      |
| L-Tyrosine           | 398     | 74      |
| L-Valine             | 803     | 230     |
| **Vitamins**         |         |         |
| Choline chloride     | 28.6    | 7.1     |
| D-Calcium pantothenate| 8.4  | 2.1    |
| Folic Acid           | 9.1     | 2.3     |
| Niacinamide          | 32.8    | 8.2     |
| Pyridoxine hydrochloride | 19.6 | 4.9   |
| Riboflavin           | 1.1     | 0.3     |
| Thiamine hydrochloride | 11.9 | 3      |
| i-Inositol           | 40      | 11.1    |
| **Inorganic Salts**  |         |         |
| Calcium Chloride     | 1800    | 1800    |
| Ferric Nitrate       | 0.2     |         |
| Magnesium Sulfate    | 813     | 813     |
| Potassium Chloride   | 5330    | 5330    |
| Sodium Bicarbonate   | 44050   | 44050   |
| Sodium Chloride      | 110340  | 118706  |
| Sodium Phosphate monobasic | 916 | 1010 |
| **Other Components** |         |         |
| D-Glucose            | 25000   | 5560    |
| Phenol Red           | 40      | 25      |
| Sodium Pyruvate      | 1000    | 100     |
| Taurine              |         | 130     |
**Supplementary Table 2** Exchange rates of metabolites in GBM cell lines. Cells were incubated for 24h -/+ U-^{13}\text{C}_5\text{Gln}, and secretion (positive values) and consumption (negative values) rates of the indicated metabolites are reported as nmol/μg prot/day. The sum of all isotopologues is reported for clarity. Mean ± S.D. n=3 independent experiments.

| Metabolite | Mean ± S.D. (nmol/μg prot/day) |
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### Supplementary Table 3 Representation of SMEM growth medium in the metabolic model Recon1.

| Metabolite          | Reaction number | Concentration (µM) |
|---------------------|-----------------|--------------------|
| L-Alanine           | 3457            | 510                |
| L-Arginine          | 165             | 64                 |
| L-Asparagine        | 1590            | 41                 |
| L-Aspartic acid     | 2387            | 6                  |
| Bicarbonate         | 2802            | 44050              |
| Calcium             | 3165            | 1800               |
| Chloride            | 163             | 125836             |
| Choline             | 662             | 7.1                |
| L-Cysteine          | 1184            | 65                 |
| D-Glucose           | 968             | 5560               |
| L-Glutamic acid     | 2567            | 98                 |
| L-Glutamine         | 1372            | 0                  |
| L-Glycine           | 2510            | 330                |
| L-Histidine         | 3719            | 120                |
| L-Isoleucine        | 2655            | 140                |
| L-Leucine           | 889             | 170                |
| L-Lysine            | 2721            | 220                |
| L-Methionine        | 2978            | 30                 |
| Myo-Inositol        | 110             | 11.1               |
| L-Ornithine         | 3153            | 80                 |
| Oxygen              | 3446            | 50000              |
| (R)-Pantothenate    | 3471            | 2.1                |
| L-Phenylalanine     | 997             | 68                 |
| Phosphate           | 1943            | 1010               |
| Potassium           | 1210            | 5330               |
| L-Proline           | 2269            | 360                |
| Pyridoxine          | 75              | 4.9                |
| Pyruvic acid        | 3125            | 100                |
| Riboflavin          | 3385            | 0.3                |
| L-Serine            | 1422            | 140                |
| Sodium              | 3670            | 163866             |
| Sulfate             | 3463            | 813                |
| Taurine             | 3174            | 130                |
| Thiamin             | 420             | 3                  |
| L-Threonine         | 2363            | 240                |
| L-Tryptophan        | 1993            | 78                 |
| L-Tyrosine          | 3206            | 74                 |
| L-Valine            | 2763            | 230                |
| Sodium Chloride     | 110340          | 118706             |
| Sodium Phosphate monobasic | 916 | 1010 |
| Other Components    |                 |                    |
| D-Glucose           | 25000           | 5560               |
| Phenol Red          | 40              | 25                 |
| Sodium Pyruvate     | 1000            | 100                |
| Taurine             |                 | 130                |

**Supplementary Table 3** Representation of SMEM growth medium in the metabolic model Recon1.
### Supplementary Table 4  Weighted costs of growth limiting metabolites.

| Metabolite                                | Shadow Price | Minimal Flux | Weighted cost |
|-------------------------------------------|--------------|--------------|---------------|
| AMP                                       | -2.09        | 1490.82      | -3113.91      |
| Glutamine                                 | -1.04        | 408.89       | -427.02       |
| CMP                                       | -2.09        | 75.41        | -157.52       |
| GMP                                       | -3.13        | 18.13        | -56.8         |
| UMP                                       | -1.04        | 28.09        | -29.33        |
| dGMP                                      | -3.13        | 2.29         | -7.18         |
| dAMP                                      | -2.09        | 3.44         | -7.18         |
| dTMP                                      | -1.04        | 3.44         | -3.59         |
| dCMP                                      | -1.04        | 2.29         | -2.39         |
| Glutamate                                 | 0            | 1767.71      | 0             |
| Aspartate                                 | 0            | 1117.08      | 0             |
| Proline                                   | 0            | 738.79       | 0             |
| glycogen, structure 1 (glycogenin)        | 0            | 401.36       | 0             |
| Alanine                                   | 0            | 284.98       | 0             |
| Serine                                    | 0            | 280          | 0             |
| Glycine                                   | 0            | 240.24       | 0             |
| Histidine                                 | 0            | 240          | 0             |
| Leucine                                   | 0            | 237.26       | 0             |
| phosphatidic acid                         | 0            | 148.71       | 0             |
| Tyrosine                                  | 0            | 148          | 0             |
| diacylglycerol                            | 0            | 122.87       | 0             |
| Phosphatidylcholine                       | 0            | 88.05        | 0             |
| Asparagine                                | 0            | 82           | 0             |
| phosphatidylserine                        | 0            | 56.53        | 0             |
| Methionine                                | 0            | 54.75        | 0             |
| phosphatidylethanolamine                  | 0            | 47.63        | 0             |
| triacylglycerol                           | 0            | 41.54        | 0             |
| Threonine                                 | 0            | 30.84        | 0             |
| Cholesterol                               | 0            | 22.44        | 0             |
| Valine                                    | 0            | 18.22        | 0             |
| Cysteine                                  | 0            | 18.09        | 0             |
| Lysine                                    | 0            | 14.02        | 0             |
| cholesterol ester                        | 0            | 9.93         | 0             |
| monoacylglycerol 2                        | 0            | 5.83         | 0             |
| Isoleucine                                | 0            | 5.61         | 0             |
| Phenylalanine                             | 0            | 5.61         | 0             |
| phosphatidylinositol                      | 0            | 4.68         | 0             |
| sphingomyelin                             | 0            | 3.65         | 0             |
| lysophosphatidylcholine                   | 0            | 1.91         | 0             |
| Arginine                                  | 0            | 1.4          | 0             |
| Tryptophan                                | 0            | 1.4          | 0             |
Supplementary Table 5 Statistics Source Data.
Supplementary note

Genome-scale constraint-based metabolic modeling.

To predict the effects of Gln deprivation on cellular growth we simulated the experiment in a computational model. Our simulations were run on the generic model of human metabolism, Recon1. Flux Balance Analysis (FBA) and Flux Imbalance Analysis (FIA) were used to query the model and predict cellular growth rate, essential genes and growth limiting metabolites.

1. Simulated experimental conditions:

In order to simulate the in vitro conditions we set the concentrations of nutrients available to the model to match those of the SMEM growth medium. The list of components used for the model growth medium and their concentrations are reported in Supplementary Table 3. To account for biomass production, we added a growth reaction representing the steady-state consumption of biomass compounds required for cellular proliferation. The stoichiometric coefficients of the growth reaction represent the relative molecular concentrations of 42 essential metabolites. The addition of a biomass reaction is common practice when modeling proliferating cells, and was described in detail in previous work.

2. Searching for conditionally essential reactions:

We queried the model for conditionally essential reaction knockouts – reactions that are non-essential on SMEM medium, yet become essential when Gln is removed from the medium. The flux through a conditionally essential reaction is thus predicted to modulate the cell’s sensitivity to Gln deprivation. We used an FBA approach to identify conditionally essential reactions. For each reaction $r$, we simulated the knockout of $r$ and used FBA to simulate the maximal cellular growth rate (denoted
Gr−) when Gln is present/absent from the growth medium (denoted Gln+, Gln−, respectively). r was defined as conditionally essential if Gr−,Gln+ > 0 and Gr−,Gln− = 0.

Our analysis revealed a single conditionally essential reaction: Glutamine synthetase (GS). The knockout of GS was also found to completely abolish the model’s ability to replenish intra-cellular Gln.

3. Finding growth limiting metabolites:

We next explored which metabolites became growth limiting during Gln deprivation. FIA allows for the prediction of growth limiting metabolites by calculating a shadow price for each metabolite m. The shadow price of m (denoted SP(m)) represents the potential increase in cellular growth resulting from the synthesis or secretion of a small quantity of m. A negative SP(m) signifies that m is growth limiting, whereas SP(m) = 0 signifies that m is not growth limiting. Positive shadow prices are rare and theoretically signify toxic metabolites, whose secretion or detoxification would require resources that could otherwise be used for growth.

Gln deprivation was simulated by removing Gln from the model’s growth medium and restricting the flux through GS to half the amount necessary to sustain maximal growth. FIA was used to calculate shadow prices for all biomass constituents, revealing a negative shadow price for all nucleotides. The shadow prices were seen to directly represent the number of Gln-derived amide nitrogens necessary for the synthesis of each nucleotide – 1 amide nitrogen for TMP and UMP, 2 for AMP and CMP, 3 for GMP. Calculated shadow prices are shown in Supplementary Table 4.

We then calculated the total flux passing through each metabolite m, denoted by F(m) and weighted the shadow prices by multiplying SP(m) by F(m). This weighting
was performed in order to determine which nucleotide imposes the greatest burden on cellular growth. The weighting has two justifications:

1. One caveat of FIA is that the shadow price of $m$ is unrelated to the amount of limiting resource (Gln-derived nitrogen in our case) invested in the synthesis of $m$. At the end of this section we provide a toy network example which better illustrates this property of FIA.

2. The total flux $F(m)$ passing through metabolite $m$ represents $m$'s direct contribution to biomass production as well as $m$'s involvement in other growth related pathways, where $m$ participates as an intermediate metabolite or a co-factor. Hence, $F(m)$ provides a proxy to the significance of $m$ to the cellular growth process. We expect some of the cellular effort to be vested in maintaining the steady state concentrations of metabolites with high $F(m)$ values.

4. Detailed calculation of the weighted shadow prices:

For each metabolite $m$, $SP(m)$ was multiplied by $F(m)$, the minimal absolute flux passing through $m$ under FBA formulation. A flux distribution with minimal absolute fluxes was calculated using the following linear problem:

\[
\text{minimize} \quad \sum_j |v_j| \\
\text{s.t.} \quad Sv = 0 \\
\quad v_{\min} \leq v \leq v_{\max} \\
\quad v_{\text{biomass}} = v_{\text{maxBiomass}}
\]

Where $v_{\text{maxBiomass}}$ is the maximal attainable growth rate calculated using FBA. The absolute flux $F(m)$ through metabolite $m$ was then calculated as:
Finally, the weighted cost of $m$ was estimated by $SP(m) \cdot F(m)$. The results of this calculation are given in Supplementary Table 4.

Following weighting AMP was shown to incur the heaviest cost to the cell, in spite of its synthesis requiring the same amount of Gln-derived nitrogens as CMP, and less than GMP. Specifically, AMP is the most limiting, due to its use as a precursor for ATP, the most abundant nucleotide in the cell, and in other AMP containing cofactors like NADH, NADPH, FADH, S-Adenosyl methionine, and Coenzyme A. This indicates that in the absence of exogenous Gln, the availability of the Gln amide group limits purine biosynthesis. Further, the reported $K_m$ for Gln for the enzyme responsible for the first step in purine biosynthesis is at least one order of magnitude higher than that for pyrimidine and protein synthesis (EC2.4.2.14: Human amidophosphoribosyltransferase, $K_m$ 1.0 - 4.5 mM; EC6.3.5.5: mammalian carbamoyl-phosphate synthase, $K_m$ 0.1 mM; EC6.1.1.18: S. Cerevisiae Glutamine-tRNA ligase, $K_m$ 0.03 mM; http://www.brenda-enzymes.org, and Human glutaminyl-tRNA Synthetase catalytically active at 0.05 mM Gln$^6$). The differences in affinity for Gln suggest that low intracellular Gln concentrations that permit protein and pyrimidine synthesis, may be limiting for purine biosynthetic pathways.

5. Toy network example

For the sake of clarity, we repeated the analysis of growth limiting metabolites on a toy network. Supplementary Figure 7A depicts a toy network with five intracellular metabolites and a growth medium comprised of two extracellular metabolites. The numbers on the edges signify the stoichiometric coefficients. The growth medium, in our example, contains 36 (arbitrary) units of metabolite $A$ and 64 units of metabolite
The aim of the analysis is to determine which metabolite incurs the greatest cost to the cell in terms of the resources spent on its synthesis. 

Supplementary Figure 7B shows an optimal flux distribution across the toy network. Reaction fluxes are shown in red. Note that in order to calculate the total turnover of a metabolite by a reaction one must multiply the reaction flux by the metabolite’s stoichiometric coefficient (i.e. multiply the red and black numbers). The flux distribution reveals that A is the limiting resource whereas B is abundant. In order to sustain maximal growth, the cell invests 24 units of A in synthesizing metabolite C and 12 units in synthesizing B, making C the most costly investment. The synthesis of E does not incur any cost in terms of the limiting resource A.

The shadow price $SP(m)$ of a metabolite $m$ in our toy network would be $SP(m) = -s/z$, where $s$ is the amount of limiting resource (metabolite A) necessary for synthesizing $m$ and $z$ is the total amount of limiting resource necessary for producing one unit of biomass. One unit of biomass requires 6 molecules of C, 2 molecules of D and 3 molecules of E. Multiplying each metabolite by its respective cost in A we get:

$$z = 2 \cdot 6 + 3 \cdot 2 + 0 \cdot 3 = 18$$ \hspace{1cm} (6)

The shadow prices are hence $SP(C) = -\frac{2}{18}$, $SP(D) = -\frac{3}{18}$, $SP(E) = \frac{0}{18}$. It can be seen that D has the highest shadow price, even though most of the limiting resource is invested in the synthesis of C.

This behavior of FIA can be corrected by taking into account the absolute flux passing through each metabolite. In the optimal flux distribution, the absolute fluxes passing through the biomass constituents are:

$$F(C) = 1 \cdot 12 + 6 \cdot 2 = 24$$ \hspace{1cm} (7)
\[ F(D) = 1 \cdot 4 + 2 \cdot 2 = 8 \]  \hspace{1cm} (8)
\[ F(E) = 1 \cdot 6 + 3 \cdot 2 = 12 \]  \hspace{1cm} (9)

(note that in the toy network all product metabolites have a stoichiometric coefficient of 1). By weighting the shadow prices according to the total flux we get \( \text{Cost}(C) = \frac{-48}{18} \), \( \text{Cost}(D) = \frac{-24}{18} \), \( \text{Cost}(E) = \frac{0}{18} \). The weighted cost is directly proportional to the amount of limiting resource invested in the synthesis of each metabolite and shows that \( C \) poses the heaviest limitation on cellular growth, as desired.
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