Brain tumor initiating cells adapt to restricted nutrition through preferential glucose uptake

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Like all cancers, brain tumors require a continuous source of energy and molecular resources for new cell production. In normal brain, glucose is an essential neuronal fuel, but the blood-brain barrier limits its delivery. We now report that nutrient restriction contributes to tumor progression by enriching for brain tumor initiating cells (BTICs) owing to preferential BTIC survival and to adaptation of non-BTICs through acquisition of BTIC features. BTICs outcompete for glucose uptake by co-opting the high affinity neuronal glucose transporter, type 3 (Glut3, SLC2A3). BTICs preferentially express Glut3, and targeting Glut3 inhibits BTIC growth and tumorigenic potential. Glut3, but not Glut1, correlates with poor survival in brain tumors and other cancers; thus, tumor initiating cells may extract nutrients with high affinity. As altered metabolism represents a cancer hallmark, metabolic reprogramming may maintain the tumor hierarchy and portend poor prognosis.

Glioblastomas (glioblastoma multiforme; GBM; World Health Organization grade IV gliomas) are the most lethal and prevalent primary malignant brain tumors in adults, with median survival remaining 14.6 months with the best available therapies. The inability to effectively manage GBMs has motivated the search for more effective treatments. Recent observations underscore the importance of inter- and intratumoral heterogeneity driven by genetic and nongenetic causes to therapeutic responses and patient outcomes. Heterogeneity within the neoplastic compartment is partially explained by the tumor initiating cell (TIC) hypothesis, which holds that a cellular hierarchy exists in some cancers, with self-renewing TICs generating progeny constituting the tumor bulk. Although the TIC hypothesis remains controversial, several groups, including our own, have demonstrated that brain tumor initiating cells (BTICs) express stem cell markers, display sustained self-renewal, differentiate toward multiple lineages and phenocopy the original tumor upon xenotransplantation. BTICs also display radio- and chemoresistance, which is thought to contribute to tumor recurrence after treatment. Thus, targeting of BTICs offers a potential model for GBM control.

Nutrient acquisition and utilization are critical for growth of tumors, and metabolic alterations in cancers are recognized as the Warburg effect: the observation that cancer cells become less reliant on oxygen-dependent mitochondrial oxidative phosphorylation and instead rely on the anaerobic but glucose-intensive glycolysis pathway for ATP generation. This metabolic reprogramming generates glycolytic end products necessary to produce biological building blocks (proteins, nucleic acids and lipids) required for tumor growth even under hypoxia. As ATP production per glucose molecule is lower with anaerobic metabolism, tumors ultimately require higher glucose flux than normal tissues.

The brain is an extremely metabolically active organ that derives energy almost entirely from glucose, and the lack of extensive energy stores in the brain necessitates tight control of blood glucose homeostasis. However, the difference in glucose uptake in normal and neoplastic brain is complex and has been exploited clinically with [18F]deoxyglucose positron emission tomography (PET) imaging. The clinical importance of glucose consumption for brain tumor growth is also suggested by reports indicating that higher glucose in brain tumor patients associates with shorter survival.

Vascular glucose delivery to the normal brain is physiologically stymied by the blood-brain barrier. In response, neurons express a specialized glucose transporter isofrom, type 3 (Glut3). Glut3 has a fivefold higher affinity for glucose than the ubiquitous glucose transporter, type 1 (Glut1), and Glut3 expression is largely restricted to cells with both a high glucose demand and a glucose-poor microenvironment. Cancer glucose uptake is thought to be primarily driven by Glut1. Limited reports demonstrate Glut3 expression in cancers, but its functional importance has been largely ignored. Recently, the

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concepts of metabolic reprogramming and oncogenic metabolites support key roles of metabolism during transformation with a similar transition to a glycolytic state during somatic cell reprogramming\textsuperscript{10,11}. These data and others suggest the importance of understanding the mechanisms driving metabolic adaptation in cancer, and specifically within the BTIC fraction, to develop treatments.

In GBMs and other solid tumors, glucose metabolism is elevated in microenvironmental conditions associated with poor vascular supply, such as hypoxia and reduced extracellular pH. BTICs are enriched in areas of necrosis\textsuperscript{12}. Both hypoxia\textsuperscript{13} and acidic stress\textsuperscript{14} induce increased BTIC functional readouts: neurosphere formation and tumorigenic potential. These data suggest that regulation of metabolic processes and resulting changes in the tumor microenvironment have substantial effects on the BTIC phenotype. As reduced blood flow in growing tumors can cause localized nutrient deprivation with very low levels of glucose, we considered whether molecular differences in BTICs permit improved competition for limited resources.

RESULTS
Nutrient restriction promotes a BTIC phenotype
To determine whether glucose deprivation influences TICs, we exposed unenriched GBM cells isolated from dissociated tumors to media containing standard (4.5 g l\textsuperscript{-1}) or restricted (0.45 g l\textsuperscript{-1}) glucose. (a-d) Quantitative real-time PCR indicated elevation in (a) OCT4 (* \(P < 0.0001\) with unpaired \(t\)-test), (b) NANOG (* \(P < 0.0001\) with unpaired \(t\)-test) and (c) SOX2 (* \(P < 0.05\) with unpaired \(t\)-test) mRNA under conditions of restricted glucose in comparison to standard culture conditions, while (d) GFAP was reduced (* \(P < 0.05\) with unpaired \(t\)-test). (e) Flow cytometry demonstrated increased percentages of CD133\textsuperscript{+} cells after culture of unenriched tumor cells from specimens (f) M12 (\(P = 0.03\) with extreme limiting dilution assay (ELDA) analysis; http://bioinf.wehi.edu.au/software/elda/), (g) 3832 (\(P = 0.00128\) with ELDA analysis) or (h) 4121 (\(P = 0.0128\) with ELDA analysis) indicate that the frequency of BTICs increased after culture in low glucose. (i) Neurosphere formation assays indicate that the percentage of wells with neurospheres increased after culture of unenriched GBM cells in restricted-glucose conditions when 10 cells were plated per well (* \(P < 0.007\) with unpaired \(t\)-test). (j,k) Kaplan-Meier survival curves of immunocompromised mice intracranially injected with (j) 4302 (\(n = 5\) per arm) or (k) M43 (\(n = 10\) per arm) cells cultured in restricted-glucose or standard-glucose conditions; * \(P < 0.05\) with log-rank analysis. Error bars, s.e.m.

Figure 1 Glucose restriction promotes a brain tumor initiating cell phenotype. Unenriched glioma cells isolated from the indicated xenografts were cultured for 7 d in standard (4.5 g l\textsuperscript{-1}) or restricted (0.45 g l\textsuperscript{-1}) glucose. (a-d) Quantitative real-time PCR indicated elevation in (a) OCT4 (* \(P < 0.0001\) with unpaired \(t\)-test), (b) NANOG (* \(P < 0.0001\) with unpaired \(t\)-test) and (c) SOX2 (* \(P < 0.05\) with unpaired \(t\)-test) mRNA under conditions of restricted glucose in comparison to standard culture conditions, while (d) GFAP was reduced (* \(P < 0.05\) with unpaired \(t\)-test). (e) Flow cytometry demonstrated increased percentages of CD133\textsuperscript{+} cells after culture of unenriched tumor cells in low glucose (* \(P < 0.008\) with unpaired \(t\)-test). An overlay of representative flow plots is shown at right. (f-h) In vitro limiting dilution assays plating decreasing numbers of unenriched tumor cells from specimens (f) M12 (\(P = 0.03\) with extreme limiting dilution assay (ELDA) analysis; http://bioinf.wehi.edu.au/software/elda/), (g) 3832 (\(P = 0.00128\) with ELDA analysis) or (h) 4121 (\(P = 0.0128\) with ELDA analysis) indicate that the frequency of BTICs increased after culture in low glucose. (i) Neurosphere formation assays indicate that the percentage of wells with neurospheres increased after culture of unenriched GBM cells in restricted-glucose conditions when 10 cells were plated per well (* \(P < 0.007\) with unpaired \(t\)-test). (j,k) Kaplan-Meier survival curves of immunocompromised mice intracranially injected with (j) 4302 (\(n = 5\) per arm) or (k) M43 (\(n = 10\) per arm) cells cultured in restricted-glucose or standard-glucose conditions; * \(P < 0.05\) with log-rank analysis. Error bars, s.e.m.

As BTICs express stem cell markers, we first analyzed the expression of a subset of BTIC markers in unenriched GBM cells derived from dissociated human tumors after culture under standard or restricted glucose for 1 week. Quantitative real-time PCR of mRNA demonstrated six- to tenfold elevation of the core stem cell transcription factor markers OCT4 (Fig. 1a) and NANOG (Fig. 1b) in the cultures with restricted glucose. SOX2 expression was also consistently, but
more modestly, increased, by greater than twofold in half the samples tested (Fig. 1c). Levels of transcripts for the astrocyte differentiation marker glial fibrillary acidic protein (GFAP) decreased with low-glucose culture (Fig. 1d). Using flow cytometry, we observed a twofold or greater increase in the percentage of cells expressing a putative BTIC immunophenotype, the cell surface marker CD133 (Prominin1), after exposure to low glucose (Fig. 1e). These data demonstrate that glucose deprivation increased the expression of BTIC markers and decreased differentiation.

As TICs are defined functionally, we next determined the effects of restricted glucose in BTIC functional assays. BTIC enriched cultures have an increased capacity to form tumor neurospheres, a phenotype associated with self-renewal and poorer clinical outcomes in GBM17. In vitro limiting dilution assays using cells isolated from GBM xenografts demonstrated that the frequency of BTICs capable of forming neurospheres increased after culture under glucose restriction (Fig. 1f–h). With a constant number of glucose-deprived cells, the percentage of wells with neurospheres also increased threefold (Fig. 1i).

The ability to propagate tumors in vivo is the gold standard for BTIC function. We determined whether culture in low glucose influenced tumorigenic potential using an orthotopic xenograft model. Low-glucose culture significantly increased in vivo tumor growth relative to that of standard-glucose culture, as demonstrated by reduced median survival and/or increased tumor incidence (Fig. 1j,k and Supplementary Table 1). Xenografts phenotypically recapitulated the histology of the original tumors, demonstrating the BTIC-associated property of tumor propagation (data not shown). These data demonstrate that glucose deprivation promotes the BTIC phenotype.

To determine the biological mechanism by which nutrient deprivation enriches for BTICs, we investigated two potential hypotheses: (i) BTICs preferentially survive low-glucose conditions, and/or (ii) non-BTICs adapt to low glucose by acquiring a more BTIC-like phenotype (Fig. 2a). We generated fluorescently labeled GBM cells and isolated differentially labeled BTICs and non-BTICs by flow cytometry for use in coculture experiments (Fig. 2b). We cocultured cells under standard or low glucose conditions at defined and biologically relevant percentages (namely, 10% BTIC:90% non-BTIC for specimens...
4121 and IN326 or 50% BTIC:50% non-BTIC for specimen IN528) and monitored the percentage of cells and their progeny over time. Imaging demonstrated strong differences in the percentage of BTIC-derived cells under restricted glucose conditions (Fig. 2c). Using flow cytometry, we confirmed that there was a significantly greater percentage change in the fraction of BTICs and their derivatives under glucose restriction (Fig. 2d–f).

To further define the mechanism responsible for the changes observed in coculture experiments, we investigated the survival of BTICs and non-BTICs in normal and low glucose. When ethidium homodimer III staining was used to determine the percentage of dead cells in matched GBM fractions, we did not observe significant increases in BTIC cell death under restricted glucose (Fig. 2g,h). However, the fraction of dead BTICs was consistently slightly elevated (Fig. 2g,h). In contrast, non-BTICs showed more than six times as many dead cells (Fig. 2g,h). Together, these data demonstrate that BTICs preferentially survive restricted glucose conditions.

BTIC markers, including NANOG, were elevated in non-BTICs that survived low glucose, as in the bulk tumor cells (data not shown). These data suggested that non-BTICs surviving low glucose adapt through acquisition of a more BTIC-like state. We therefore performed modified in vitro limiting dilution assays with non-BTICs plated directly into wells containing normal or low-glucose stem cell media (Fig. 2i,j). After an initial incubation period of 7 d, we restored normal glucose levels and assessed neurosphere formation 2 weeks later. Using this strategy, we excluded the potential for BTIC enrichment due to selection to affect the capacity to form neurospheres. We continued to observe a significant increase in the frequency of sphere-forming GBM cells with low-glucose exposure (Fig. 2i,j).

In an alternative approach, we evaluated the adaptation of non-BTICs to low glucose at the single-cell level using time-lapse microscopy with reporter cells expressing NANOG-promoter driven GFP. Imaging demonstrated the presence of infrequent NANOG-promoter–GFP negative GBM cells that acquired NANOG promoter activation after more than 60 h of culture in low glucose (Fig. 2k–m and Supplementary Video 1). Over the same time course, we did not observe NANOG-promoter–GFP expression under normal glucose (data not shown). These data demonstrate that non-BTICs can adapt to nutrient restriction through BTIC marker expression.

Preferential glucose uptake by BTICs
De novo synthesis of molecular building blocks for cell growth requires glucose for energy production.18. Highly efficient glucose uptake could therefore provide a competitive advantage contributing to tumor maintenance, particularly with restricted glucose levels. A glucose oxidase colorimetric assay following a pulse of glucose demonstrated BTICs had higher levels of glucose than matched non-BTICs (Fig. 3a). To directly compare the uptake of glucose in GBM tumor cell...
Glut3 in BTICs: glucose transport (*P < 0.001 with unpaired t-test) or (e) Glut1 (*P < 0.001 with unpaired t-test) demonstrates elevated Glut3 protein in BTICs. (f) Flow cytometry confirms that shRNAs directed against GLUT3 (shRNA1 and shRNA2) can reduce the expression of Glut3 protein relative to a non-targeting control shRNA (*P < 0.001 with unpaired t-test).

In the representative flow histogram (right), the gray line represents the isotype control. (g) Knockdown of Glut3 using shRNA in BTICs isolated from 3832, 4121 or M43 specimens reduces the uptake of a fluorescent glucose analog, demonstrating a requirement for Glut3 in BTIC glucose transport (*P < 0.005 with ANOVA comparison). (h) There is no requirement for Glut3 for glucose uptake in matched non-BTICs (3832, P = 0.69; 4121, P = 0.12; M43, P = 0.68). To best show the impact of GLUT3-directed shRNAs in (g,h), values were normalized to non-targeting shRNAs in each group. Error bars, s.e.m.

Glut3 expression in BTICs is often increased in comparison to matched non-BTICs. Knockdown of Glut3 expression significantly impaired uptake of 2-NBDG in comparison to that in the nontargeting control (shRNA1, shRNA2). In unenriched GBM cultures, we observed Glut3 expression increase with extended cell culture (Supplementary Fig. 2e), consistent with results in the flow cytometry assays. Together, these data indicate that BTICs preferentially take up glucose relative to their non-BTIC counterparts.

BTICs express elevated glucose transporter type 3

Cellular uptake of glucose occurs through facilitated diffusion using a family of solute carriers, the Glut family of proteins. The major Glut isoforms in normal brain are Glut1 and Glut3, which are differentially expressed: Glut1 is broadly expressed in both glia and neurons, whereas the higher-affinity Glut3 is restricted to neurons21 (Supplementary Fig. 2a,b). Limited previous reports on Glut expression in brain tumors including GBMs also indicate that Glut1 and Glut3 are likely to be the predominant isoforms expressed22,23. To determine whether differential Glut isoform expression in BTICs contributes to preferential glucose uptake, we examined mRNA levels for glucose transporter isoforms. As GLUT1 (SLC2A1) and GLUT3 (SLC2A3) expression increase with extended cell culture (Supplementary Fig. 2c,d), we performed experiments within five passages after tumor dissociation. We observed GLUT1 and GLUT3 expression in both BTICs and non-BTICs, with minimal GLUT2 (SLC2A2) and GLUT4 (SLC2A4) levels (Fig. 4a-c). Glucose transporter isoform expression was most significantly different for GLUT3 (Fig. 4a-c), but we often observed significantly increased mRNA for GLUT1, as reported12 (Fig. 4a,b). To extend these observations of mRNA difference into protein analysis, we examined Glut3 (Fig. 4d) and Glut1 (Fig. 4e) expression via flow cytometry. Fluorescence intensity for Glut3 (Fig. 4d) was consistently elevated in BTICs in comparison to matched non-BTICs.

Protein expression of Glut1 (Fig. 4d) was sometimes increased in BTICs to the level of statistical significance, but the biological significance of this difference in Glut1 expression is likely to be less than for Glut3 (Fig. 4e). We observed a 20% change in Glut1 expression between BTICs and non-BTICs, whereas there was 300% change in Glut3 expression (Fig. 4d,e). We also evaluated the expression of Glut3 and the BTIC marker Sox2 using coimmunofluorescence (Supplementary Fig. 2e). In unenriched GBM cultures, we observed colocalization of Glut3 and Sox2, suggesting that Glut3 protein is expressed in BTICs (Supplementary Fig. 2e). These data suggest that Glut proteins can be differentially expressed in the brain tumor hierarchy, potentially contributing to differences in glucose uptake efficiency.

Glut3 mediates BTIC glucose uptake and tumorigenic potential

To functionally demonstrate the importance of Glut3 in BTIC glucose uptake, we used a targeting approach based on lentivirally expressed short hairpin RNAs to reduce GLUT3 mRNA (Supplementary Fig. 2f) and protein (Fig. 4f and data not shown). Targeting of GLUT3 had no effect on GLUT1 expression (Supplementary Fig. 2f). Expression of GLUT3 shRNA in BTICs significantly impaired uptake of 2-NBDG in comparison to that in the nontargeting control (Fig. 4g). In contrast, there was no significant effect of Glut3 knockdown on glucose uptake in matched non-BTICs (Fig. 4h). The data demonstrate that Glut3 is preferentially required for the elevated uptake of glucose in BTICs.

We next evaluated the phenotypic consequences of reduced uptake of glucose in BTICs with impaired Glut3 expression. Knockdown of Glut3 significantly decreased the growth of BTICs (Fig. 5a,b), whereas there was no effect on non-BTICs (Supplementary Fig. 2g,h), further
GLUT3 was strongly correlated with tumor grade in two data sets (Fig. 6b,c and Supplementary Figs. 3a, 4a and 5a). Neither GLUT2 nor GLUT4 correlated with grade; GLUT1 slightly correlated with tumor grade in one data set (1.1-fold), although the change in GLUT3 expression was higher (1.68-fold) and strongly significant (Fig. 6c). GLUT3 levels are increased upon disease recurrence (Fig. 6d), unlike those of other GLUT isoforms (Supplementary Figs. 3b, 4b and 5b). Further, only GLUT3 was a negative prognostic factor for long term (>3 year) survival (Fig. 6e and Supplementary Figs. 3c, 4c and 5c). GLUT3 expression threefold higher in tumors isolated from patients who died within 3 years compared to those who survived, whereas there was no significant difference in the expression of GLUT1, GLUT2 or GLUT4 (Fig. 6e and Supplementary Figs. 3c, 4c and 5c). GLUT3 was also the only glucose transporter isoform that correlated with poorer survival at 1 year (data not shown).

To further evaluate the potential correlation of GLUT3 expression with patient outcome, we generated Kaplan-Meier survival curves from the Freije, Phillips and Nutt data sets24,25,27, as well as the US National Cancer Institute’s Repository for Molecular Brain Neoplastic Data (REMBRANDT)28 and The Cancer Genome Atlas (TCGA)29. In all data sets, GLUT3 expression informed poor prognosis (Fig. 7a–e), whereas GLUT1, GLUT2 or GLUT4 did not correlate with patient outcomes (Supplementary Figs. 3d–h, 4d–h and 5d–h). To determine
whether GLUT3 was more closely associated with prognosis in one of the GBM subtypes, we segregated data in the TCGA on the basis of Verhaak classification\(^{36}\). Whereas we did not observe a significant association of GLUT3 with survival in mesenchymal-subtype tumors (\(P = 0.26\); Fig. 7f), GLUT3 correlated with survival in classical (Fig. 7g) and proneural (Fig. 7h) subtypes. GLUT1, GLUT2 and GLUT4 did not correlate with survival in any GBM subtype (Supplementary Figs. 3i–k, 4i–k and 5i–k). Together these data demonstrate that, among the GLUT isoforms, GLUT3 expression is the only consistent indicator of brain tumor patient outcomes.

The association between GLUT3 and survival was the strongest in the proneural GBM subtype (Fig. 7h), and this tumor subgroup is highly associated with glioma-CpG island methylator phenotype (GCIMP)\(^{31}\) and mutational status of the metabolic enzyme isocitrate dehydrogenase (IDH1)\(^{32}\). GLUT3 levels were lowest in GCIMP-positive or IDH1-mutant tumors, indicators of good prognosis. However, GLUT3 remained informative for poor prognosis in proneural GCIMP-negative or IDH1-wild-type tumors (Supplementary Fig. 6). These data further implicate Glut3 as an important regulator of GBM patient survival.

Tumor microenvironments, including hypoxia, are negative prognostic indicators of therapeutic response and patient survival. In the glioma TCGA data set, high levels of a hypoxia marker, carbonic anhydrase IX (CA9)\(^{33}\), correlated with poor patient survival (Supplementary Fig. 7a). As GLUT3 and GLUT1 are reported to be hypoxia response genes, we determined whether the contribution of GLUT3 to patient survival was a surrogate for hypoxia. While GLUT3 (Supplementary Fig. 7b) and GLUT1 (Supplementary Fig. 7c) correlated with CA9 expression, GLUT3 (Supplementary Fig. 8) but not GLUT1 (Supplementary Fig. 9) was informative for patient survival independent of hypoxia. Collectively, these data support a unique role of Glut3 in tumor growth and the malignancy of gliomas.

**GLUT3 correlates with pluripotency and cancer survival**

GLUT3 was strongly associated with BTIC function and patient outcome. We therefore interrogated GLUT3 in relation to a stem-cell state and patient survival in other solid tumors that follow a TIC model. As noted above, core stem cell regulators—Nanog, Sox2 and Oct4—are expressed by BTICs and are induced upon nutrient restriction, during which Glut3 is critical. Thus, the role of Glut3 in BTICs could be a phenocopy of roles in pluripotency and reprogramming. Indeed, analysis of induced pluripotency data sets demonstrated that embryonic stem cells or induced pluripotent stem cells expressed markedly elevated levels of GLUT3 in comparison to those in fibroblasts\(^{34,35}\) (Fig. 8a,b and Supplementary Fig. 10d).

Glut3 could affect patient outcomes in TIC-containing cancers beyond the brain. Previous reports have suggested GLUT3 can be expressed in some cancers\(^{36–39}\), but Glut3 biological function and significance for patient outcomes is largely unknown, although GLUT3 association with poor survival has been reported in lung\(^{37}\), oral squamous\(^{38}\) and laryngeal\(^{39}\) carcinomas. Using Oncomine, we found that...
GLUT3 expression correlated with poor survival in a broad range of tumor types, including those of the breast (Fig. 8f–h), ovary (Fig. 8i) and lung (Fig. 8j,k). These data suggest that many cancers dysregulate Glut3 to drive tumor growth and indicate a potential role for Glut3 in the TIC fraction of these tumors. The data also suggest that targeting of Glut3 could represent an important anticancer strategy.

**DISCUSSION**

Metabolic reprogramming is a hallmark of cancer required for cellular transformation. Cancer cells are generally highly metabolically active, requiring both a constant source of energy and carbon for anabolic processes such as cell division and growth factor production. These needs are met through a shift to anaerobic glycolysis, which provides a cancer cell with both energy and carbon sources in the form of pyruvate. However, the glucose demand of cancer cells is dramatically increased due to the usage of the glycolytic end products for processes other than energy generation. These observations are challenging to resolve in light of the frequent impaired nutrient availability for cancers. Our current results suggest that the stem-like cells in malignant primary brain tumors co-opt high affinity transporter systems used by neurons to maintain access to available nutrients even in scarcity. The neuronal glucose transporter Glut3 is a high-affinity glucose transporter (its Michaelis constant (Km) is approximately one-fifth that of Glut1), permitting neurons to outcompete for nutrients where environmental concentrations are low, preserving their function and survival40. Our data demonstrate that BTICs have elevated levels of Glut3 that permit preferential glucose uptake, suggesting BTICs have a competitive advantage particularly important in relatively harsh microenvironments. More broadly, BTICs have differential molecular profiles that permit metabolic adaptations influencing GBM cell behaviors and maintaining tumor growth.

Exploiting the difference in glucose uptake in normal and neoplastic tissues with PET imaging permits noninvasive diagnosis or monitoring of tumor progression. These clinical observations in combination with our results indicating increased glucose uptake in BTICs suggest that PET imaging could be used to visualize the BTIC fraction. However, glucose metabolic rate and standardized uptake value do not significantly correlate with survival in BTICs. Although glucose metabolic rate correlates most strongly with cell proliferation rather than with Glut expression in glioma42. As we have found a relative difference in glucose uptake, with preferential but not exclusive uptake in BTICs, as well as Glut expression in both BTICs and non-BTICs, it is unlikely that any changes in PET imaging would be sufficient to define a TIC compartment.

Metabolism has been poorly linked to cancer cellular hierarchies, but metabolic changes are known to drive epigenetic modifications that influence cell fate. For example, mutations in IDH1 (refs. 43, 44) in low-grade astrocytomas and secondary GBMs (but not the primary GBMs predominately studied here) result in production of a new metabolic end product (2-hydroxyglutarate) that leads to hypermethylation of DNA and repression of differentiation32,45. Targeting mutant IDH1 activity induces cellular differentiation and reduces tumor growth46, further supporting the potential of antimetabolic therapies as a strategy for targeting GBMs. Although IDH1 mutation contributes to GBM development, patients with IDH1 mutation have...
Figure 8 GLUT3 expression correlates with induced pluripotency and predicts poor survival in multiple tumor types beyond the brain. (a) Analysis of the Yamanaka data set for induced pluripotent stem cell (iPSC) generation from mouse embryonic fibroblasts (mEF) demonstrates a significant increase in GLUT3 expression with reprogramming. Mouse embryonic stem cells (mESC) also express higher GLUT3 than fibroblasts (*P = 0.01, **P < 0.01 with ANOVA comparison to mouse embryonic fibroblasts). (b) Analysis of the Thomson data set for iPSC generation from human foreskin fibroblasts (parent) demonstrates increases in GLUT3 with reprogramming. Human embryonic stem cell lines (H1L and H7) also express higher GLUT3 than fibroblasts (*P = 0.01, **P < 0.01 with ANOVA comparison to parental cells). RMA, robust multi-array average. (c–k) Analysis of carcinoma data sets available through Oncomine indicates a significant correlation between high GLUT3 expression and poor survival for breast (c–e), colorectal (f–h), ovarian (i) and lung (j,k) cancers. (c) van de Vijver breast carcinoma data set (n = 39 for GLUT3 low, n = 138 for GLUT3 medium, n = 112 for GLUT3 high; P = 0.0107 with log-rank analysis). (d) Kao breast carcinoma data set (n = 145 GLUT3 low, n = 181 GLUT3 high; P = 0.0466 with log-rank analysis). (e) Sorlie breast carcinoma data set (n = 54 GLUT3 low, n = 18 GLUT3 high; P = 0.06 with log-rank analysis). (f) Smith 2 colorectal adenocarcinoma data set (n = 9 GLUT3 low, n = 34 GLUT3 medium, n = 11 GLUT3 high; P = 0.004 with log-rank analysis). (g) Staub colon carcinoma data set (n = 4 GLUT3 low, n = 42 GLUT3 medium, n = 16 GLUT3 high; P = 0.0105 with log-rank analysis). (h) TCGA colon carcinoma data set (n = 85 GLUT3 low, n = 68 GLUT3 high; P = 0.013 with log-rank analysis). (i) Bonome ovarian carcinoma data set (n = 24 GLUT3 low, n = 117 GLUT3 medium, n = 18 GLUT3 high; P = 0.0135 with log-rank analysis). (j) Bild lung adenocarcinoma data set (n = 10 GLUT3 low, n = 33 GLUT3 medium, n = 7 GLUT3 high; P = 0.0001 with log-rank analysis). (k) Raponi squamous cell lung carcinoma data set (n = 18 GLUT3 low, n = 90 GLUT3 medium, n = 21 GLUT3 high; P < 0.04 with log-rank analysis). Error bars, s.e.m.

a relatively longer median survival. As our data indicate that IDH1 mutation is associated with lower levels of Glut3, it is likely that glucose metabolism differs in these tumors.

Although we have not fully explored the mechanisms regulating Glut3 upregulation in BTICs, the differences in Glut3 expression with GCIMP status indicate the possibility that aberrant methylation of GLUT3 could be an important mechanism controlling its expression in cancer. Recent publications indicate a role for DNA methylation in the regulation of Glut3 expression in neurons and placenta47,48. As cancers display a stem-cell epigenetic signature49,50, critical roles of metabolism in influencing epigenetics likely affect TIC phenotypes. Indeed, induced pluripotent stem cells expressed markedly elevated levels of GLUT3 in comparison to parental fibroblasts, suggesting that upregulation of Glut3 is directly associated with acquisition of a stem cell state.

Cancer cell pro-tumorigenic properties are modeled in cell culture, but ex vivo conditions used to propagate cell lines are quite different from those found in vivo. To identify new cancer treatment targets, we must therefore establish formulations for media to better mimic tumor microenvironments. Once targets are identified, therapies should be directed, to reduce toxicity, toward those that drive tumor progression without being functionally required for non-neoplastic cells. The brain is a very metabolically demanding organ, but the increased glucose demand of cancer cells suggests there may be a therapeutic window for targeting Glut3. However, Glut3 is expressed in neurons throughout the normal brain, suggesting the potential for toxicity. Unlike cancer cells, neurons are postmitotic, suggesting that anti-Glut3 combinatorial therapies with conventional drugs targeting rapidly proliferating cells may have a therapeutic index, with efficacy against BTICs while sparing neurons. In addition, the data correlating Glut3 with poor prognosis in tumor types outside the brain suggest that a Glut3 inhibitor that does not cross the blood-brain barrier would be useful for treating many aggressive cancers with minimal toxicity. We anticipate that understanding how TICs can outcompete for critical resources to maintain or promote tumor growth will represent a significant advancement in our understanding of malignant tumors, including GBM, and our ability to treat this devastating disease.
METHODS

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

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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COMPETING FINANCIAL INTERESTS

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ONLINE METHODS

Cell isolation and culture. Glioblastoma (GBM) cells were derived from specimens of neurosurgical resection directly from patients in accordance with a Duke University or Cleveland Clinic Institutional Review Board-approved protocol in which informed consent was obtained by the tumor bank, which provided de-identified excess tissue to the laboratory. Unenriched cells, BTICs and/or non-BTICs were separated from GBM surgical specimens or xenografts as previously described. Because of concerns about selection of cells in culture resulting in upregulation of Glut3 (Supplementary Fig. 2c,d), all cells were used within five-tissue-culture passages after dissociation. The cancer stem cell phenotype of CD133+ cells was confirmed by functional assays of self-renewal, stem cell marker expression and tumor propagation. The CD133-depleted cells did not share these properties and were used in matched assays as non-BTICs. CD133-enriched BTICs were cultured in Neurobasal medium with B27 (without vitamin A, Invitrogen), basic fibroblast growth factor (20 ng/ml) and epidermal growth factor (20 ng/ml), on Petri dishes. CD133-depleted non-BTICs were cultured in Dulbecco’s modified Eagle medium (DMEM) with 10% FBS, on tissue-culture coated plates. Unenriched populations were cultured in BTIC medium with 1% FBS added, on tissue-culture coated plates. The glucose concentration of all standard medium formulations used was 4.5 g/l. For experiments with standard and restricted glucose, a restricted glucose environment was produced through a combination of one part standard medium to nine parts glucose-free medium, otherwise supplemented identically, resulting in a final medium glucose concentration one-tenth standard medium glucose, at 0.45 g/l. Due to poor viability of trypsin-EDTA-treated glucose-restricted cultures, all cultures were passaged in Dulbecco’s modified Eagle medium (DMEM) with 10% FBS, on tissue-culture coated plates. The glucose concentration of all standard medium formulations used was 4.5 g/l. For experiments with standard and restricted glucose, a restricted glucose environment was produced through a combination of one part standard medium to nine parts glucose-free medium, otherwise supplemented identically, resulting in a final medium glucose concentration one-tenth standard medium glucose, at 0.45 g/l. Due to poor viability of trypsin-EDTA-treated glucose-restricted cultures, all cultures were passaged using mechanical disaggregation and lifted from tissue culture plates.

Brain tumor specimen characteristics. On the basis of microarray analysis, specimens were subtyped as follows: proneural (3832, IN528), classical (4121, GBM43, GBM12, PBI) and mesenchymal (IN326).

Quantitative RT-PCR. Total cellular RNA was isolated with the RNeasy kit (Qiagen) and reverse-transcribed into cDNA using the SuperScript III Reverse Transcription Kit (Invitrogen). Real-time PCR was performed on an Applied Biosystems 7900HT system using SYBR-Green Mastermix (SA Biosciences) and gene-specific primers as follows: ACTB (β-actin) forward 5′-AGA AAA TCT GCC ACC ACA CC-3′ and reverse 5′-AGA GCC GTA CAG GAG TAG CA-3′; OCT4 5′-TCTCCCATGATTAAAACCTGAG-3′ and reverse 5′-CCTTTGTTCCCAATTTCTC-3′; NANOG forward 5′-GAAATACCTGAGCTCCACC-GC-3′ and reverse 5′-GGGTCACACTT GCTAATC-3′; SOX2 forward 5′-CAACCTGGCCCTCTC-3′ and reverse 5′-CTCCGGTGTTTGTGCAGG-3′; GLUT1 forward 5′-GGCTGGGCT ATCTTGGCGGTG-3′ and reverse 5′-CTGGAAAGCATGCAGCCCAA-3′; GLUT2 forward 5′-AAGCTCTGAGGATCTCAATTGTGCCCTG-3′ and reverse 5′-ATGTGAAAGGTGTTTGAGAGAG-3′; GLUT3 forward 5′-AGCTTCTGGTATGATCTGCGG-3′ and reverse 5′-ATGTTTGCCCAATATTCTGGGCTG-3′; and reverse 5′-TAAGGACCCCATAGCATCGCAAAA-3′.

Flow cytometry Glut surface staining. For FACS staining of surface Glut expression, live cells were incubated with either Glut3 antibody (R&D, MAB1415) or Glut1 antibody (Abcam AB40084) and with CD133 (AC133-APC, Miltenyi) for 45 min at dilutions specified in the manufacturer’s protocols (2.5 μg Glut3 antibody per million cells, 1 μg Glut1 antibody per million cells).

In vitro limiting dilution neurosphere formation assay. For in vitro limiting dilution assays, propidium iodide–negative cells were sorted by FACS with decreasing dilution analysis was performed using software available at http://bioinf.wehi.edu.au/software/elda/. Neurosphere formation assays were also performed similarly to those in our previous report, with 10 cells with per well plated in 96-well plates and the percentage of wells with neurospheres measured after 10 d.

Competition mixing assay. Unsorted glioma cells were infected with either RFP or GFP lentivirus. Following infection, cells were sorted for color, viability using LIVE/DEAD Fixable Blue Dead Cell Stain kit (Invitrogen) and CD133-APC status by flow cytometry and directly plated in Neurobasal stem cell medium with 1% FBS on plates coated with Geltrix (Invitrogen). Cells were imaged on days 4 and 7 and collected for flow cytometry analysis on day 7.

Cell death assay. An Apoptosis and Necrosis Quantitation kit was obtained from Biotium and assay performed according to the manufacturer’s protocol, with cell fixation following staining.

pGZ-Nanog non-BTIC adaptation imaging. pGreen2Neo-Nanog plasmid was obtained from System Biosciences, prepared into lentiviral particles and used to infect cells. Infected cells were sorted via flow cytometry to obtain only GFP expressing cells. Fourteen days after sorting, cells were plated on Geltrix in Neurobasal stem cell medium with 1% FBS and either full or restricted glucose. Twenty-four hours after plating, images were taken on a Leica DMIRB Inverted Microscope equipped for time-lapse microscopy with a Roper Scientific CoolSNAP HQ2 Cooled CCD camera (Roper Scientific, Tucson AZ, USA), temperature controller (37 °C) and CO2 (5%) incubation chamber (Leica Microsystems GmbH), PeCon incubator (PeCon GmbH, Erbach, Germany), Prior motorized stage with linearly encoded controller with x-y-z drive for time-lapse imaging of multiple fields and heating insert for six-well plates (Prior Scientific Inc., Rockland, MA, USA), Uniblitz shutter (Vincent Associates, Rochester, NY, USA) and MetaMorph Software (Molecular Devices, Downingtown, PA, USA). Bright-field images were taken every 10 min and green fluorescence images were taken every 20 min for 72 h.

Low-glucose non-BTIC adaptation neurosphere formation assay. For the demonstration of non-BTIC adaptation to low glucose, a slightly modified version of the above neurosphere formation assay was employed. CD133-negative, propidium iodide–negative cells were sorted in decreasing numbers of cells (as above) into 96-well plates containing either stem cell medium or restricted-glucose stem cell medium. Cells were left in this medium for 7 d, at which point glucose was added to all wells to eliminate any possible effect of glucose restriction on proliferation; which would affect the sphere formation results. Fourteen days after glucose addition (21 d after plating), spheres were counted as above. The frequency of adaptive non-BTICs was calculated as the difference in sphere formation between the normal glucose condition and the 7-d restricted glucose condition.

Non-fluorescent glucose uptake assay. Following 45 min of glucose starvation, BTIC or non-BTIC cells were incubated in 0.45 g/l glucose for 30 min, then collected for lysis via mechanical lifting. Cells were lysed in RIPA buffer (Sigma) and the protein was quantitated as a loading control via Bradford assay. A glucose colorimetric detection kit was obtained (Arbor Assays) and assays performed according to the manufacturer’s protocol, with the exception of an increased incubation time to account for the use of whole-cell lysate.

Fluorescent glucose uptake assay. Following 30 min of glucose starvation, GBM cells were incubated for 30 min in the presence of a fluorescent glucose analog, 2-[N-(7-nitrobenz-2-oxa-1,3-diaxol-4-yl)amino]-2-deoxyglucose (2-NBDG). For studies comparing uptake in CD133+ and CD133− cells, unenriched cells were co-incubated with AC133-APC antibody and analyzed via FACS. For studies with shRNA-expressing BTICs, cells were washed and fluorescence analyzed using a VICTOR plate reader.

Ex vivo glucose uptake imaging assay. Before transplantation onto brain slices for imaging, BTICs were labeled with Cell Tracker Red CMPTX (Invitrogen) and non-BTICs were labeled with Cell Trace Far Red DDAA-SE (Invitrogen). For ex vivo glucose analysis, slice cultures were prepared from mice according to prior publications and 5,000 cells total transplanted (at a ratio of 1:1, BTIC:non-BTIC). Transplanted cells were incubated overnight to ensure integration and survival in the brain slices and before imaging; slices were incubated in 50 μg/ml NDBG for 30 min before image acquisition. Imaging was done using a Leica multiphoton microscope as previously described with a 20× liquid immersion objective, NA = 1.0. Images were acquired at 820 nm and processed using Imaris software (Bitplane).

Immunofluorescence staining. For immunostaining analysis at the single-cell level, live cells isolated via a Ficoll gradient were collected on Cell-Tack (BD)
Vectors and lentiviral transfection. Lentiviral clones expressing SLC2A3 shRNAs (TRC0000043616 and TRC0000043615) and control shRNA (SHC002) were purchased from Sigma-Aldrich. Viral particles were produced in 293T cells with the pPACK set of helper plasmids (System Biosciences) in stem cell medium. Both shRNA constructs were designed against the GLUT3 coding sequence. The shRNA sequences were as follows: GLUT3 shRNAn1, 5′-CCGGCTTGGTCTTTGTTAGCCTTCTCAGAAAGGCTACAAAGACCAAGTTTTTG-3′; GLUT3 shRNAn2, 5′-CCGGAGTAGCTAAGTCGGTTGAAATCTCGAGATTTCAACCGAC TTAGCIACTTTTTTG-3′.

In vivo tumor initiation assay. All animal procedures were performed in accordance with Cleveland Clinic IACUC approved protocols. Animals were housed in a temperature-controlled vivarium with a 14 h light, 10 h dark cycle at no more than five animals per cage. For nutrient deprivation studies, unenriched GBM cells were cultured in standard or low-glucose conditions for 7 d and 5,000 viable cells were intracranially injected into athymic nude mice as previously described12. For shRNA studies, BTICs were transduced with lentiviral vectors expressing GLUT3 shRNA or non-targeting control shRNA and 1,000 viable cells were intracranially injected into female athymic nude mice between 4 and 6 weeks of age. Animals were maintained until development of neurological signs (for example, lethargy, ataxia, seizures and/or paralysis), when brains were collected. If no neurologic signs developed within 105 d, animals were subsequently sacrificed and the experiment terminated. Brains were fixed in 4% formaldehyde, sunk in 30% sucrose, cryopreserved in OCT and cryosectioned.

Retrospective analysis of GLUT3 gene expression in human gliomas. Correlations between glioma grade, patient survival, tumor recurrence and GLUT3 gene expression were determined through analysis of TCGA, Sun, Nutt, Freij and Phillips brain data sets, respectively, which are available through Oncomine (Compendia Biosciences, http://www.oncomine.org/). High and low groups were defined as above and below the mean, respectively. For analysis with high, medium and low groups, high was defined as greater than 1 s.d. above the mean, low as greater than 1 s.d. below the mean and medium as within 1 s.d. of the mean. The National Cancer Institute's Repository for Molecular Brain Neoplasia Data (REMBRANDT, https://caintegrator.nci.nih.gov/rembrandt/) was also evaluated for correlations between glioma patient survival and gene expression with up- or downregulation being defined as a twofold change relative to mean values.

Statistical analysis. All grouped data are presented as mean ± s.e.m. Difference between groups was assessed by ANOVA or Student's t-test using GraphPad Prism software. For survival analysis, Kaplan-Meier curves were generated using either MedCalc or Prism software and log-rank analysis performed. All experiments were repeated in each specimen presented in at least duplicate with triplicate technical replicates. We used short-term passaged cells, and numbers of BTICs isolated from any one specimen or xenograft were low (usually 10% or less of the unenriched GBM cells after tumor dissociation). Therefore, we have not repeated all experiments with every sample used in the entire manuscript. We used isolated cells from different specimens to address specific questions in separate replicated experiments. We observed consistent results across specimens when similar experiments were performed as shown. Data distribution was assumed to be normal, but this was not formally tested. Data and animals were not randomized but were collected and processed in appropriate experimental arms. Data collection and analysis were not performed blind to the conditions of the experiments.

Sample sizes. For mouse experiments, sample sizes were calculated using the formula \( n = 1 + 2C(\sigma/d)^2 \), where \( n \) = number of animals per arm, \( C = 7.85 \) (significance level of 5% with a power of 80%), \( \sigma = s.d. \) and \( d = \) difference to be detected. For other experiments, no statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those in our previous publications.

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Corrigendum: Brain tumor initiating cells adapt to restricted nutrition through preferential glucose uptake

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In the version of this article initially published online, author Bryan W. Day did not appear. The error has been corrected for the print, PDF and HTML versions of this article.