Glucose transporter GLUT1 is a transmembrane protein responsible for the uptake of glucose into the cells of many tissues through facilitative diffusion. Plasma membrane (PM) localization is essential for glucose uptake by GLUT1. However, the mechanism underlying GLUT1 PM localization remains enigmatic. We find that GLUT1 is palmitoylated at Cys207, and S-palmitoylation is required for maintaining GLUT1 PM localization. Furthermore, we identify DHHC9 as the palmitoyl transferase responsible for this critical posttranslational modification. Knockout of DHHC9 or mutation of GLUT1 Cys207 to serine abrogates palmitoylation and PM distribution of GLUT1, and impairs glycolysis, cell proliferation, and glioblastoma (GBM) tumorigenesis. In addition, DHHC9 expression positively correlates with GLUT1 PM localization in GBM specimens and indicates a poor prognosis in GBM patients. These findings underscore that DHHC9-mediated GLUT1 S-palmitoylation is critical for glucose supply during GBM tumorigenesis.
GLUT1, encoded by SLC2A1, is a transporter facilitating the uptake of glucose in many tissues and belongs to the facilitative glucose transporter family that consists of 12 members. To support rapid proliferation, cancer cells take up more glucose for glycolysis even in the presence of oxygen, a phenomenon known as the Warburg effect. Elevated expression levels of GLUT1 were observed in many malignant tumors and correlated with poor clinical outcomes in patients. Despite its upregulation in many types of human cancer, the mechanisms underlying GLUT1-promoted tumor malignant progression remain largely unknown.

A previous study on the palmitoylation of the blood-brain barrier capillary proteins has shown that GLUT1 is palmitoylated. Palmitoylation of cysteine residues, the major modality of thio-cyclation on cellular proteins by the 16-carbon fatty acid palmitate, is catalyzed by palmitoyl acyltransferases (PATs). Over 800 putative palmitoylated proteins have been identified in mouse adipose tissues and adipocytes. Human PATs contain a conserved zinc finger DHHC motif (Asp–His–His–Cys) within a cysteine-rich domain (CRD), thus belonging to the zinc finger DHHC domain-containing protein family, which comprises 23 enzymes named DHHC1 to DHHC24 (without DHHC10). The hydrophobic palmitoyl moiety of palmitoylated proteins serves as a lipid anchor to facilitate the interaction between proteins and membranes that is critical for subcellular trafficking and membrane localization of intracellular proteins. For example, H-RAS and N-RAS are palmitoylated by DHHC9/GCP16 PAT complex, leading to plasma membrane (PM) localization of these proteins. Loss-of-function mutations in DHHC9 have been identified in patients with X-linked intellectual disability (XLID) and associate with an increased epilepsy risk. In addition, DHHC9-mediated palmitoylation maintains protein stability and cell surface distribution of PD-L1, leading to immune escape of breast cancer cells.

In this study, we demonstrate that GLUT1 palmitoylation occurs on GBM cell lines and plays an important role in GLUT1 PM localization. Further mammalian PATs screening assay show that DHHC9 is the dominant palmitoyltransferase for GLUT1. DHHC9 palmitoylates GLUT1 at Cys207 to maintain PM localization of GLUT1, leading to a high level of glycolysis, thereby promoting GBM tumorigenesis.

Results
S-palmitoylation maintains PM localization of GLUT1. To determine whether subcellular localization of GLUT1, the widely expressed glucose transporter responsible for the constant uptake of glucose, is regulated by S-palmitoylation, we examined its cellular distribution upon treatment with 2-bromopalmitate (2-BP), a pan-inhibitor of PATs. Immunofluorescence (IF) analyses of U87 and T98G human GBM cells with an anti-GLUT1 antibody and co-staining the cells with cholera toxin subunit B (CTB) conjugated with Alexa Fluor 594, a tracer for the PM, showed that 2-BP treatment induced redistribution of GLUT1 protein from the PM to the cytoplasm. Similarly, PM localization of GLUT1, as evidenced by increased cytoplasmic localization of eGFP-GLUT1 in the cells, was inhibited by 2-BP treatment (Supplementary Fig. 1a). Cell fractionation analyses confirmed that treatment of U87 and T98G cells with 2-BP substantially reduced the levels of endogenous GLUT1 associated with the PM fractions but did not affect total GLUT1 protein levels.

To determine whether GLUT1 is palmitoylated, we performed a metabolic incorporation assay using a biorthogonal fatty acid analog (alkynyl palmitic acid) with click chemistry conjugation. Endogenous GLUT1, but not another glucose transporter GLUT3 in GBM cells, is S-palmitoylated through thioester bonds that are cleavable by treatment with hydroxylamine (HAM) (Fig. 1c) and Supplementary Fig. 1b). To quantify the palmitoylation levels of GLUT1, we performed an acyl-PEG exchange (APE) assay, which enables the separation and semi-quantification of palmitoylated protein. Endogenous GLUT1 was highly palmitoylated (~90%), and its palmitoylation levels were dramatically reduced upon treatment with 2-BP (Fig. 1d). Omission of HAM treatment abolished the palmitoylation of GLUT1, which confirmed that GLUT1 is S-palmitoylated through thioester bonds (Fig. 1d).

Moreover, GLUT1 can be labeled efficiently by alkynyl palmitic acid, alkynyl myristic acid, and alkynyl stearic acid probes, but much less efficiently labeled by an alkynyl arachidonic acid probe (Supplementary Fig. 1c), suggesting that palmitoylation, myristoylation, and stearoylation are the major acylation forms of GLUT1. In addition, results from pulse-chase experiments showed that palmitoylation of GLUT1 is a dynamic process with an approximate turnover half-life of 1 h (Supplementary Fig. 1d).

Together, these findings indicate that GLUT1 is palmitoylated and that this post-translational modification is required for maintaining GLUT1 PM localization.

GLUT1 is palmitoylated at Cys207 and S-palmitoylation is required for maintaining GLUT1 PM localization. To determine the site of GLUT1 palmitoylation, we mutated every cysteine to serine in the GLUT1 protein, and their S-palmitoylation levels were characterized by using the alkynyl palmitic acid incorporation assay. The GLUT1 C207S mutation abolished palmitoylation without altering its protein expression level (Fig. 2a), which suggests that C207 is required for GLUT1 palmitoylation. Notably, this palmitoylation site at C207 of GLUT1 is located in the intracellular loop adjacent to the PM (Supplementary Fig. 2a) and is highly conserved among different species (Supplementary Fig. 2b). Similar to the 2-BP treatment, the eGFP-GLUT1 C207S mutant displayed cytoplasmic distribution in U87 and T98G cells (Supplementary Fig. 2c).

In line with this finding, reconstituted expression of Flag-tagged sgRNA-resistant (r) GLUT1 C207S in endogenous GLUT1 knockout (KO) U87 and T98G cells markedly blocked the incorporation of alkynyl palmitic acid onto GLUT1 (Fig. 2b, c). These results were validated by using the APE assays, which showed that palmitoylation levels of GLUT1 were dramatically reduced by reconstituted expression of rGLUT1 C207S as compared with wild-type (WT) rGLUT1 (Fig. 2d). Similar to that observed in 2-BP-treated U87 and T98G cells (Fig. 1a, b), the rGLUT1 C207S variant protein showed a cytoplasmic distribution (Fig. 2e) and was depleted from the PM fractions of U87 and T98G cells (Fig. 2f).

DHHC9 mediates GLUT1 S-palmitoylation and maintains GLUT1 PM localization. To identify the potential palmitoyl acyltransferases that regulate GLUT1, a set of lentiviral plasmids expressing guide RNAs (gRNAs) targeting PATs were constructed and used in a clustered regularly interspaced short palindromic repeats (CRISPR) screen. Expression of DHHC9 gRNA abolished the incorporation of alkynyl palmitic acid onto GLUT1 (Supplementary Fig. 3a), suggesting that DHHC9 is one of the PATs that regulate GLUT1 palmitoylation in GBM cells. In reconfirmation assays, depleting DHHC9 with short hairpin RNAs (shRNAs) abrogated GLUT1 palmitoylation (Supplementary Fig. 3b). Co-immunoprecipitation (Co-IP) analyses showed that DHHC9 and GLUT1 form a complex in U87 and T98 cells (Supplementary Fig. 3c). Furthermore, purified DHHC9 directly bound to purified GLUT1 in an in vitro binding assay (Supplementary Fig. 3d). In addition, IF analyses with specificity-validated antibodies (Supplementary Fig. 3e) demonstrated that endogenous DHHC9 and GLUT1 colocalized at the PM of
U87 and T98 cells (Supplementary Fig. 3f). Consistently, robust reconstituted GFP fluorescence was observed at the PM of U87 and T98 cells in a split-GFP system\(^{20}\) coexpressing C-terminal split-GFP (S1-10)-tagged DHHC9 and split-GFP (S11)-tagged GLUT1 (Supplementary Fig. 3g, h), demonstrating that DHHC9 and GLUT1 colocalize at the PM of these cells. Taken together, these data suggest that DHHC9 interacts and palmitoylates GLUT1 at the PM of GBM cells.

PAT-catalyzed S-palmitoylation of proteins utilizes palmitoyl-CoA as the palmitate donor. Thus, we performed an in vitro palmitoylation assay by mixing highly purified recombinant DHHC9 and GCP16, which have been reported to constitute a PAT\(^{13,14}\), with GLUT1 in the presence of palmitoyl alkyne-CoA as the palmitate donor (Fig. 3a). WT DHHC9 in complex with GCP16, but not single WT DHHC9 or catalytically inactive DHHC9 C169S (Supplementary Fig. 4a) in complex with GCP16, was able to incorporate palmitoyl alkyne (Supplementary Fig. 4b). Importantly, WT GLUT1, but not GLUT1 C207S, was palmitoylated by WT DHHC9 in complex with GCP16, but not DHHC9 C169S in complex with GCP16 (Fig. 3a). Similar results were also observed in an in vitro palmitoylation assay using palmitoyl-CoA as the palmitate donor and by detecting GLUT1 and DHHC9 palmitoylation in the APE assays (Fig. 3b).

To further validate our findings in GBM cells, we used CRISPR/Cas9 genome-editing technology to knockout endogenous DHHC9 and reconstitutively expressed Flag-tagged WT rDHHC9 or rDHHC9 C169S mutant (Fig. 3c). Endogenous DHHC9 KO substantially blocked GLUT1 palmitoylation (Fig. 3d, e). These effects were abrogated by reconstituted expression of WT rDHHC9, but not the catalytically inactive rDHHC9 C169S mutant (Fig. 3d, e). As expected, endogenous DHHC9 KO rendered GLUT1 largely cytoplasmic (Supplementary Fig. 4c), resembling the distribution observed after 2-BP treatment (Fig. 1a). Consistently, cellular fractionations of U87 and T98G cells confirmed that DHHC9 KO substantially suppressed GLUT1 PM association (Fig. 3g), and this suppression was abrogated by reconstituted expression of WT DHHC9 in complex with GCP16, but not DHHC9 C169S in complex with GCP16 (Fig. 3a). Similar results were also observed in an in vitro palmitoylation assay using palmitoyl-CoA as the palmitate donor and by detecting GLUT1 and DHHC9 palmitoylation in the APE assays (Fig. 3b).
rDHHC9, but not rDHHC9 C169S. Similarly, reducing the expression of DHHC9 by adenovirus-mediated shRNAs significantly suppressed GLUT1 PM localization in PDX cells (Supplementary Fig. 4d–f). Collectively, these data suggest that DHHC9 palmitoylates GLUT1 to maintain its PM localization.

We next investigated whether DHHC9-mediated GLUT1 S-palmitoylation regulates GLUT1 plasma membrane localization in normal astrocytes. Notably, DHHC9 and GLUT1 are highly expressed in GBM cells compared with non-transformed normal human astrocytes (NHAs) (Supplementary Fig. 5a). Endogenous DHHC9 depletion by adenovirus-mediated shRNAs markedly blocked palmitoylation (Supplementary Fig. 5b, c) and PM association (Supplementary Fig. 5d) of GLUT1 in NHAs. These results suggest that DHHC9-mediated GLUT1 palmitoylation is also required for maintaining GLUT1 PM localization in normal human astrocytes.

Given that PM localization of GLUT1 depends on protein kinase B (PKB, also known as AKT) and Protein kinase C (PKC)
To further examine whether GLUT1 palmitoylation is affected by activation of these kinases. We pretreated U87 cells with LY294002 and Gö−6983 to inhibit the kinase activity of AKT and PKC, respectively. Alkyl palmitic acid incorporation assay demonstrated that inhibition of AKT and PKC did not alter GLUT1 palmitoylation (Supplementary Fig. 5e), suggesting that GLUT1 palmitoylation is independent of AKT and PKC activation in GBM cells.

**Fig. 3 DHHC9 palmitoylates GLUT1 at Cys207 to maintain GLUT1 PM localization.**

- **a** In vitro palmitoylation analysis was performed by mixing purified WT DHHC9/GCP16 and DHHC9 C169S/GCP16 with purified WT GLUT1 or GLUT1 C207S in the presence of palmitoyl alkyne-CoA. GLUT1 palmitoylation levels were analyzed by click reaction and streptavidin bead pulldown, followed by immunoblotting.
- **b** In vitro palmitoylation analysis was performed by mixing purified WT DHHC9/GCP16 and DHHC9 C169S/GCP16 with purified WT GLUT1 or GLUT1 C207S in the presence of palmitoyl-CoA. The palmitoylation levels of GLUT1 were analyzed by APE assays. The top band indicates the palmitoylated GLUT1 (PEG-GLUT1).
- **c** Flag-tagged WT rDHHC9 or rDHHC9 C169S was reconstitutively expressed in GBM cells with the knockout of endogenous DHHC9. Immunoblotting was performed using indicated antibodies.
- **d** DHHC9-knockout U87 or T98G cells rescued with Flag-tagged WT rDHHC9 or rDHHC9 C169S was metabolically labeled with 50 μM of alkynyl PA for 4 h. Palmitoylation levels of GLUT1 were analyzed by click reaction and streptavidin bead pulldown, followed by immunoblotting.
- **e** APE assay was performed to analyze the GLUT1 palmitoylation in DHHC9-knockout U87 or T98G cells with reconstituted expression of Flag-tagged WT rDHHC9 or rDHHC9 C169S. The top band indicates the palmitoylated GLUT1 (PEG-GLUT1).
- **f** DHHC9-knockout U87 or T98G cells with or without reconstituted expression of Flag-tagged WT rDHHC9 or rDHHC9 C169S were incubated with 1μg/ml−1 of CTB conjugated with Alexa Fluor 594 for 5 min at 37 °C. GLUT1 cellular localization was visualized by immunofluorescent staining using the antibody against GLUT1 and the PM was marked by Alexa Fluor 594-conjugated CTB. PM plasma membrane. Scale bar, 20 μm.
- **g** Levels of GLUT1 in the PM and ICM fractions were analyzed by immunoblotting in DHHC9-knockout U87 or T98G cells rescued with Flag-tagged WT rDHHC9 or rDHHC9 C169S. ATP1A1, calnexin, and GAPDH served as the marker of the PM, ICM, and cytosol fraction, respectively. PM plasma membrane, ICM intracellular membrane, WCL whole-cell lysate. Representative results were obtained from at least three independent experiments with similar results. Source data are provided as a Source Data file.
glucose uptake, we performed the glucose uptake assays by using a glucose structurally similar compound 2-deoxyglucose (2-DG) (Fig. 4a). DHHC9 KO or GLUT1 KO significantly suppressed glucose uptake in U87 and T98G cells (Fig. 4a). Notably, this suppression was ablated by reconstituted expression of WT rDHHC9 or rGLUT1, but not rDHHC9 C169S or rGLUT1 C207S, respectively (Fig. 4a). In line with this finding, we detected a decreased glycolytic rate (Fig. 4b), glucose consumption (Fig. 4c), and lactate production (Fig. 4d) in DHHC9-KO or GLUT1-KO U87 and T98G cells and these decreases were restored by reconstituted expression of WT rDHHC9 or rGLUT1, but not rDHHC9 C169S or rGLUT1 C207S, respectively (Fig. 4b–d). These results show that DHHC9-mediated GLUT1 S-palmitoylation promotes glycolysis in GBM cells. In contrast, endogenous DHHC9 depletion by adenovirus-mediated shRNAs did not significantly alter the glucose uptake and glycolytic rate in NHAs (Supplementary Fig. 6a–c), suggesting that NHAs may use mechanisms beyond DHHC9-mediated GLUT1 S-palmitoylation to support their glycolysis.

To further determine the functional consequences of DHHC9-mediated GLUT1 S-palmitoylation, we performed growth and colony-formation analyses of U87 and T98G cells. As expected, DHHC9 KO or GLUT1 KO inhibited proliferation (Fig. 4e) and colony formation (Fig. 4f, g) of U87 and T98G cells. Reconstituted expression of WT rDHHC9 or rGLUT1 restored cell proliferation (Fig. 4e) and colony formation (Fig. 4f, g). In contrast, reconstituted expression of rDHHC9 C169S or rGLUT1 C207S did not have these effects on cells (Fig. 4e–g). These results demonstrate that DHHC9-mediated GLUT1 S-palmitoylation promotes the growth and colony formation of GBM cells.

**DHHC9-mediated GLUT1 S-palmitoylation promotes GBM tumorigenesis.** To investigate the functions of DHHC9-mediated GLUT1 S-palmitoylation in GBM development, we intracranially injected athymic nude mice with luciferase-expressing U87 cells with or without knockout of endogenous DHHC9 or GLUT1 and reconstituted expression of their WT counterparts, rDHHC9 C169S or rGLUT1 C207S (Fig. 5a). Bioluminescent imaging demonstrated that injection of DHHC9-KO or GLUT1-KO U87 cells resulted in significant inhibition of brain tumor growth in mice (Fig. 5a), which was accompanied by a considerably prolonged survival time (Fig. 5b). These alterations were abrogated by reconstituted expression of WT rDHHC9 or rGLUT1, but not rDHHC9 C169S or rGLUT1 C207S (Fig. 5a, b).

Immunohistochemical (IHC) analyses with anti-DHHC9, anti-GLUT1, anti-Ki67, and anti-cleaved PARP1 antibodies revealed that tumor samples derived from U87 cells with the knockout of endogenous DHHC9 or GLUT1 displayed no expression of DHHC9 or GLUT1, respectively, had decreased expression of proliferation marker Ki67 and increased positive rate of apoptotic marker cleaved PARP1 (Fig. 5c). Reconstituted expression of WT rDHHC9 or rGLUT1, but not rDHHC9 C169S or rGLUT1 C207S, restored Ki67 expression, and abrogated the increased PARP1 cleavage (Fig. 5c). Notably, we found that knockout of endogenous DHHC9, similar to rGLUT1 C207S expression, significantly reduced S-palmitoylation (Supplementary Fig. 7a) and PM localization of GLUT1 (Fig. 5c). These effects were abrogated by reconstituted expression of WT rDHHC9, but not rDHHC9 C169S (Fig. 5c and Supplementary Fig. 7a). In addition, 2-DG uptake was markedly suppressed in tumors derived from U87 cells with the knockout of endogenous DHHC9 or GLUT1, and these suppression effects were abrogated by reconstituted expression of WT rDHHC9 or rGLUT1, but not rDHHC9 C169S or rGLUT1 C207S (Supplementary Fig. 7b). These results show that DHHC9-mediated GLUT1 S-palmitoylation promotes GBM tumorigenesis.

**DHHC9 expression positively correlates with GLUT1 plasma membrane localization in GBM specimens and indicates clinical aggressiveness of GBM.** To determine the clinical significance of the observed DHHC9-regulated GLUT1 PM localization, we next performed IHC analyses in GBM samples from 68 patients with anti-DHHC9 and anti-GLUT1 antibodies (Fig. 6a), revealing that DHHC9 expression levels were positively correlated with the percentage of GLUT1 PM localization (Fig. 6a). Quantification of the staining on a scale of 0–8 indicated that these correlations were significant (Fig. 6b). In addition, we performed the survival analyses in these patients, all of whom had received standard therapies, with stratification by levels of DHHC9 expression and PM-localized GLUT1 in tumor tissues (Fig. 6c). The median overall survival (OS) duration was 1057 and 1380 days for patients whose tumors had low levels of DHHC9 expression and PM-localized GLUT1, respectively, and 778 and 436 days for those whose tumors had high levels of DHHC9 expression and PM-localized GLUT1, respectively (Fig. 6c). Multivariate analyses revealed that a high level of DHHC9 expression and PM-localized GLUT1 was an independent, unfavorable prognostic indicator for OS of GBM patients after adjusting for patient age, sex, and total resection status, all of which are relevant clinical covariates (Supplementary Table 1). Taken together, these analyses reveal that a high level of DHHC9 expression and PM-localized GLUT1 significantly correlate with the clinical aggressiveness of GBM.

**Discussion**

A previous study has shown that the P485L mutation creating a dileucine motif in the cytosolic tail causes GLUT1 internalization from the PM, leading to deficiency of glucose uptake in mammalian cells. This phenomenon suggested that PM localization, which is independent of gene expression levels, is important for the biological functions of GLUT1. We demonstrate here that DHHC9 palmitoylates GLUT1 at Cys207 to maintain PM localization of GLUT1. Furthermore, PM-localized GLUT1 increases glucose uptake, glycolytic rate, and lactate production, consequently promoting GBM cell proliferation and GBM tumorigenesis (Fig. 6d). These findings expand the layers of protein biological functions regulated by post-translational modification, highlighting the importance of protein subcellular localization during cancer progression.

Protein S-palmitoylation was discovered as a post-translational modification that is well-known to regulate membrane distribution of proteins and cell signaling. However, very little is understood about how S-palmitoylation contributes to metabolic homeostasis. This study demonstrates that DHHC9-mediated S-palmitoylation of glucose transporter GLUT1 promotes glycolysis in human GBM cells, implying that S-palmitoylation-regulated membrane localization of cellular proteins may be required for metabolic reprogramming in cancer cells.

Our findings demonstrate that knockout of palmitoyltransferase DHHC9 or disruption of DHHC9-mediated GLUT1 S-palmitoylation by site-specific point mutation inhibited growth and colony formation of GBM cells in vitro and GBM tumorigenesis in vivo. These results indicate that DHHC9 contributes to the malignant progression of GBM through facilitating GLUT1 palmitoylation.

GBM cells had a dramatic decrease in glucose uptake and glycolytic rate under the condition of DHHC9 KO. In contrast, NHAs, which have lower expression of DHHC9 compared to GBM cells, did not show significant alteration in glucose uptake or glycolytic rate when endogenous DHHC9 was depleted. These findings suggest that GBM cells have increased dependence...
Fig. 4 DHHC9-mediated GLUT1 S-palmitoylation promotes glycolysis, growth and colony formation of GBM cells. Flag-tagged WT rDHHC9 or rDHHC9 C169S, WT rGLUT1, or rGLUT1 C207S were reconstitutively expressed in endogenous DHHC9-knockout or GLUT1-knockout U87 or T98G cells, respectively. Data represent the mean ± SD of three independent experiments. *P value was determined by the two-tailed Student’s t test. (a) The indicated cells were treated with 1 mM of 2-DG for 10 min. Uptake of 2-DG was measured using a glucose uptake kit and normalized to cell number. RLU relative luminescence units. *P = 1.15E-05, **P = 4.81E-05, 6P = 3.25E-05, 5P = 1.28E-05, #P = 2.32E-05, ##P = 1.85E-05, 6&P = 1.04E-05, 5&P = 3.60E-05. (b) The indicated cells were incubated with 5.5 mM glucose spiked with 10 μCi of D-[5-3H] glucose for 1 h. The glycolytic rate was measured by monitoring the conversion of D-[5-3H] glucose to 3H2O and normalized to cell numbers. c.p.m. counts per minute. *P = 2.85E-04, **P = 2.54E-04, 6P = 2.44E-04, 5P = 1.21E-04, #P = 1.94E-05, ##P = 2.81E-04, 6&P = 7.95E-05, 5&P = 2.23E-04. (c) The indicated cells were treated with DMEM without serum for 16 h. The media were collected for analysis of glucose consumption. *P = 1.68E-03, **P = 1.40E-03, 6P = 2.40E-04, 5P = 5.93E-04, #P = 1.03E-03, ##P = 3.33E-04, 6&P = 2.60E-04, 5&P = 3.03E-04. (d) The indicated cells were treated with DMEM without serum for 6 h. The media were collected for analysis of lactate production. *P = 2.45E-04, **P = 5.98E-05, 6P = 1.79E-04, 5P = 9.70E-05, 6&P = 2.07E-05, 5&P = 2.37E-05, 5&P = 2.55E-05. (e) The indicated cells (2 x 10^5) were seeded in six-well plates and cultured for 3 days. The cells were then collected and counted. *P = 1.26E-04, **P = 4.26E-04, 6P = 1.91E-04, 5P = 1.16E-04, #P = 4.96E-05, ##P = 1.30E-04, 6&P = 5.21E-04, 5&P = 9.62E-05, 6&P = 2.29E-04. (f) One thousand U87 or T98G cells were seeded in a 60-mm dish and cultured for 14 days. The cells were then fixed by 4% paraformaldehyde and stained with crystal violet (left panels). Colony-formation number in each dish was counted (right panels). *P = 6.01E-04, **P = 2.52E-03, 6P = 1.27E-03, 5P = 1.08E-03, 6&P = 8.97E-04, 5&P = 8.04E-04, 5&P = 2.29E-04. Source data are provided as a Source Data file.
on DHHC9-mediated GLUT1 S-palmitoylation, unlike normal astrocytes which primarily depend on other mechanisms to maintain their glycolysis, highlighting that DHHC9 could be a potential target for cancer cell-specific inhibition of glycolysis.

Therefore, the development of small molecules against DHHC9 palmitoyltransferase activity may provide an alternative approach to treat human GBM. Importantly, high DHHC9 expression in tumor tissues appeared to correlate with poor survival of GBM.

**Fig. 5 DHHC9-mediated GLUT1 S-palmitoylation promotes GBM tumorigenesis.**

- **a, b** Luciferase-expressing DHHC9-knockout U87 cells rescued with Flag-tagged WT rDHHC9 or rDHHC9 C169S and GLUT1-knockout GBM cells rescued with Flag-tagged WT rGLUT1 or rGLUT1 C207S were intracranially injected into athymic nude mice (n = 5). Luminescence intensity derived from tumors was measured and relative luminescence intensity is shown (a). Data were collected from n = 5 mice per group, and the mean ± SD values are shown (a). The survival times of the mice (n = 10) were recorded (b). *P = 2.57E-06, #P = 1.16E-05, $P = 9.63E-06, \&P = 1.82E-05, \*P = 4.00E-06, \#P = 3.00E-06, \&P = 5.00E-06, \*P = 4.00E-06. **P = 3.87E-19, \#P = 1.44E-18, \&P = 3.33E-12, \*P = 8.58E-14, \#P = 1.15E-18, \&P = 4.84E-18, $\&P = 5.63E-13, \$\$P = 1.14E-13. P values were determined by the two-tailed Student’s t test (a, c) and the two-tailed log-rank test (b). a.u., arbitrary unit (a).

Source data are provided as a Source Data file.
patients, suggesting that DHHC9 could serve as a prognostic indicator for GBM patients.

**Methods**

**Materials.** Rabbit monoclonal antibodies recognizing GLUT1 (ab115370), tubulin (ab176506), Ki67 (ab92742) and ATP1A1 (ab76020), and mouse monoclonal antibody recognizing GAPDH (ab8245) were obtained from Abcam. Mouse monoclonal antibodies recognizing GCP16 (sc-101278) and GLUT3 (sc-74399), n-dodecyl-β-D-maltoside (DDM) (sc-281071), and n-Nonyl-β-D-glucopyranoside (sc-281084) were purchased from Santa Cruz Biotechnology. Mouse monoclonal antibody recognizing GAPDH (ab8245), nickel affinity gel (PA5-56868), 10 kDa spin column (#88513), streptavidin agarose beads (#SA10004), EDTA-free protease inhibitors cocktail (#A32965), cholera toxin (PDX cells derived from a GBM primary tissue were obtained from Drs. Chunsheng Kang and Chuan Fang (Tianjin Medical University) and used in previous publications24,25. The GBM tissue was surgically removed from a 46-year-old male Chinese patient. Informed consent was obtained and the use of a patient specimen and relevant database was approved by the Human Research Ethics Committee of Tianjin Medical University. is routiney tested for mycoplasma contamination. Cell lines were authenticated by Short Tandem Repeat (STR) pro-
Glycolytic rate assays. Glycolytic rate assay was performed according to the published procedure with slight modifications. Briefly, GBM cells (5 × 10⁴) seeded in 12-well plates and grown for 12 h and incubated with 2 ml of Krebs buffer (126 mM NaCl, 2.5 mM KCl, 25 mM NaHCO₃, 1.2 mM NaH₂PO₄, 1.2 mM MgCl₂, 2.5 mM CaCl₂) without glucose for 30 min at 37 °C. The Krebs buffer was removed and 2 ml of Krebs buffer containing 5.5 mM glucose spiked with 10 μCi of [³²P]-5-[³²P]-glucose was then added into each well followed by incubation for 1 h at 37 °C. The Krebs buffer containing 10 μCi of [³²P]-5-[³²P]-glucose and without any buffer was added into an equal volume of 2 N HCl in an uncapped PCR tube, which was then transferred into a 1.5 ml Eppendorf tube containing 0.5 ml of unlabeled distill water. The Eppendorf tubes were sealed to allowed diffusion of ³²H₂O into unlabeled water for 24 h at 37 °C. The amount of diffused ³²H₂O was measured using a scintillation counter and normalized to the cell number counted in each sample.

Glucose uptake assay. Glucose uptake was measured by a kit obtained from Promega (#J1342) according to the manufacturer’s instructions. Briefly, 10,000 GBM cells were seeded into 96-well plates and grew for 12 h. The cells were washed twice with PBS and incubated with 1 ml of 2-DG for 10 min at 37 °C. The uptake was terminated by the addition of an acidic detergent solution (stop buffer) and neutralization buffer was then added to neutralize the acid. The 2-DG6P detection reagent containing glucose-6-phosphate dehydrogenase, NADP⁺, reductase, recombinate lucerase, and proluciferin substrate was added to the sample wells. The plate was incubated for 1 h at 25 °C and luminescence intensity was read on a luminesimeter with 0.3-1 s integration. The glucose uptake level was normalized to the cell number counted in a duplicate sample.

Production and purification of recombinant proteins. Baculovirus expression system (Thermo Fisher Scientific, MA) was used for the production of 6His-tagged GLUT1 and GST-tagged DHHC9 or GCP16 proteins. The cells were harvested 48 h after viral infection and homogenized in the buffer (25 mM Tris [pH 8.0], 150 mM NaCl, 0.1% Triton X-100, containing protease inhibitor cocktail and 0.2% 2-mercaptoethanol). The lysates were loaded onto a Ni-NTA column (GE Healthcare Life Sciences, PA) followed by washing with washing buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Triton X-100, 50 mM imidazole) and elution buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5% glycerol, 0.4% (w/v) n-Nonyl-phthalate-2-sulfonate) followed by a Cu(I)-assisted click reaction with biotin picoyl azide (50 μM) to biotinylate the proteins with the incorporation of palmitoyl alkyne. The samples were loaded onto 10 kDa spin columns to remove the free biotin picoyl azide using a buffer containing 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.5 mM EDTA, 0.1% Triton X-100, 0.1% SDS, and 0.5% NP40. Biotinylated proteins were captured by streptavidin agarose beads prior to boiling in SDS-PAGE sample buffer without DTT for 10 min at 95 °C. Immunoblotting was performed to analyze the palmitoylation of the target protein.

GST pull-down assay. His-tagged purified proteins (200 ng/sample) were mixed with 100 ng of GST fusion proteins or GST protein as a control in a binding buffer (50 mM Tris-HCl [pH 7.5], 1% Triton X-100, 100 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 100 μM PMFS, 100 μM leupeptin) for 1 h and incubated with glutathione agarose beads for an additional 30 min at 4 °C. The bound protein complexes were retained by washing the glutathione beads with binding buffer three times and detected by immunoblot.

Conflonal microscopic analysis. To label the plasma membrane of cells, GBM cells with or without expression of eGFP-tagged proteins were seeded into eight-well chamber slides (12 h before staining) and then stained with Alexa Fluor 594 (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 100 μM PMFS, 100 μM leupeptin) for 1 h and incubated with glutathione agarose beads for an additional 30 min at 4 °C. The bound protein complexes were retained by washing the glutathione beads with binding buffer three times and detected by immunoblot.

Glucose-6-phosphate dehydrogenase (G6PDH) was also expressed in seven-well plates and grown for 12 h. Equal amounts of purified G6PDH (200 ng/sample) were mixed with an equal volume of 0.2 N HCl in an uncapped PCR tube, which was then transferred into 1.5-ml Eppendorf tube containing 0.5 ml of unlabeled distill water. The Eppendorf tubes were sealed and allowed diffusion of ³²H₂O into unlabeled water for 24 h at 37 °C. After the addition of ³²H₂O to the samples, the Eppendorf tubes were incubated at 37 °C for 1 h. Glucose-6-phosphate dehydrogenase (G6PDH) in the buffer was then added to each sample and followed by incubation for 1 h at 37 °C. The radioactivity was measured using a scintillation counter. The specific activity of each sample was calculated as the ratio of radioactivity to the glucose uptake count rate from the Glucose uptake assay.

Glucose fed uptake assay. Glucose uptake assay was performed according to the published procedure with slight modifications. Briefly, glucose was added to the 96-well plates containing GBM cells seeded in each well. The plates were incubated for 1 h at 37 °C. The amount of glucose fed uptake was measured using a scintillation counter and normalized to the cell number counted in a duplicate sample.

Production and purification of recombinant proteins. Baculovirus expression system (Thermo Fisher Scientific, MA) was used for the production of 6His-tagged GLUT1 and GST-tagged DHHC9 or GCP16 proteins. The cells were harvested 48 h after viral infection and homogenized in the buffer (25 mM Tris [pH 8.0], 150 mM NaCl, 0.1% Triton X-100, containing protease inhibitor cocktail and 0.2% 2-mercaptoethanol). The lysates were loaded onto a Ni-NTA column (GE Healthcare Life Sciences, PA) followed by washing with washing buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5% glycerol, 0.4% (w/v) n-Nonyl-phthalate-2-sulfonate) and concentrated to about 10 mg ml⁻¹ followed by loading onto a Superdex-200 gel filtration column (GE Healthcare Life Sciences, PA) to remove the contaminated proteins. The peak fractions were collected and separated by SDS-PAGE. High-sensitivity colloidal Coomassie Blue (C250) staining was performed to examine the purification efficiency.

Transient transfection. The plasmids were transiently transfected into GBM cells with Lipofectamine 3000 (Invitrogen, CA) according to the manufacturer’s protocol. Cells were harvested and analyzed 36 h after transfection.

Immunoprecipitation and immunoblotting. Extraction of proteins from cultured cells was performed with a modified lysis buffer (50 mM Tris-HCl [pH 7.5], 1% SDS, 0.5% Triton X-100, 10% glycerol, 0.05 mM EDTA, 0.1% PMSF, 100 μM leupeptin). Cell debris was removed by centrifugation at 13,400 × g for 10 min at 4 °C. The supernatant was then mixed with protein A or G agarose beads for an additional 30 min at 4 °C. The bound protein complexes were retained by washing the glutathione beads with binding buffer three times and detected by immunoblot.

Plasmid and intracellular membrane fractionation. Plasma membrane (PM) and intracellular membrane (ICM) fractions were isolated using a PM/ICM protein extraction kit (#ab65400) obtained from Abcam according to the manufacturer’s instructions. Briefly, cells (5 × 10⁴) cultured on 13-cm dishes were rinsed twice with ice-cold PBS and immediately incubated with 2 ml of homogenization buffer on ice and harvested using a cell scraper. Resuspended cells were homogenized in a Dounce homogenizer for 30–50 times on ice and spun at 700g for 5 min at 4 °C. The supernatants were transferred to new vials and centrifuged at 10,000g for 30 min at 4 °C. The pellet and supernatant contained total cellular membrane proteins and cytosolic proteins, respectively. The pellets were resuspended in 200 μl of upper-phase solution and then mixed with 200 μl of lower-phase solution. The samples were incubated on ice for 5 min and centrifuged at 1000g for 5 min at 4 °C; upper-phase solution and lower-phase solution containing PM proteins and ICM proteins, respectively, were collected and transferred to new tubes. Extraction repeats were repeated by adding fresh upper-phase solution to the lower-phase solution and vice versa. The resulting upper-phase solution and the lower-phase solution from the repeated extraction were combined and diluted with 5×volume of water, followed by centrifugation at 15,000×g for 30 min at 4 °C. The pellets containing PM or ICM proteins were harvested and then analyzed by immunoblotting.

Lentiviral production and generation of stable cell lines. Guide RNAs targeting SLC22A1 (GLUT1 coding gene) or DHHCs were designed using Cas9 target design online tool (http://www.genome-engineering.org). Three different promising guide RNAs were chosen and cloned into the lentiviral vector with a selectable marker of puromycin. The empty vector lentiviral vector was used as a control. Presence of the guide RNA was confirmed by RT-PCR. Lentiviral vectors were produced by cotransflecting 293FT cells with lentiCRISPRv2 plasmid containing expression cassettes of hSpCas9 and the chimeric guide RNA, and two packaging plasmids pMD2.G (#12259) and psPA2 (Addgene #12260). Infectious lentiviruses were harvested 72 h after transfection, centrifuged to remove cell debris, and filtered through a 0.45-μm filter (Millipore, MA). GBM cells were infected with lentiviruses at a multiplicity of infection [MOI] of 1 and then selected by 1 μg ml⁻¹ of puromycin for 7 days. The knockout efficiency was evaluated by immunoblotting. For rescued expression of GLUT1 or DHHC9 in GLUT1 and DHHC9-knockout cells, respectively, Flag-tagged sgRNA-resistant (r) wild-type GLUT1 or rDHHC9 or rmutant (r) GLUT1 (2075g and rDHHC9 C1698) were cloned into pCDH lentiviral vector selectable marker of hygromycin. Lentiviruses were produced by the pMD2.G/pspA2 packaging system. Infection of cells was
performed in 6-well plates at a MOI of 1, and selected under 200 μg ml⁻¹ hygromycin for 10 days. The rescued protein expression was evaluated by immunoblotting using specific antibodies.

The pGIPZ lentiviral vector was used for the construction of the control shRNA and shRNAs targeting DHHC9. The lentiviruses were produced as described above. GBM cells were infected with the lentiviruses following with selection with 1 μg ml⁻¹ of puromycin for 7 days. The knockdown efficiency was evaluated by immunoblotting using specific antibodies.

Supplementary Table 2 contains the detailed information of the nucleotide sequences of guide RNAs (gRNAs) or shRNAs targeting indicated genes.

**Adenovirus preparation and infection.** Recombinant adenoviruses expressing the control shRNA or DHHC9 shRNAs were produced by RapaD adenoviral expression system (Cell Biosabs, CA) according to the manufacturer’s instructions with some modifications. Briefly, an Xhol/EcoR1 fragment containing a U6-shRNA cassette was amplified from plKO.1 vector using high-fidelity PCR and ligated into Xhol/EcoR1-digested adenoviral shuttle vector pacAdS K-Npa. Adenoviruses were produced by cotransfection of the Pac1-digested pacAdS U6-shRNA and pacAd5 9.2-100 plasmid into Adeno-X 293 cells (Clontech Laboratories, CA). Infectious adenoviruses were collected 8 days after transfection, centrifuged to remove cell debris, filtered through a 0.45-μm filter (Millipore), and used to infect the Adeno-X 293 cells to produce high-titer adenoviral particles. Adenoviruses were purified using an Adenovirus Purification Kit (Clontech Laboratories) and titration was determined by an Adeno-X Rapid Titer Kit (Clontech Laboratories) according to the manufacturer’s instructions. For adenovirus infection, PDX cells or NHA cells were infected with indicated adenoviruses at a multiplicity of infection (MOI) of 30 and replaced with fresh media at 12 h after infection.

**Glucose consumption**

The brain of each mouse was then harvested, and 20 mg of tumor tissue was dissected for homogenization in 0.2 ml of lysis buffer, which was prepared by mixing two volumes of PBS with one volume of Stop buffer provided by the glucose uptake assay kit (Promega #J1342). Homogenate was cleared by centrifugation at 10,000×g for 10 min. The supernatant of each sample (75 μl) was transferred to a 96-well plate and mixed with 25 μl of neutralization buffer followed by the addition of 100 μl of 2-DG/6P detection reagent. The plate was incubated for 1 h at 25 °C, and luminescence intensity was read by a luminometer with 0.3–1 s integration.

**Bioluminescent imaging.** Bioluminescent imaging of mice was performed according to the published procedure with slight modifications. Briefly, 1×10⁶ human GBM cells (5 × 10⁵) suspended in 5 μl of PBS was subcutaneously injected into the neck region of each mouse. Images of the mice were acquired 10–20 min after D-luciferin administration, and the peak photon flux within a region of interest was recorded and quantified using an IVIS Lumina System coupled with the Living Image data-acquisition software program (Xenogen Corporation, Alameda, CA).

**Statistical analysis.** Statistical analyses were performed using IBM SPSS Statistics software. The two-tailed unpaired or paired Student’s t test was used to analyze means ± SD between the control and experimental groups. The survival rate analyses were performed using the two-tailed log-rank test. The correlation between DHHC9 and PM-localized GLUT1 staining was determined by the two-tailed Pearson correlation coefficient. Multivariate analysis of overall survival in GBM patients was performed using Cox’s regression model. P values less than 0.05 were considered significant.
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