Cancer Letters 454 (2019) 98–107

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

# NT5DC2 promotes tumorigenicity of glioma stem-like cells by upregulating fyn

Saisai Guo, Haowen Ran, Dake Xiao, Haohao Huang, Lanjuan Mi, Xinzheng Wang, Lishu Chen, Da Li, Songyang Zhang, Qiuying Han, Tao Zhou, Ailing Li**, Jianghong Man*

*State Key Laboratory of Proteomics, Institute of Basic Medical Sciences, National Center of Biomedical Analysis, Beijing, 100850, China*

ARTICLE INFO

*Keywords:*
Glioblastoma
Glioma Stem-like Cell
NT5DC2
Fyn
Tumorigenesis

ABSTRACT

Glioblastoma (GBM) is an incurable primary brain tumor that is highly resistant to current treatments. Glioma stem-like cells (GSCs) are an aggressive population of glioma cells that not only initiate malignant growth, but also promote therapeutic resistance. Thus, targeting GSCs is critical for improving GBM treatment and ensuring complete eradication of the tumor. Here, we show that NT5DC2 (5′-Nucleotidase Domain Containing 2), a functionally unknown protein, plays a crucial role in GSC tumor initiation via upregulating Fyn expression. NT5DC2 is preferentially expressed in GSCs relative to the non-stem tumor cells. Knockdown of NT5DC2 significantly inhibits the GSC tumorsphere formation and cell viability in vitro, and tumorigenesis in vivo, thus, prolonging animal survival. Moreover, disruption of NT5DC2 in GSCs markedly reduces the expression of Fyn, a Src family proto-oncogene that has been implicated in the regulation of GBM progression. Importantly, the expression of NT5DC2 strongly correlated with increased aggression of human gliomas, but not that of other brain tumors. Taken together, our results uncover the function of NT5DC2 in GSC maintenance and highlight NT5DC2 as a promising therapeutic target for GBM.

## 1. Introduction

Glioblastoma multiform (GBM) is the most aggressive and lethal primary brain tumor, with a dismal prognosis. Patients with GBM have a short life expectancy, with a typical survival of less than 16 months, despite the therapeutic advances in treatments [1,2]. Glioma stem-like cells (GSCs) are a small fraction of tumor cells, with self-renewal, multilineage differentiation, and potent tumorigenic capacity. GSCs are known to promote cancer metastasis, angiogenesis, immune evasion, therapeutic resistance, and tumor recurrence [3–6], which suggests that targeting GSCs could be a promising strategy to improve GBM treatment. Thus, identification of potential therapeutic target genes that are preferentially expressed in GSCs is essential.

The non-receptor tyrosine kinase Src family kinases (SFKs) are deregulated in many types of cancers, and collectively regulate a variety of cellular functions such as proliferation, invasion, motility, survival, differentiation, and angiogenesis during tumor development [7,8]. Currently, ten SFKs have been identified: c-Src, Yes, Fyn, Lyn, Fgr, Lck, Hck, Yrk, Blk, and Frk [9]. Like c-Src, upregulation of Fyn has been shown in various tumors, including GBM, melanoma, and squamous cell carcinoma [10]. Recent studies have shown that activation of c-Src and Fyn mediates EGFR signaling, one of the most important gene aberrations in GBM tumorigenesis [11,12], to promote tumor progression and invasion [13]. However, several aspects of the function of Fyn in GSCs need further investigation.

5′-Nucleotidase Domain Containing 2 (NT5DC2) belongs to the NT5DC family, which includes members NT5DC1-4. Although studies have shown that NT5DC family members have a haloacid dehalogenase (HAD) motif localized in the N-terminal of these proteins, the physiological roles of NT5DC family proteins are poorly understood [14]. NT5DC2 has been previously found to be associated with schizophrenia [15]. However, to date, little is known about the function of NT5DC2. In this study, we found that NT5DC2 induced the expression of Fyn in GSCs and promoted GSCs tumorigenesis. We found that NT5DC2 was preferentially expressed in GSCs and significantly correlated with the expression of multiple stem cell markers. Knockdown of NT5DC2 strongly inhibited GSC tumor initiation and prolonged animal survival. Importantly, we found that NT5DC2 associated with Fyn and stabilized

* Corresponding author. State Key Laboratory of Proteomics, Institute of Basic Medical Sciences, National Center of Biomedical Analysis, Taiping Rd., Haidian District, Beijing, 100850, China.
** Corresponding author. State Key Laboratory of Proteomics, Institute of Basic Medical Sciences, National Center of Biomedical Analysis, Taiping Rd., Haidian District, Beijing, 100850, China.
*E-mail addresses:* alli@ncba.ac.cn (A. Li), jhman@ncba.ac.cn (J. Man).

https://doi.org/10.1016/j.canlet.2019.04.003
Received 20 December 2018; Received in revised form 24 March 2019; Accepted 4 April 2019

the Fyn protein in GSCs. In addition, the high expression of NT5DC2 was strongly correlated with increased aggression of human gliomas, indicating that NT5DC2 is a potential therapeutic target for GBM.

## 2. Materials and methods

### 2.1. Cell culture

All patient-derived GSCs were isolated and functionally validated as previously described [16–20], and were maintained in the Neurobasal™ Medium (Gibco, 12348017) supplemented with B-27™ Supplement (Gibco, 12587010), sodium pyruvate (Macgene, CC007), L-glutamine (Macgene, CC009), penicillin-streptomycin (Macgene, CC004), 10 ng/mL basic fibroblast growth factor (βFGF), (R&D Systems, 4114-TC) and 10 ng/mL epidermal growth factor (EGF) (R&D Systems, 236-EG). These cells were validated by a series of functional assays and express GSC markers such as SOX2, OLIG2, and CD133 etc. These assessed cells were used for sphere-forming and in vitro limiting dilution assay and orthotopic mouse xenografts in immunocompromised mice. When differentiated, GSCs were cultured in DMEM (Macgene, CM10013) with 10% fetal bovine serum (Excell Bio, FNA500) and 1% penicillin-streptomycin for more than 7 days.

### 2.2. Orthotopic mouse xenografts

All mice used in this study were maintained in the pathogen-free barrier animal facility at the National Center of Biomedical Analysis. We used 4-week-old, female, BALB/c nude mice purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. Animal care was monitored daily by certified veterinary staff and laboratory personnel. To establish the GBM xenografts, GSCs were transduced with non-targeting shRNA or NT5DC2 shRNAs through lentiviral infection. Forty-eight hours after lentiviral infection, GSCs were selected in a culture containing puromycin (1 μg/mL) for another 24 hours. Next, $5 \times 10^4$ GSCs were implanted into the right frontal lobes of nude mice. For survival experiments, animals were maintained until the manifestation of neurological signs. To compare tumor growth, groups of mice expressing shNT5DC2 or shNT were harvested on the same day (day 30). Mouse brains were fixed in 4% paraformaldehyde, followed by 30% sucrose cryoprotection for 48 hours, before they were embedded by O.C.T. compound (Sakura, 4583). Histological analysis was performed on 8-μm sections. Hematoxylin and eosin (H&E) staining were performed as described previously [18]. All animal procedures were in accordance with the Institutional Animal Care and Use Committee of the National Center of Biomedical Analysis.

### 2.3. Immunohistochemistry

Immunohistochemical staining of tissue sections was performed as described previously [16,18]. Tissue microarrays GL803b were purchased from US Biomax, including those for normal brain, low-grade and high-grade gliomas, oligodendroglioma, medulloblastoma, and ependymoma. Tissue microarrays were heated at 65 °C for 1 hour, and then dewaxed with xylene and ethanol. For antigen retrieval, tissue microarrays were treated with citrate buffer at 95 °C. Endogenous peroxidase were inactivated with 3% $H_2O_2$. Tissue microarrays were blocked by goat serum (ZSGB-BIO, ZLI-9022) for 1 hour at room temperature and then incubated overnight with an anti-NT5DC2 antibody (Abcam, ab173963) at 4 °C. The tissue microarrays were then incubated with horseradish-peroxidase-conjugated anti-rabbit IgG (ZSGB-BIO, PV-6001), and detected using DAB (ZSGB-BIO, ZLI-9017). The nuclei were stained with hematoxylin (ZSGB-BIO, ZLI-9610). NT5DC2 staining was scored from 0 to 3 (corresponding to negative, weak, moderate, and strong staining, respectively).

### 2.4. Western blot

The cells were collected, washed with PBS (PH 7.4), and lysed in M2 buffer (20 mM Tris pH 7.6, 0.5% NP40, 250 mM NaCl, 2.5 mM EDTA, 3 mM EGTA, and EDTA-free protease inhibitor cocktail). Protein concentration in the supernatants was measured by the Bradford 1X dye reagent (BIO-RAD, 500–0205). Proper amounts of protein lysates were electrophoresed on 8%–12% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride (PVDF) membranes (PALL, BSP0161), and probed with anti-NT5DC2 (Abcam, ab173963), anti-SOX2 (Santa Cruz, sc-365823), anti-Olig2 (Santa Cruz, sc-48817), anti-GFAP (Dako, Z-0334), anti-GAPDH (prepared in our lab), anti-Tubulin (Sigma, T5168), anti-cleaved Caspase 3 (Cell signaling technologies, 9664S), anti-PARP1 (Cell signaling technologies, 9542S), anti-Flag tag (Sigma, F3165), and anti-Fyn (Cell signaling technologies, 4023T) antibodies.

### 2.5. Immunofluorescence staining

Immunofluorescent (IF) staining of cells and tissue sections was carried out as previously reported [16,18]. All types of GSCs were isolated to the single-cell level. A proper number of GSCs were resuspended and plated on Matrigel-coated plate. For mouse GBM xenografts, the tissue samples were treated with citrate buffer at 95 °C for antigen retrieval. The cells and tissue samples were fixed with 4% PFA and permeabilized with PBS containing 0.3% Triton X-100 (Sigma, V900502) for 20 min at 37 °C. The cells were blocked with PBS containing 5% BSA (GenStar VA10411-500 g) and 0.1% Triton X-100 for 1 hour at room temperature and incubated using primary antibodies at 4 °C overnight. Relative antibodies were used in IF include: anti-Flag tag (Sigma, F3165), anti-SOX2 (Santa Cruz, sc-17320), anti-Olig2 (R&D Systems, AF2418), anti-NT5DC2 (Abcam, ab173963). After washing thrice with PBS, the cells or tissue sections were incubated with the appropriate secondary antibody at room temperature for 1 hour. The nuclei were stained using PBS containing 0.1% Hoechst (ThermoFisher, H3570).

### 2.6. Cell proliferation and colony formation

All types of GSCs were isolated to the single-cell level. For the gene knock down assay, 48 hours after lentiviral infection, the GSCs were selected in a culture medium containing puromycin (1 μg/mL) for another 24 hours. Approximately $2 \times 10^3$ GSCs were inoculated into each well of a 96-well plate. Cell viability were detected by Celtiter-Glo Luminescent Cell Viability Assay (Promega, G7572) on the 0, 2nd, 4th, and 6th day, and sphere numbers were counted at 5th day.

### 2.7. In vitro limiting dilution assay

Limiting dilution assay was performed as described previously [21]. All types of GSCs were isolated to the single-cell level. GSCs were implanted into a 96-well plate at a gradient of 20, 50, 100, 150, or 200 cells per well, with 10 replicates for each gradient. The number of tumorspheres in each well was determined after incubation for 7 days, and the sphere formation efficiency was calculated using the Extreme Limiting Dilution Analysis [22] (http://bioinf.wehi.edu.au/software/elda/).

### 2.8. DNA constructs and production of lentivirus

Human Flag-NT5DC2 was constructed by PCR-based amplification and cloned into the pCDH-MCS-T2A-Puro-MSCV lentiviral vector (System Biosciences). NT5DC2-specific shRNA and Fyn-specific shRNA (synthesized from ThermoFisher) inserted into the pLKO.1 vector. HEK293T cells were transfected with target plasmids, psPAX2 and VSVG by MaxiCaP (Macgene, CTK001). Following target plasmids were used: pLKO.1-shNT, pLKO.1-shNT5DC2 #1(GTGGCCTCCACTATGACA

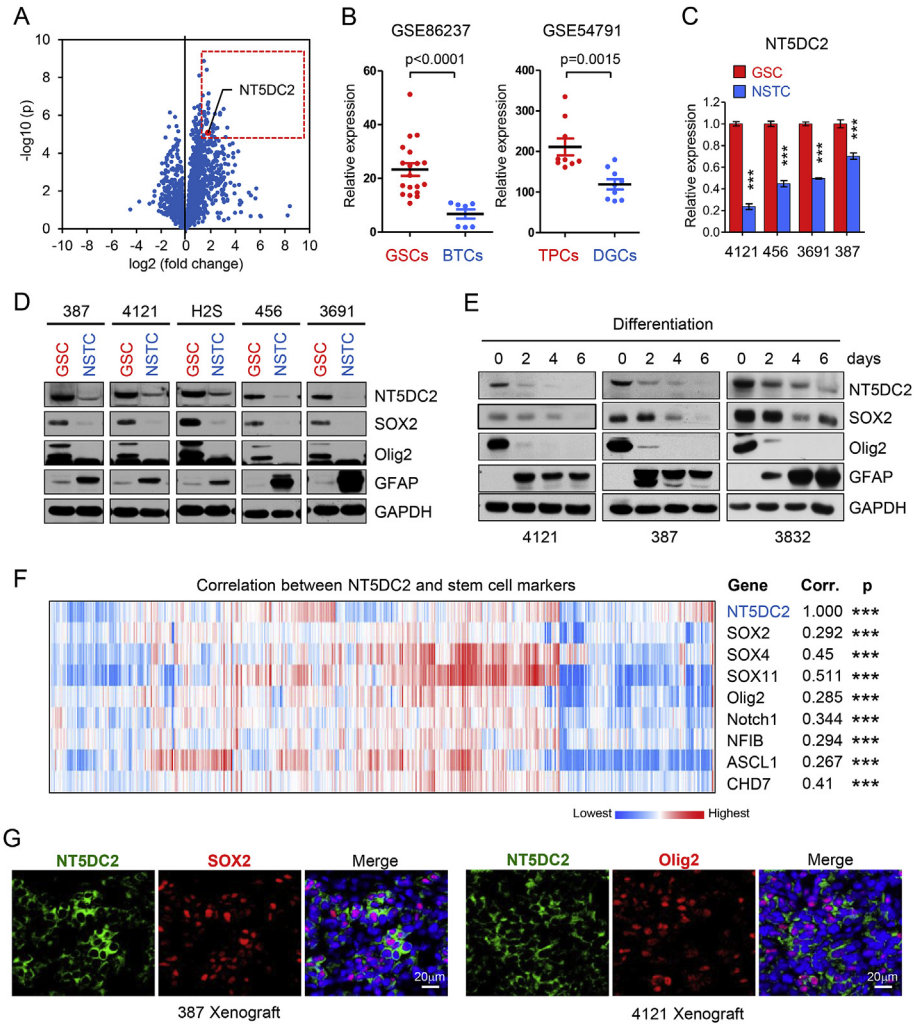


**Fig. 1. NT5DC2 is Preferentially Expressed in Glioma Stem-like Cells.** (A) NT5DC2 was found to be highly expressed in GSCs in the GSE86237 database. Volcano plot analysis of the expression difference between GSCs and GBM bulk tumor cells (BTCs) in GSE86237 database. Transcripts in red square were significantly upregulated in GSCs relative to BTCs (Fold change > 2.0 and p value < 1.0E-05). Significance testing was done by unpaired Student's t-test. (B) The relative NT5DC2 expression in GSCs vs. BTCs from the GSE86237 database (left), and tumor propagating cells (TPCs) vs. differentiated glioma cells (DGCs) from GSE54791 database (right). Significance testing was done by unpaired Student's t-test with Welch's correction. (C) mRNA expression of NT5DC2 was analyzed by qRT-PCR in GSCs and matched NSTCs. Data are shown as means ± s.d. Significance testing was done by unpaired Student's t-test. ***p < 0.001. (D) Immunoblot (IB) analysis of proteins NT5DC2, SOX2, Olig2, GFAP, and GAPDH in GSCs, and matched NSTCs. (E) Differentiation was induced in 4121, 387, and 3832 GSCs with DMEM containing 10% fetal bovine serum (FBS). IB analysis of NT5DC2, SOX2, Olig2, and GFAP during serum-induced GSC differentiation. (F) Heat map of the correlation analysis of expression between NT5DC2 and multiple stem cell markers in the TCGA GBM database. Correlation was derived from the average linkage hierarchical clustering and all data were normalized against the median-centered intensity, with lowest expression in blue and highest expression in red. ***p < 0.001. (G) Co-Immunofluorescence (Co-IF) staining of NT5DC2 (green) and SOX2 or Olig2 (red) in mouse GBM xenografts. Nuclei were counterstained with Hoechst (blue). Representative images are shown.

TTC), #2 (CAGAAGGGATTCGGAAGTATG), #3(TACCCAGCACATCCC ACTATA), and pLKO.1-shFyn #1(GTGCCAACAATCCTAGTGCTT), #2( CATCGAGCGCATGAATTATAT). 60–72 hours after medium renewed, medium was collected and filtered by filter membrane with aperture of 0.45 μm. The filtrate was incubated with 5X PEG buffer overnight at 4 °C and then were centrifuged at 7000 g. Supernatant was discarded and precipitate was re-suspended by 4 °C PBS. The solution was centrifuged at 12000g. Supernatant was divided into four equal volumes. Each volume was used to infect $1.5 \times 10^6$ GSCs.

### 2.9. Apoptosis assays

The rate of apoptotic GSCs was determined by TACS Annexin V-FITC Apoptosis Detection Kit (R&D Systems, 4830-01-K). All types of GSCs were isolated to single-cell and experiments were performed as described in the instruction manual. All samples were detected by flow cytometry and data were analyzed by BD CSampler Plus.

### 2.10. RNA isolation and real-time PCR

Total RNA was extracted from cells using RNeasy Mini Kit (QIAGEN, 74104), Then, 500 ng of RNA was reverse transcribed to cDNA for each sample with PrimeScript™ RT Master Mix (Takara Bio Inc., RR036A). Real-time quantitative PCR was per-formed using the SYBR Green Master Mix (ThermoFisher, A25742). mRNA expression was based on Ct values and GAPDH or ACTIN was used for normalization. The relevant primer sequences used to detect the mRNA levels are presented in Table S1.

### 2.11. RNA sequencing

RNA-seq analysis of shNT and shNT5DC2 (#1 GTGGCCTCCACTAT GACATTC) was performed using an Illumina HiSeq 2500 instrument at the Oebiotech Corp. Clean data were obtained by Trimmomatic. Bowtie2 and eXpress were used to calculate the FPKMs (Fragments Per kb Per Million Reads) of known genes. Differentially expressed genes were identified using the DESeq R package functions estimate Size Factors and nbinomTest.

### 2.12. Cell invasion assay

Cell invasion ability was performed with a diameter of 6.5 mm and pore size of 8 μm (Corning, NY), according to the manufacturer's protocol. Cells were transduced with NT or Fyn shRNAs and then $2 \times 10^4$ pretreated cells were added into the inserts. The inserts were placed in the culture media containing 5% FBS as a chemoattractant. After 24 hours, the invasion cells were stained with crystal violet.

### 2.13. Statistical analysis

All grouped data are reported as means ± standard deviation (SD). All in vitro experiments were repeated at least three times, and differences between the groups were compared using unpaired t-tests, one-way analysis of variance (ANOVA), and two-way ANOVA. For the in

vivo studies, log-rank survival analysis was carried out. Graph pad Prism was used for all statistical analyses.

## 3. Results

### 3.1. NT5DC2 is preferentially expressed in glioma stem-like cells

To determine the novel potential targets of glioma stem-like cells (GSCs), we analyzed a Gene Expression Omnibus (GEO) database (GSE86237) [23] that includes 18 GSCs and 7 bulk tumor cells (BTCs) isolated from human GBM patients. A total of 45 genes were identified to be highly expressed (Fold change > 2.0 and p value < 1.0E-05) in GSCs relative to BTCs (Fig. 1A). Among the top 20 candidates, 9 genes, whose expression was significantly and negatively correlated with GBM patient survival, were analyzed using REMBRANDT GBM database (Supplemental Fig. 1A). We then focused on NT5DC2, a functionally unknown gene. NT5DC2 was one of the most highly expressed genes in GSCs and was significantly correlated with GBM survival (Fig. 1A and Supplemental Fig. 1A). Moreover, NT5DC2 expression was found to be strongly correlated with malignant progression of glioma (Fig. 6). In both GSE86237 database and another GSC database, GSE54791 [24], which includes 3 stem–like tumor-propagating cells (TPC) and their matched differentiated glioma cells (DGC), NT5DC2 was highly expressed in GSCs or TPCs relative to BTCs or DGCs (Fig. 1B).

Next, we validated the preferential expression of NT5DC2 in multiple GSC lines isolated from GBM patients that are propagated in mouse GBM xenografts and have been functionally validated in our previous studies [16–20]. NT5DC2 gene transcript levels was assessed using quantitative real-time polymerization chain reaction (qRT-PCR) in four GSCs and their matched NSTCs. We found that NT5DC2 was significantly upregulated in four GSCs relative to NSTCs (Fig. 1C). The expression of NT5DC2 was further demonstrated at protein level by immunoblotting in five GSCs and NSTCs (Fig. 1D). SOX2 (sex determining region y-box 2) and Olig2 (oligodendrocyte lineage transcription factor 2) were examined as the putative stem cell markers, and GFAP (glial fibrillary acidic protein) was examined as the differentiated marker in the experiments (Fig. 1D and Supplementary Fig. S1B). Moreover, induction of differentiation in GSCs by serum treatment resulted in a rapid loss of the expression of NT5DC2 as well as SOX2 and Olig2 (Fig. 1E and Supplementary Fig. S1C). We also queried The Cancer Genome Atlas (TCGA) GBM dataset and observed that there was a significant correlation for gene expression between NT5DC2 and multiple putative stem cell markers, including SOX2, SOX4, and SOX11, in GBM (Fig. 1F), which suggest a role of NT5DC2 in GSCs.

Next, we ectopically expressed a flag-tagged NT5DC2 in GSC, and found that NT5DC2 was localized mainly in the cell cytoplasm (Supplementary Fig. S1D). To further investigate the correlation between NT5DC2 and GSCs, we performed a co-immunofluorescent (CO-IF) staining with mouse orthotopic GBM xenografts, and found that NT5DC2 staining was strong in a subpopulation of glioma cells expressing the GSC markers, including SOX2 and Olig2 (Fig. 1G). Together, these findings suggest that NT5DC2 is preferentially expressed in GSCs.

### 3.2. Targeting NT5DC2 reduces cell growth in GSCs and tumorsphere formation, and inhibits tumor growth

To investigate the biological significance of NT5DC2 in the maintenance of GSC phenotype, we knocked down NT5DC2 expression with three non-overlapping short hairpin ribonucleic acids (shRNAs) in four GSC lines. Knockdown of NT5DC2 expression in GSCs was confirmed at the protein level by immunoblotting (Fig. 2A and Supplementary Fig. S2A). The tumorsphere formation assay showed that disruption of NT5DC2 by shRNAs strongly inhibited the sphere formation of GSCs, as revealed by the decreased number and size of tumorspheres (Fig. 2B and Supplementary Figs. S2B and C). In addition, knockdown of NT5DC2 significantly inhibited the cell viability of GSCs, but had a limited impact on NSTCs (Fig. 2C and Supplementary Figs. S2D–F). Moreover, the effects of NT5DC2 on GSC maintenance were further examined by in vitro limiting dilution assay, the standard in vitro experiment to assess self-renewal capacity [25] (Fig. 2D). However, knockdown of NT5DC2 did not significantly impact the expression of stem cell markers such as SOX2 and Olig2 in GSCs (Fig. 2A), indicating that NT5DC2 has no effect on stem cell identity.

To assess the functional consequences of NT5DC2 knockdown on GSC tumorigenic potential in vivo, we examined the tumor initiation capacity of GSCs transduced with two independent NT5DC2 shRNAs (shND5DC2#1 or shND5DC2#2), or non-targeting shRNA (shNT) as control. Two GSC lines expressing shNT or shNT5DC2 were transplanted into the brain of immunocompromised nude mice. When the first few shNT mice showed neurologic signs around day 30, a subset of mice in each group was sacrificed. shNT mice had developed huge tumors in the brain, whereas the shNT5DC2 groups failed to form tumors in both GSC orthotopic tumor models (Fig. 3A and Supplementary Figs. S3A and B). As a result, the mice intracranially injected with the GSCs expressing shNT5DC2 showed a significantly longer survival relative to shNT mice (Fig. 3B).

Although downregulation of NT5DC2 in GSC significantly inhibited GSC tumor initiation, all mice died by the end of the experiment in our study. To investigate if there was a recovered expression of NT5DC2 in tumor cells during GSC tumorigenesis in vivo, we performed the IF staining of NT5DC2 in these GBM xenograft tissue. Our data showed that a subpopulation of tumor cells was NT5DC2-positive in the NT5DC2 knocked-down xenografts (Supplementary Fig. S3C), suggesting that some GSCs might have escaped the shRNA interference and re-expressed NT5DC2 in the tumor. In addition, we found that NT5DC2 knockdown significantly reduced the proportion of SOX2-or Olig2-positive cells in the tumors (Fig. 3C). Moreover, our data suggested that NT5DC2 deletion in GSCs increased the cell apoptosis, as assessed by annexin V and phosphatidylinositol (PI) flow cytometry (Fig. 3D), and by cleaved poly-ADP ribose polymerase (PARP) and caspase3 immunoblotting (Fig. 3E). Together, these data suggest that NT5DC2 plays a crucial role in GSC maintenance in vitro and tumorigenic capacity in vivo.

### 3.3. Knockdown of NT5DC2 reduces Fyn expression and impairs GSC maintenance

To gain further mechanistic insights into the function of NT5DC2 in GSC maintenance, we performed transcriptome analysis in three GSCs transduced with shNT or shNT5DC2, using RNA sequencing (RNA-seq). The analysis showed that NT5DC2 knockdown resulted in a downregulation of 73, 114, and 257 genes (fold change > 1.5, p < 0.05) in 3832, 456, and 4121 GSCs respectively, compared with the shNT GSCs (Fig. 4A). Seventeen genes were common decreased in three different GSC lines with NT5DC2 deletion (Fig. 4A and B). Interestingly, Fyn, an important member of the Src family kinases (SFKs), which has been implicated in GBM progression and invasion [10,13], was identified in these downregulated genes (Fig. 4B). We further validated some of these gene expression levels, including that of Fyn, in three GSCs, and found that NT5DC2 knockdown significantly decreased the expression of these candidates (Fig. 4C and Supplementary Fig. S4A).

Src family kinases (SFKs) comprise ten family members that share similar functions in tumor development, but vary widely in expression across different types of tumor [7]. To further explore the roles of Fyn in GSCs, we performed qRT-PCR for these SFK members in three GSCs, and found that the expression of Fyn was much higher than other SFKs in GSCs (Fig. 4D), indicating a primary function of Fyn among the SFKs in GSCs. Notably, the knockdown of NT5DC2 significantly reduced the expression of Fyn, but not of other SFKs, such as Yes, Lyn, or c-Src, in GSCs (Fig. 4E), which reveals Fyn as a potential target gene for NT5DC2 in GSCs. The association between NT5DC2 and Fyn expression was also

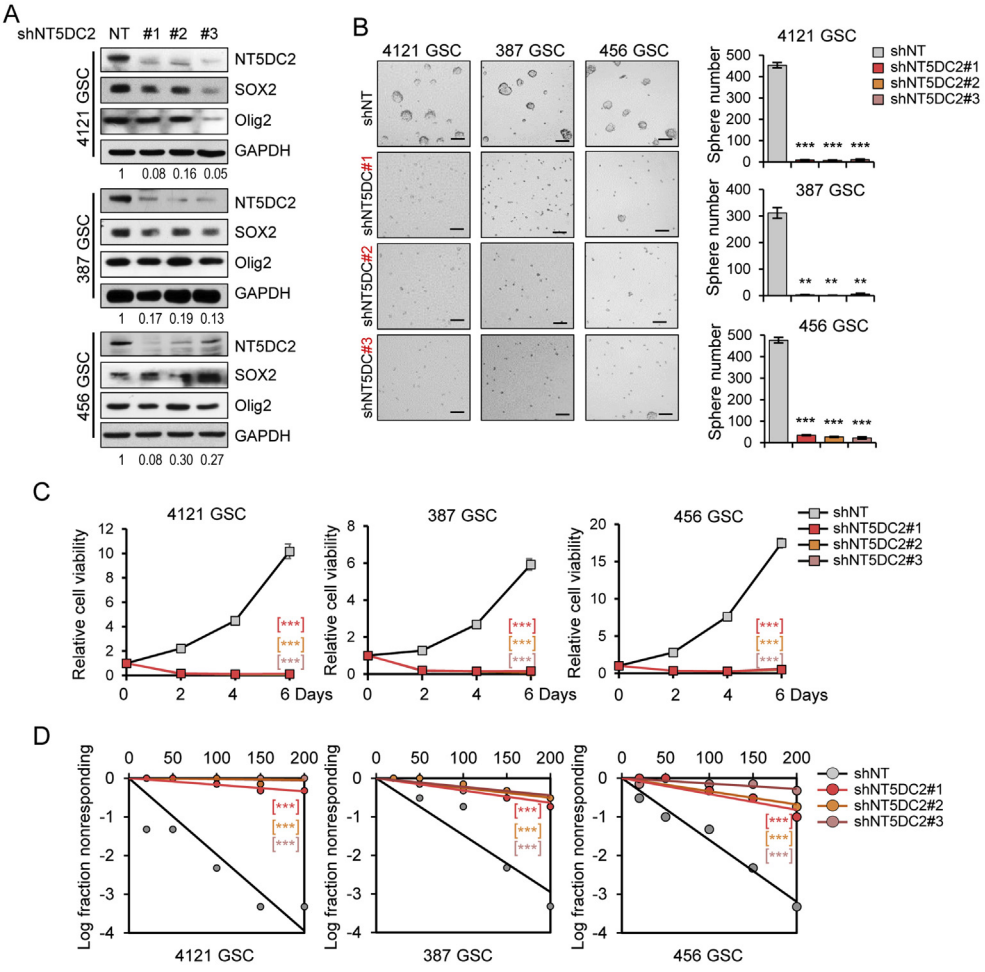


**Fig. 2. NT5DC2 is Critical for GSC Viability and Self-Renewal**. (A) GSCs (4121, 387, and 456) were transduced with three different shNT5DC2 sequences. NT5DC2, SOX2, Olig2, and GAPDH were assessed by IB. The relative amount of NT5DC2 normalized to GAPDH was assessed by ImageJ and is shown under each GSC line. (B) Tumorsphere formation assay of GSCs expressing shNT5DC2 or shNT. Tumorsphere images assessed by bright-field microscopy (left) and sphere number are shown (right). Scale bar represents 100 μm. Data are shown as means ± s.d. Unpaired Student's t-test, **p < 0.01, ***p < 0.001. (C) GSC viability was assessed in three GSC lines (4121, 387, and 456) by cell titer assay. GSCs transduced with shNT5DC2 resulted in a decrease in cell viability. Data are shown as means ± s.d. Two-way ANOVA, ***p < 0.001. (D) In vitro limiting dilution assay of three GSC lines (4121, 387, and 456) expressing shNT or shNT5DC2. Data are shown as means ± s.d. Significance testing was done by Extreme Limiting Dilution Analysis (ELDA), ***p < 0.001.

analyzed in human GBMs in TCGA database and the study by Gravendeel et al. [26,27] (Supplementary Fig. S4B). Importantly, disrupting Fyn by shRNAs strongly inhibited the GSC tumorsphere formation and cell viability (Fig. 4F–H and Supplementary Figs. S4C–F). Taken together, these data suggest that NT5DC2 might be required for the expression of Fyn in GSC, which is essential for the maintenance of GSCs.

### 3.4. NT5DC2 associates with Fyn and Promotes Fyn Stability in GSCs

The regulation of Fyn expression by NT5DC2 was validated by immunoblotting. We found that NT5DC2 knockdown resulted in a strong reduction of Fyn expression at the protein level (Fig. 5A). Interestingly, overexpression of NT5DC2 in GSCs increased the expression of Fyn at the protein level, but not at the mRNA level (Fig. 5B and C), suggesting a direct regulation of Fyn protein expression by NT5DC2. To further investigate the mechanism of Fyn protein regulation by NT5DC2, we performed a co-immunoprecipitation assay with Flag-NT5DC2, and found that NT5DC2 interacted with Fyn in GSCs (Fig. 5D). Previous studies have suggested that Fyn is degraded by ubiquitin–dependent proteasome pathway [28,29]. We hypothesized that the binding of NT5DC2 might protect Fyn protein from ubiquitin proteasome degradation. We found that treatment with MG132, a proteasome inhibitor, significantly rescued the reduction in Fyn protein expression in NT5DC2 knocked-down GSCs (Fig. 5E). These results suggested that the association between NT5DC2 and Fyn increased the stability of Fyn protein in GSCs.

To determine whether Fyn can mediate the effect of NT5DC2 in GSC, we overexpressed Fyn in GSCs transduced with shNT or shNT5DC2. Our data showed that ectopic expression of Flag-Fyn had no obvious effect on cell viability in GSCs. However, the suppression of GSC viability and tumor sphere formation by NT5DC2 knockdown was strongly rescued by the ectopic expression of Flag-Fyn (Fig. 5F and G), indicating that Fyn was at least partly responsible for the impact of NT5DC2 on GSC. To further test whether Fyn also contributes to the expression of NT5DC2, we knocked down Fyn expression in three different GSC lines, and found that deletion of Fyn had no impact on the protein or mRNA expression of NT5DC2 in GSCs (Supplemental Figs. S5A and B). Since previous studies have suggested that Fyn plays important roles in glioma invasion [13,30,31], we next performed the transwell assay to test the impact of Fyn on GSC invasion. We found that GSC invasion was significantly inhibited in cells transduced with Fyn shRNAs (Supplemental Fig. S5C). However, our data also showed that deletion of Fyn strongly decreased the cell viability of GSCs (Fig. 4H). It remains to be examined whether Fyn can directly regulate the cell motility of GSCs. Together, these results suggest that Fyn associates with NT5DC2, and mediates, at least partly, the function of NT5DC2 in GSCs.

### 3.5. Elevated NT5DC2 expression correlates with glioma progression

To further determinate the clinical relevance of NT5DC2 in glioma, we interrogated several brain tumor datasets, including those for TCGA GBM, TCGA diffuse glioma (GBMLGG), and REMBRANDT gliomas. The expression of NT5DC2 was significantly elevated in GBM compared with the normal brain tissue (Fig. 6A). GBM has been recently classified into proneural, neural, classical, and mesenchymal subgroups by expression profiling [11]. NT5DC2 displayed a strong association with the proneural subtype relative to the other three subtypes (Fig. 6B). In

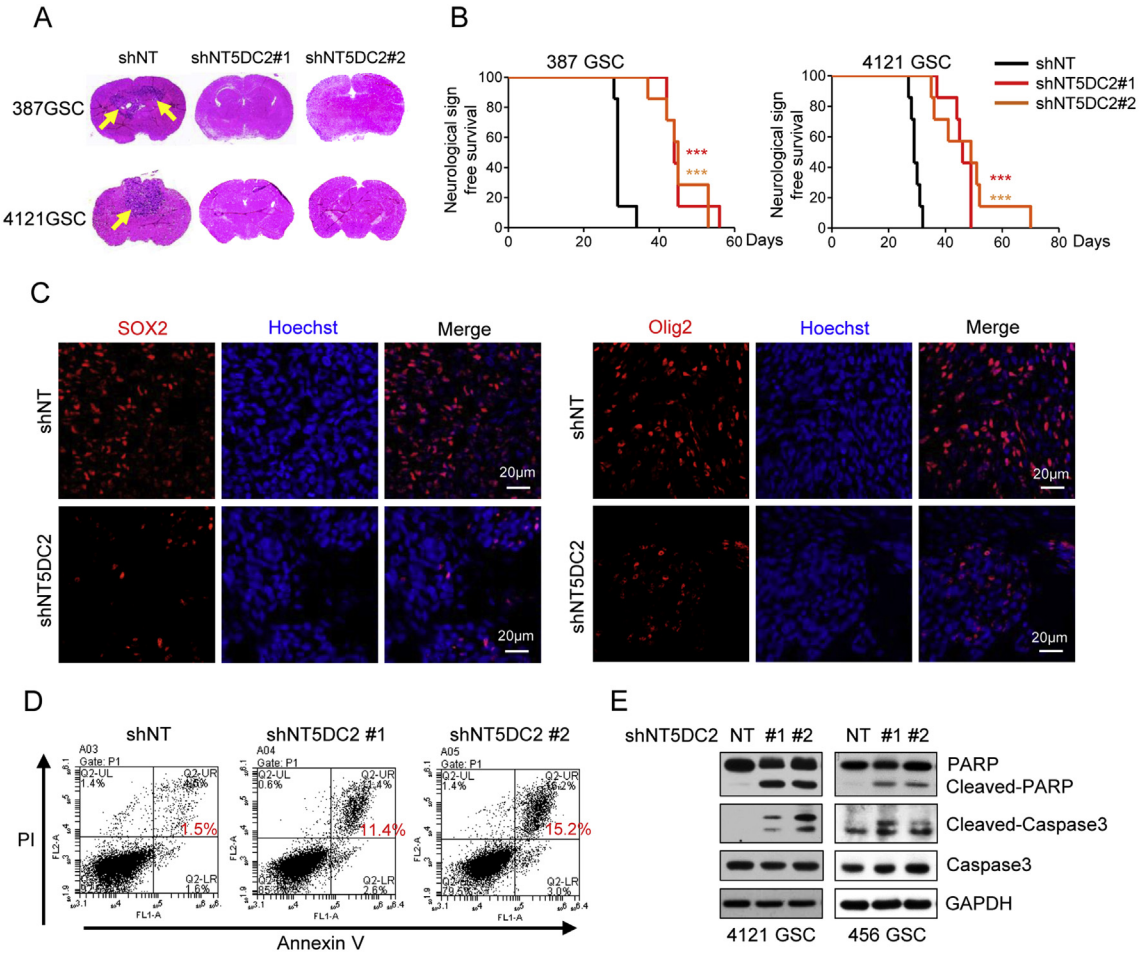


**Fig. 3. Disruption of NT5DC2 Potently Inhibits GSC Tumor Growth and Prolongs Animal Survival.** (A) and (B) Two GSC lines (387 or 4121) were transduced with shNT or shNT5DC2 through lentiviral infection and then intracranially transplanted into the brains of nude mice ($5 \times 10^4$ cells per mouse). Seven mice per group were used. Representative images of cross-sections (H&E-stained) of mouse brains 30 days after transplantation are shown (A). Kaplan-Meier survival curves of mice intracranially implanted with GSCs expressing shNT or shNT5DC2 are shown (B). Significance testing was done by log-rank test, ***$p < 0.001$. (C) Co-Immunofluorescence (Co-IF) staining of SOX2 or Olig2 (red) in mouse GBM xenografts derived from GSCs transduced with shNT or shNT5DC2. Nuclei were counterstained with Hoechst (blue). Representative images are shown. (D) Apoptosis assay was performed with 4121 GSCs expressing shNT or shNT5DC2s using an Annexin V/PI apoptosis kit. (E) IB analysis of cleaved-caspase3 and cleaved-PARP proteins in GSCs transduced with shNT or shNT5DC2.

addition, the expression of NT5DC2 increased strongly with the glioma grade (Fig. 6C), and high level of NT5DC2 was significantly correlated with worse prognosis in all glioma patients (Fig. 6D) and GBM patients (Fig. 6E).

Recently, gliomas have been categorized into two groups, based on the mutational status of isocitrate dehydrogenase (IDH) [32]. Patients with IDH mutant gliomas show better prognosis compared with patients with wild-type IDH tumors [33,34]. However, we did not observe any difference for the expression of NT5DC2 between IDH mutant and IDH wt GBM patients (Supplementary Fig. S6A). We further performed the IHC staining with NT5DC2 antibody in a human glioma tissue array, and found that NT5DC2 was highly expressed in high-grade gliomas but no expression was detected in normal brain tissue (Fig. 6F and Supplementary Fig. S6B). Meanwhile, other brain tumors such as oligoastrocytoma, medulloblastoma, and ependymoma were negative for NT5DC2 expression (Supplementary Fig. S6C). Together, these data suggest that NT5DC2 expression is increased in more aggressive gliomas, and it may serve as a prognostic marker for GBM.

## 4. Discussion

GBM is an incurable primary brain tumor with dismal prognosis, despite the development of aggressive therapies [1,35]. GBM tumors exhibit remarkable cellular heterogeneity, and a small population of GSCs resides at the apex of the hierarchy. GSCs have been demonstrated to contribute to GBM treatment failure, owing to their self-renewal properties and resistance to radio-chemotherapy [2,3], indicating that elimination of GSCs may significantly improve GBM treatment and help overcome the therapeutic resistance. However, a GSC-targeting drug is still unavailable for clinical practice, and development of therapeutics based on GSC-specific targets is urgently needed.

To date, little is known about the function of NT5DC2 (5′-Nucleotidase Domain Containing 2), one of the members of the NT5DC family, a family of haloacid dehalogenase-type phosphatases [14]. Two published databases of GSCs [23,24] suggest that the expression of NT5DC2 is much higher in GSCs or TPCs than in the bulk tumor cells or differentiated glioma cells. Through bioinformatic analysis, we also found a strong correlation between the expression of NT5DC2 and multiple stem cell markers in the TCGA GBM database. In this study, we identified NT5DC2 as a preferentially expressed protein in GSCs, compared with that in their non-stem tumor cells (NSTCs) counterparts. Deletion of NT5DC2 strongly inhibits GSC tumorsphere formation and cell viability. Notably, although NT5DC2 knockdown has a limited impact on NSTCs, this effect was significant, suggesting that the functions of NT5DC2 may not be restricted to GSC, but instead, that its effects are more evident in GSCs solely because these cells show a much

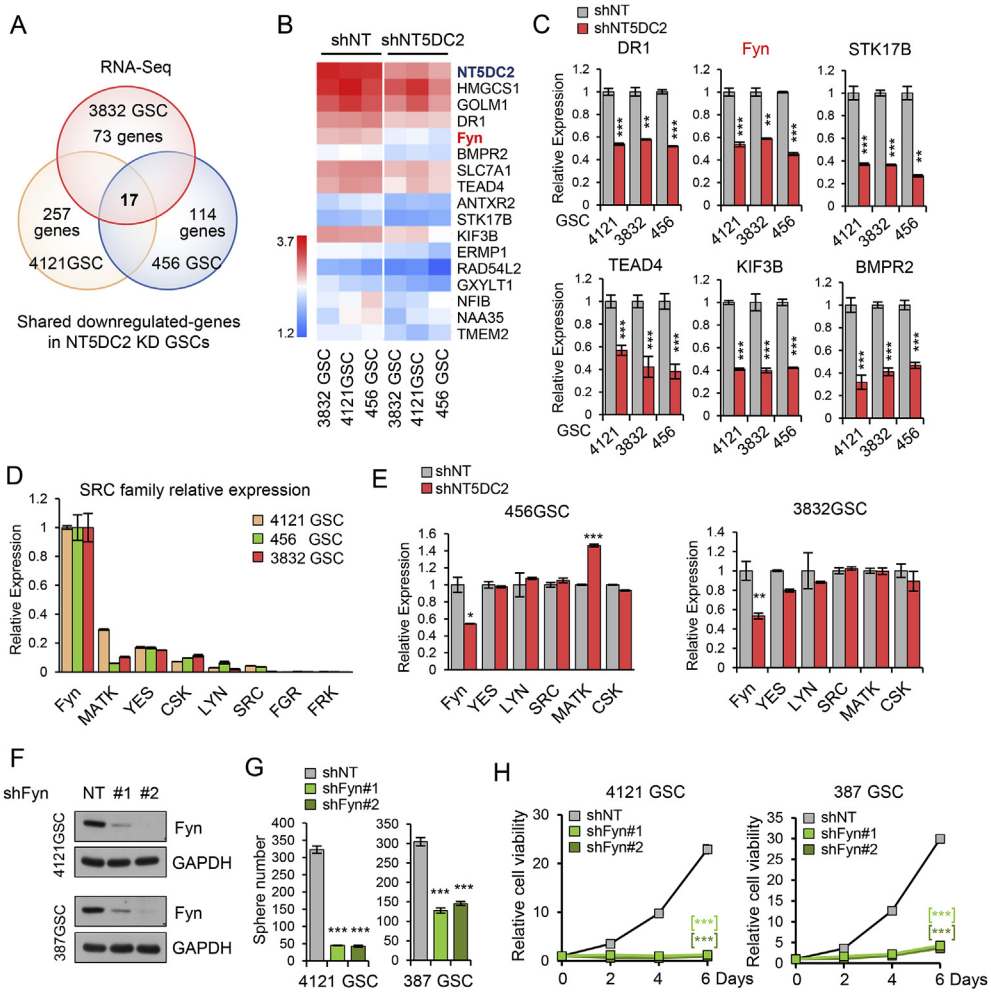


**Fig. 4. Knockdown of NT5DC2 Reduces Fyn Expression and Impairs GSC Maintenance**. (A) Venn diagram demonstrates the overlapping genes (fold change > 1.5, $p < 0.05$) in 3832, 4121, and 456 GSCs with knocked-down NT5DC2. (B) Heatmap shows expression profiles of the 17 downregulated genes in three different GSCs by RNA-seq transcriptomic analysis. Data were generated using three biological replicates, and the color represents the Z scores of gene expression. (C) Validation of expression of candidate genes DR1, Fyn, STK17B, TEAD4, KIF3B, and BMPR2, after knockdown of NT5DC2 in three GSCs, by qRT-PCR. Data are shown as means ± s.d. Unpaired Student's t-test, $^{**}p < 0.01$, $^{***}p < 0.001$. (D) The expression of Fyn was much higher than other SFK members in 4121, 456, and 3832 GSCs. Data are shown as means ± s.d. (E) Knockdown of NT5DC2 significantly downregulated the expression of Fyn on mRNA levels, but not that of other SFKs in GSCs. Data are shown as means ± s.d. Unpaired Student's t-test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (F) GSCs (387 and 4121) were transduced with shNT or two different sequences of shFyn and the effects of Fyn knockdown were assessed by IB. GAPDH was used as a control. (G) and (H) Knockdown of Fyn resulted in a decrease in tumorsphere numbers (G) and cell viability (H) in 4121 and 387 GSCs. Data are shown as means ± s.d. Unpaired Student's t-test (G) or two-way ANOVA (H), $^{***}p < 0.001$.

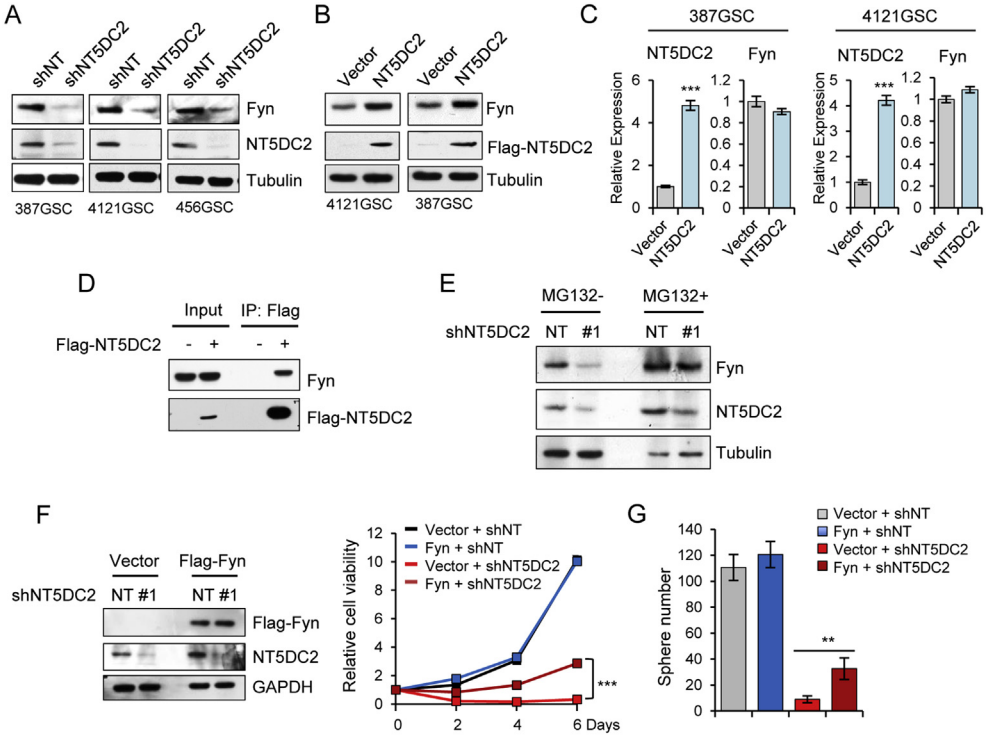


**Fig. 5. NT5DC2 Associates with Fyn and Promotes Fyn Stability in GSCs**. (A) Knockdown of NT5DC2 strongly decreased the expression of Fyn in GSCs (387, 4121, and 456), as assessed by IB. (B) Overexpression of Flag-NT5DC2 increased the expression of Fyn in 387 and 4121 GSCs, as assessed by IB. (C) Overexpression of Flag-NT5DC2 had no impact on mRNA expression of Fyn in 387 and 4121 GSCs, as assessed by RT-qPCR. Data are shown as means ± s.d. Unpaired Student's t-test, $^{***}p < 0.001$. (D) Immunoassay of GSCs transfected with Flag-NT5DC2. Lysates were immunoprecipitated with anti-Flag and then analyzed by IB with anti-Fyn and anti-Flag antibodies. (E) The 387 GSCs transduced with NT5DC2 shRNAs were treated with MG132 for 12 hours. The expression levels of Fyn, NT5DC2, and Tubulin were assessed by IB. (F and G) Cell viability (F) and tumor sphere number (G) of 4121 GSCs ectopically expressed with Flag-Fyn under the scenario of reduced NT5DC2. Data are shown as means ± s.d. Unpaired Student's t-test, $^{**}p < 0.01$.

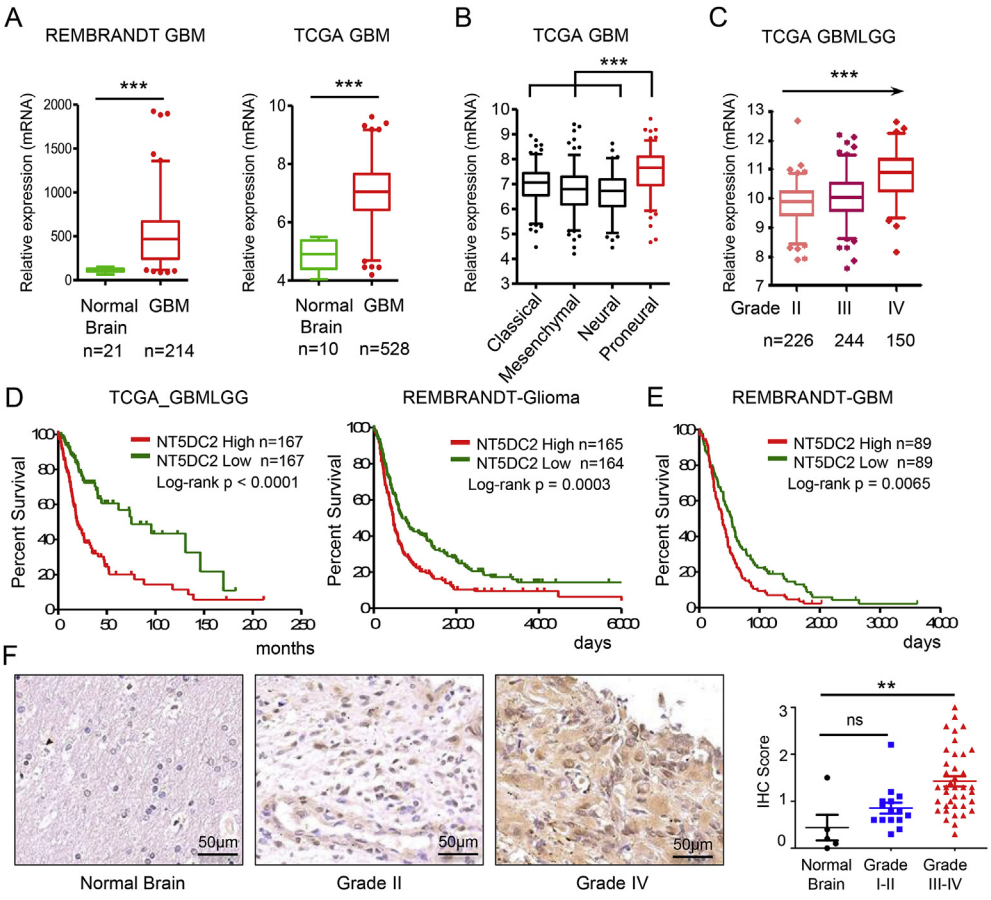


**Fig. 6. Expression Level of NT5DC2 is Associated with Poor Survival in Glioblastoma Patients**. (A) Boxplot of NT5DC2 expression in non-tumor and glioblastoma (GBM) from the REMBRANDT (left) and TCGA GBM database (right). Unpaired Student's t-test with Welch's correction, ***p < 0.001. (B) Boxplot of NT5DC2 expression in different subtypes of GBM from the TCGA database. Unpaired Student's t-test, ***p < 0.001. (C) Boxplot of NT5DC2 expression in different grades of gliomas from the TCGA GBMLGG database. One-way ANOVA, ***p < 0.001. (D) and (E) Kaplan–Meier survival analysis of NT5DC2 expression and the progression-free survival of glioma patients from the TCGA GBMLGG and REMBRANDT database (D), and that of GBM patients from the REMBRANDT database (E). Log rank test. (F) Immunohistochemical (IHC) staining of NT5DC2 in normal brain, low-grade glioma, and high-grade glioma. Representative images are shown (left). Histoscore analysis showed that NT5DC2 levels were higher in high-grade glioma compared with normal brain and low-grade glioma. Unpaired Student's t-test, **p < 0.01.

higher expression than NSTC. Importantly, deletion of NT5DC2 compromises the GSC tumor initiation in vivo and prolongs animal survival. We found that NT5DC2 is overexpressed in partial astrocytoma and most cases of GBM, but not in other types of brain tumor, including medulloblastoma and ependymoma. In addition, as NT5DC2 expression correlates positively with glioma tumor grade and predicts unfavorable prognosis for GBM patients, it may serve as a potential target for anti-GSC treatment and prognostic determination of GBM.

Src family kinases (SFKs) are best known as key regulators of tumor development, including GBM, melanoma, and squamous cell carcinoma [7,8,13]. Previous studies show that multiple SFKs are overexpressed in GBM, which may also be involved in GBM pathogenesis [36,37]. Activation of Src-dependent signaling pathway in glioma cells promotes glioma tumorigenesis and therapeutic resistance, suggesting Src as a possible therapeutic target in glioblastoma [38–40]. Fyn is a member of SFKs that has been shown to play a role in glioma invasion [13,30,31], but the function and regulation of Fyn in GSCs remains largely unknown. Our data show that Fyn is highly expressed in GSCs relative to other Src family genes, including c-Src, Lyn, and Yes, suggesting its crucial role in the maintenance of GSC properties. We found that disrupting Fyn significantly inhibits GSC tumorsphere formation and cell viability, something that has not been clearly demonstrated in glioma stem cells before. In addition, we also tested the impact of Fyn on GSC motility in our study. Knockdown of Fyn was found to indeed inhibit the invasion of GSCs. However, at the same time, our data suggested that deletion of Fyn results in a serve inhibition of cell viability of GSCs. The direct regulation of GSC invasion by Fyn remains to be further explored.

Our study showed that abrogation of NT5DC2 significantly reduces Fyn expression in GSCs, indicating that NT5DC2 may function through regulating Fyn. Importantly, we found that NT5DC2 binds to Fyn in GSC and this association stabilizes the Fyn protein. Blockage of ubiquitin proteasome pathway by MG132 significantly rescues the reduction of Fyn protein due to the knockdown of NT5DC2 in GSC. Interestingly, we found that overexpression of NT5DC2 increases the protein expression of Fyn, but not its mRNA expression, suggesting that the decrease of Fyn mRNA in NT5DC2 knocked-down GSCs might be an indirect effect. The molecular mechanism by which NT5DC2 stabilizes Fyn protein from ubiquitin-mediated degradation needs further investigation in future studies.

Together, our study unveils a novel function of NT5DC2, such as promotion of GSC maintenance and tumor growth through stabilization of Fyn protein, indicating that NT5DC2 may be an attractive therapeutic target to improve GBM therapy.

## Conflicts of interest statement

The authors declare no competing financial interests.

## Acknowledgements

We appreciate Dr. Jeremy Rich (University of California San Diego School of Medicine) and Dr. Jennifer S. Yu (Cleveland Clinic) for GSCs. This research was supported by grants from the National Key R&D Program of China (2017YFA0505602, 2017YFC1601100, 2017YFC1601101, 2017YFC1601102 and 2017YFC1601104), National Natural Science Foundation of China (no. 81872408 and no. 81572889).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.04.003.

## References


[1] R. Stupp, M.E. Hegi, W.P. Mason, M.J. van den Bent, M.J. Taphoorn, R.C. Janzer, S.K. Ludwin, A. Allgeier, B. Fisher, K. Belanger, P. Hau, A.A. Brandes, J. Gijtenbeek, C. Marosi, C.J. Vecht, K. Mokhtari, P. Wesseling, S. Villa, E. Eisenhauer, T. Gorlia, M. Weller, D. Lacombe, J.G. Cairncross, R.O. MirimanoffR. European Organisation for, T. Treatment of Cancer Brain, G. Radiation Oncology, G. National Cancer Institute of Canada Clinical Trials, Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial, the Lancet, Oncology 10 (2009) 459–466.

[2] D.A. Reardon, P.Y. Wen, Glioma in 2014: unravelling tumour heterogeneity-implications for therapy, Nature reviews, Clin. Oncol. 12 (2015) 69–70.

[3] J.D. Lathia, S.C. Mack, E.E. Mulkearns-Hubert, C.L. Valentim, J.N. Rich, Cancer stem cells in glioblastoma, Genes Dev. 29 (2015) 1203–1217.

[4] L. Cheng, Z. Huang, W. Zhou, Q. Wu, S. Donnola, J.K. Liu, X. Fang, A.E. Sloan, Y. Mao, J.D. Lathia, W. Min, R.E. McLendon, J.N. Rich, S. Bao, Glioblastoma stem cells generate vascular pericytes to support vessel function and tumor growth, Cell 153 (2013) 139–152.

[5] W. Zhou, S.Q. Ke, Z. Huang, W. Flavahan, X. Fang, J. Paul, L. Wu, A.E. Sloan, R.E. McLendon, X. Li, J.N. Rich, S. Bao, Periostin secreted by glioblastoma stem cells recruits M2 tumour-associated macrophages and promotes malignant growth, Nat. Cell Biol. 17 (2015) 170–182.

[6] X. Wang, B.C. Prager, Q. Wu, L.J.Y. Kim, R.C. Gimple, Y. Shi, K. Yang, A.R. Morton, W. Zhou, Z. Zhu, E.A.A. Obara, T.E. Miller, A. Song, S. Lai, C.G. Hubert, X. Jin, Z. Huang, X. Fang, D. Dixit, W. Tao, K. Zhai, C. Chen, Z. Dong, G. Zhang, S.M. Dombrowski, P. Hamerlik, S.C. Mack, S. Bao, J.N. Rich, Reciprocal signaling between glioblastoma stem cells and differentiated tumor cells promotes malignant progression, Cell stem cell 22 (2018) 514–528 e515.

[7] L.C. Kim, L. Song, E.B. Haura, Src kinases as therapeutic targets for cancer, Nature reviews, Clin. Oncol. 6 (2009) 587–595.

[8] S. Zhang, D. Yu, Targeting Src family kinases in anti-cancer therapies: turning promise into triumph, Trends Pharmacol. Sci. 33 (2012) 122–128.

[9] S.M. Thomas, J.S. Brugge, Cellular functions regulated by Src family kinases, Annu. Rev. Cell Dev. Biol. 13 (1997) 513–609.

[10] Y.D. Saito, A.R. Jensen, R. Salgia, E.M. Posadas, Fyn: a novel molecular target in cancer, Cancer 116 (2010) 1629–1637.

[11] R.G. Verhaak, K.A. Hoadley, E. Purdom, V. Wang, Y. Qi, M.D. Wilkerson, C.R. Miller, L. Ding, T. Golub, J.P. Mesirov, G. Alexe, M. Lawrence, M. O'Kelly, P. Tamayo, B.A. Weir, S. Gabriel, W. Winckler, S. Gupta, L. Jakkula, H.S. Feiler, J.G. Hodgson, C.D. James, J.N. Sarkaria, C. Brennan, A. Kahn, P.T. Spellman, R.K. Wilson, T.P. Speed, J.W. Gray, M. Meyerson, G. Getz, C.M. Perou, D.N. Hayes, N. Cancer Genome Atlas Research, Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1, Cancer Cell 17 (2010) 98–110.

[12] C.W. Brennan, R.G. Verhaak, A. McKenna, B. Campos, H. Noushmehr, S.R. Salama, S. Zheng, D. Chakravarty, J.Z. Sanborn, S.H. Berman, R. Beroukhim, B. Bernard, C.J. Wu, G. Genovese, I. Shmulevich, J. Barnholtz-Sloan, L. Zou, R. Vegesna, S.A. Shukla, G. Ciriello, W.K. Yung, W. Zhang, C. Sougnez, T. Mikkelsen, K. Aldape, D.D. Bigner, E.G. Van Meir, M. Prados, A. Sloan, K.L. Black, J. Eschbacher, G. Finocchiaro, W. Friedman, D.W. Andrews, A. Guha, M. Iacocca, B.P. O'Neill, G. Foltz, J. Myers, D.J. Weisenberger, R. Penny, R. Kucherlapati, C.M. Perou, D.N. Hayes, R. Gibbs, M. Marra, G.B. Mills, E. Lander, P. Spellman, R. Wilson, C. Sander, J. Weinstein, M. Meyerson, S. Gabriel, P.W. Laird, D. Haussler, G. Getz, L. Chin, T.R. Network, The somatic genomic landscape of glioblastoma, Cell 155 (2013) 462–477.

[13] K.V. Lu, S. Zhu, A. Cvrljevic, T.T. Huang, S. Sarkaria, D. Ahkavan, J. Dang, E.B. Dinca, S.B. Plaisier, I. Oderberg, Y. Lee, Z. Chen, J.S. Caldwell, Y. Xie, J.A. Loo, D. Seligson, A. Chakravari, F.Y. Lee, R. Weinmann, T.F. Cloughesy, S.F. Nelson, G. Bergers, T. Graeber, F.B. Furnari, C.D. James, W.K. Cavenee, T.G. Johns, P.S. Mischel, Fyn and SRC are effectors of oncogenic epidermal growth factor receptor signaling in glioblastoma patients, Cancer Res. 69 (2009) 6889–6898.

[14] A. Seifried, J. Schultz, A. Gohla, Human HAD phosphatases: structure, mechanism, and roles in health and disease, FEBS J. 280 (2013) 549–571.

[15] S. Ripke, C. O'Dushlaine, K. Chambert, J.L. Moran, A.K. Kahler, S. Akterin, S.E. Bergen, A.L. Collins, J.J. Crowley, M. Fromer, Y. Kim, S.H. Lee, P.K. Magnusson, N. Sanchez, E.A. Stahl, S. Williams, N.R. Wray, K. Xia, F. Bettella, A.D. Borglum, B.K. Bulik-Sullivan, P. Cormican, N. Craddock, C. de Leeuw, N. Durmishi, M. Gill, V. Golimbet, M.L. Hamshere, P. Holmans, D.M. Hougaard, K.S. Kendler, K. Lin, D.W. Morris, O. Mors, P.B. Mortensen, B.M. Neale, F.A. O'Neill, M.J. Owen, M.P. Milovancevic, D. Posthuma, J. Powell, A.L. Richards, B.P. Riley, D. Ruderfer, D. Rujescu, E. Sigurdsson, T. Silagadze, A.B. Smit, H. Stefansson, S. Steinberg, J. Suvisaari, S. Tosato, M. Verhage, J.T. Walters, C. Multicenter Genetic Studies of Schizophrenia, D.F. Levinson, P.V. Gejman, K.S. Kendler, C. Laurent, B.J. Mowry, M.C. O'Donovan, M.J. Owen, A.E. Pulver, B.P. Riley, S.G. Schwab, D.B. Wildenauer, F. Dudbridge, P. Holmans, J. Shi, M. Albus, M. Alexander, D. Campion, D. Cohen, D. Dikeos, J. Duan, P. Eichhammer, S. Godard, M. Hansen, F.B. Lerer, K.Y. Liang, W. Maier, J. Mallet, D.A. Nertney, G. Nestadt, N. Norton, F.A. O'Neill, G.N. Papadimitriou, R. Ribble, A.R. Sanders, J.M. Silverman, D. Walsh, N.M. Williams, B. Wormley, C. Psychosis Endophenotypes International, M.J. Arranz, S. Bakker, S. Bender, E. Bramon, D. Collier, B. Crespo-Facorro, J. Hall, C. Iyegbe, A. Jablensky, R.S. Kahn, L. Kalaydjieva, S. Lawrie, C.M. Lewis, K. Lin, D.H. Linszen, I. Mata, A. McIntosh, R.M. Murray, R.A. Ophoff, J. Powell, D. Rujescu, J. Van Os, M. Walshe, M. Weisbrod, D. Wiersma, C. Wellcome Trust Case Control, P. Donnelly, I. Barroso, J.M. Blackwell, E. Bramon, M.A. Brown, J.P. Casas, A.P. Corvin, P. Deloukas, A. Duncanson, J. Jankowski, H.S. Markus, C.G. Mathew, C.N. Palmer, R. Plomin, A. Rautanen, S.J. Sawcer, R.C. Trembath, A.C. Viswanathan, N.W. Wood, C.C. Spencer, G. Band, C. Bellenguez, C. Freeman, G. Hellenthal, E. Giannoulatou, M. Pirinen, R.D. Pearson, A. Strange, Z. Su, D. Vukcevic, P. Donnelly, C. Langford, S.E. Hunt, S. Edkins, R. Gwilliam, H. Blackburn, S.J. Bumpstead, S. Dronov, M. Gillman, E. Gray, N. Hammond, A. Jayakumar, O.T. McCann, J. Liddle, S.C. Potter, R. Ravindrarajah, M. Ricketts, A. Tashakkori-Ghanbaria, M.J. Waller, P. Weston, S. Widaa, P. Whittaker, I. Barroso, P. Deloukas, C.G. Mathew, J.M. Blackwell, M.A. Brown, A.P. Corvin, M.I. McCarthy, C.C. Spencer, E. Bramon, A.P. Corvin, M.C. O'Donovan, K. Stefansson, E. Scolnick, S. Purcell, S.A. McCarroll, P. Sklar, C.M. Hultman, P.F. Sullivan, Genome-wide association analysis identifies 13 new risk loci for schizophrenia, Nat. Genet. 45 (2013) 1150–1159.

[16] J. Man, J. Shoemake, W. Zhou, X. Fang, Q. Wu, A. Rizzo, R. Prayson, S. Bao, J.N. Rich, J.S. Yu, Sema3C promotes the survival and tumorigenicity of glioma stem cells through Rac1 activation, Cell Rep. 9 (2014) 1812–1826.

[17] J. Man, J.D. Shoemake, T. Ma, A.E. Rizzo, A.R. Godley, Q. Wu, A.M. Mohammadi, S. Bao, J.N. Rich, J.S. Yu, Hyperthermia sensitizes glioma stem-like cells to radiation by inhibiting AKT signaling, Cancer Res. 75 (2015) 1760–1769.

[18] J. Man, X. Yu, H. Huang, W. Zhou, C. Xiang, H. Huang, L. Miele, Z. Liu, G. Bebek, S. Bao, J.S. Yu, Hypoxic induction of vasorin regulates Notch1 turnover to maintain glioma stem-like cells, Cell stem cell 22 (2018) 104–118 e106.

[19] C.E. Eyler, Q. Wu, K. Yan, J.M. MacSwords, D. Chandler-Militello, K.L. Misuraca, J.D. Lathia, M.T. Forrester, J. Lee, J.S. Stamler, S.A. Goldman, M. Bredel, R.E. McLendon, A.E. Sloan, A.B. Hjelmeland, J.N. Rich, Glioma stem cell proliferation and tumor growth are promoted by nitric oxide synthase-2, Cell 146 (2011) 53–66.

[20] S. Bao, Q. Wu, R.E. McLendon, Y. Hao, Q. Shi, A.B. Hjelmeland, M.W. Dewhirst, D.D. Bigner, J.N. Rich, Glioma stem cells promote radioresistance by preferential activation of the DNA damage response, Nature 444 (2006) 756–760.

[21] Y. Shi, Y.F. Ping, W. Zhou, Z.C. He, C. Chen, B.S. Bian, L. Zhang, L. Chen, X. Lan, X.C. Zhang, K. Zhou, Q. Liu, H. Long, T.W. Fu, X.N. Zhang, M.F. Cao, Z. Huang, X. Fang, X. Wang, H. Feng, X.H. Yao, S.C. Yu, Y.H. Cui, X. Zhang, J.N. Rich, S. Bao, X.W. Bian, Tumour-associated macrophages secrete pleiotrophin to promote PTPRZ1 signalling in glioblastoma stem cells for tumour growth, Nat. Commun. 8 (2017) 15080.

[22] Y. Hu, G.K. Smyth, ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays, J. Immunol. Methods 347 (2009) 70–78.

[23] X. Jin, L.J.Y. Kim, Q. Wu, L.C. Wallace, B.C. Prager, T. Sanvoranart, R.C. Gimple, X. Wang, S.C. Mack, T.E. Miller, P. Huang, C.L. Valentim, Q.G. Zhou, J.S. Barnholtz-Sloan, S. Bao, A.E. Sloan, J.N. Rich, Targeting glioma stem cells through combined BMI1 and EZH2 inhibition, Nat. Med. 23 (2017) 1352–1361.

[24] M.L. Suva, E. Rheinbay, S.M. Gillespie, A.P. Patel, H. Wakimoto, S.D. Rabkin, N. Riggi, A.S. Chi, D.P. Cahill, B.V. Nahed, W.T. Curry, R.L. Martuza, M.N. Rivera, N. Rossetti, S. Kasif, S. Beik, S. Kadri, I. Tirosh, I. Wortman, A.K. Shalek, O. Rozenblatt-Rosen, A. Regev, D.N. Louis, B.E. Bernstein, Reconstructing and reprogramming the tumor-propagating potential of glioblastoma stem-like cells, Cell 157 (2014) 580–594.

[25] E. Pastrana, V. Silva-Vargas, F. Doetsch, Eyes wide open: a critical review of sphere-formation as an assay for stem cells, Cell stem cell 8 (2011) 486–498.

[26] L.A. Gravendeel, M.C. Kouwenhoven, O. Gevaert, J.J. de Rooi, A.P. Stubbs, J.E. Duijm, A. Daemen, F.E. Bleeker, L.B. Bralten, N.K. Kloosterhof, B. De Moor, P.H. Eilers, P.J. van der Spek, J.M. Kros, P.A. Sillevis Smitt, M.J. van den Bent, P.J. French, Intrinsic gene expression profiles of gliomas are a better predictor of survival than histology, Cancer Res. 69 (2009) 9065–9072.

[27] L.A. Gravendeel, J.J. de Rooi, P.H. Eilers, M.J. van den Bent, P.A. Sillevis Smitt, P.J. French, Gene expression profiles of gliomas in formalin-fixed paraffin-embedded material, Br. J. Canc. 106 (2012) 538–545.

[28] N. Rao, A.K. Ghosh, P. Douillard, C.E. Andoniou, P. Zhou, H. Band, An essential role of ubiquitination in Cbl-mediated negative regulation of the Src-family kinase Fyn, Signal Transduct. 2 (2002) 29–39.

[29] K. Kaabeche, J. Lemonnier, S. Le Mee, J. Caverzasio, P.J. Marie, Cbl-mediated degradation of Lyn and Fyn induced by constitutive fibroblast growth factor receptor-2 activation supports osteoblast differentiation, J. Biol. Chem. 279 (2004) 36259–36267.

[30] N. Dey, H.E. Crosswell, P. De, R. Parsons, Q. Peng, J.D. Su, D.L. Durden, The protein phosphatase activity of PTEN regulates SRC family kinases and controls glioma migration, Cancer Res. 68 (2008) 1862–1871.

[31] R.K. Goel, K.E. Lukong, Understanding the cellular roles of Fyn-related kinase (FRK): implications in cancer biology, Cancer Metastasis Rev. 35 (2016) 179–199.

[32] M. Ceccarelli, F.P. Barthel, T.M. Malta, T.S. Sabedot, S.R. Salama, B.A. Murray, O. Morozova, Y. Newton, A. Radenbaugh, S.M. Pagnotta, S. Anjum, J. Wang, G. Manyam, P. Zoppoli, S. Ling, A.A. Rao, M. Grifford, A.D. Cherniack, H. Zhang, L. Poisson, C.G. Carlotti Jr., D.P. Tirapelli, A. Rao, T. Mikkelsen, C.C. Lau, W.K. Yung, R. Rabadan, J. Huse, D.J. Brat, N.L. Lehman, J.S. Barnholtz-Sloan, S. Zheng, K. Hess, G. Rao, M. Meyerson, R. Beroukhim, L. Cooper, R. Akbani, M. Wrensch, D. Haussler, K.D. Aldape, P.W. Laird, D.H. Gutmann, T.R. Network, H. Noushmehr, A. Iavarone, R.G. Verhaak, Molecular profiling reveals biologically discrete subsets and pathways of progression in diffuse glioma, Cell 164 (2016) 550–563.

[33] J.E. Eckel-Passow, D.H. Lachance, A.M. Molinaro, K.M. Walsh, P.A. Decker, H. Sicotte, M. Pekmezci, T. Rice, M.L. Kosel, I.V. Smirnov, G. Sarkar, A.A. Caron, T.M. Kollmeyer, C.E. Praska, A.R. Chada, C. Halder, H.M. Hansen, L.S. McCoy, P.M. Bracci, R. Marshall, S. Zheng, G.F. Reis, A.R. Pico, B.P. O'Neill, J.C. Buckner, C. Giannini, J.T. Huse, A. Perry, T. Tihan, M.S. Berger, S.M. Chang, M.D. Prados,

J. Wiemels, J.K. Wiencke, M.R. Wrensch, R.B. Jenkins, Glioma groups based on 1p/19q, IDH, and TERT promoter mutations in tumors, N. Engl. J. Med. 372 (2015) 2499–2508.

[34] H. Noushmehr, D.J. Weisenberger, K. Diefes, H.S. Phillips, K. Pujara, B.P. Berman, F. Pan, C.E. Pelloski, E.P. Sulman, K.P. Bhat, R.G. Verhaak, K.A. Hoadley, D.N. Hayes, C.M. Perou, H.K. Schmidt, L. Ding, R.K. Wilson, D. Van Den Berg, H. Shen, H. Bengtsson, P. Neuvial, L.M. Cope, J. Buckley, J.G. Herman, S.B. Baylin, P.W. Laird, K. Aldape, N. Cancer Genome Atlas Research, Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma, Cancer Cell 17 (2010) 510–522.

[35] E.G. Van Meir, C.G. Hadjipanayis, A.D. Norden, H.K. Shu, P.Y. Wen, J.J. Olson, Exciting new advances in neuro-oncology: the avenue to a cure for malignant glioma, Ca - Cancer J. Clin. 60 (2010) 166–193.

[36] M.R. Stettner, W. Wang, L.B. Nabors, S. Bharara, D.C. Flynn, J.R. Grammer, G.Y. Gillespie, C.L. Gladson, Lyn kinase activity is the predominant cellular SRC kinase activity in glioblastoma tumor cells, Cancer Res. 65 (2005) 5535–5543.

[37] S. Kleber, I. Sancho-Martinez, B. Wiestler, A. Beisel, C. Gieffers, O. Hill, M. Thiemann, W. Mueller, J. Sykora, A. Kuhn, N. Schreglmann, E. Letellier, C. Zuliani, S. Klussmann, M. Teodorczyk, H.J. Grone, T.M. Ganten, H. Sultmann, J. Tuttenberg, A. von Deimling, A. Regnier-Vigouroux, C. Herold-Mende, A. Martin-Villalba, Yes and PI3K bind CD95 to signal invasion of glioblastoma, Cancer Cell 13 (2008) 235–248.

[38] H. Feng, B. Hu, K.W. Liu, Y. Li, X. Lu, T. Cheng, J.J. Yiin, S. Lu, S. Keezer, T. Fenton, F.B. Furnari, R.L. Hamilton, K. Vuori, J.N. Sarkaria, M. Nagane, R. Nishikawa, W.K. Cavenee, S.Y. Cheng, Activation of Rac1 by Src-dependent phosphorylation of Dock180(Y1811) mediates PDGFRalpha-stimulated glioma tumorigenesis in mice and humans, J. Clin. Investig. 121 (2011) 4670–4684.

[39] M.S. Ahluwalia, J. de Groot, W.M. Liu, C.L. Gladson, Targeting SRC in glioblastoma tumors and brain metastases: rationale and preclinical studies, Cancer Lett. 298 (2010) 139–149.

[40] C.M. Park, M.J. Park, H.J. Kwak, H.C. Lee, M.S. Kim, S.H. Lee, I.C. Park, C.H. Rhee, S.I. Hong, Ionizing radiation enhances matrix metalloproteinase-2 secretion and invasion of glioma cells through Src/epidermal growth factor receptor-mediated p38/Akt and phosphatidylinositol 3-kinase/Akt signaling pathways, Cancer Res. 66 (2006) 8511–8519.