β1,4-Galactosyltransferase V activates Notch1 signaling in glioma stem-like cells and promotes their transdifferentiation into endothelial cells

Malignant glioblastoma multiforme is one of the most aggressive human cancers, with very low survival rates. Recent studies have reported that glioma stem cells mediate glioma tumor angiogenesis and potentially providing new therapeutic options for glioblastoma treatment. Glioma malignancy is strongly associated with altered expression of N-linked oligosaccharide structures on the cell surface. We have previously reported that β1,4-galactosyltransferase V (β1,4GalTV), which galactosylates the GlcNAcβ1–6Man arm of the branched N-glycans, is highly expressed in glioma and promotes glioma cell growth in vitro and in vivo. However, the mechanism by which β1,4GalTV stimulates glioma growth is unknown. Here we demonstrate that short hairpin RNA–mediated β1,4GalTV knockdown inhibits the tumorigenesis of glioma stem-like cells and reduces their transdifferentiation into endothelial cells. We also found that β1,4GalTV overexpression increased glioma stem-like cell transdifferentiation into endothelial cells and that this effect required β1,4GalTV galactosylation activity. Moreover, β1,4GalTV promoted β1,4-galactosylation of Notch1 and increased Notch1 protein levels. Of note, ectopic expression of activated Notch1 rescued the inhibitory effect of β1,4GalTV depletion on glioma stem-like cell transdifferentiation. In summary, our findings indicate that β1,4GalTV stimulates transdifferentiation of glioma stem-like cells into endothelial cells by activating Notch1 signaling. These detailed insights shed important light on the mechanisms regulating glioma angiogenesis.

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Malignant glioblastoma multiforme (GBM) is one of the most aggressive human cancers, with a median survival of about 1 year (1). A number of anti-angiogenic treatment regimens are currently under clinical investigation (2). However, some studies have shown that tumor cells become more aggressive after anti-angiogenic therapy (3, 4), implying the existence of VEGF-independent angiogenesis. Supporting this notion, a series of recent papers has shown that glioma stem-like cells could transdifferentiate into tumor vascular endothelial cells (5–7), which might be one of the resistance mechanisms against anti-VEGF therapy. Thus, clarifying the mechanisms of transdifferentiation of glioma stem-like cell into endothelial cell helps to develop new anti-angiogenic therapeutic strategies.

Increased expression of highly branched N-glycans of cell surface glycoproteins is one of the most malignant prominent transformations characterized in the sugar chains of glycoproteins (8, 9). Recent experimental evidence from our laboratory and others has suggested that changes in the N-glycan structure of glycoproteins in glioma cells affect cell migration and tumor malignancy (10–13). For example, β1,4-galactosyltransferase V (β1,4GalTV), which galactosylates the GlcNAcβ1–6 branch arm of the highly branched N-glycan, is highly expressed in glioma (10, 14–16). Furthermore, β1,4GalTV promotes glioma cell growth and invasiveness (11, 17, 18). Recently, we have confirmed that β1,4GalTV could regulate the self-renewal of glioma-initiating cells (19). N-acetylglucosaminyltransferase V promotes the self-renewal and tumorigenicity of colon cancer stem cells (20). The N-glycan inhibitor tunica-mycin inhibits the self-renewal ability and tumorigenesis of glioma stem-like cells (21). Thus, N-glycans perform critical roles in cancer stem cells.

N-glycosylation frequently regulates cell behavior by regulating membrane protein signaling (22). Notch1 signaling is widely known to regulate cell differentiation, proliferation,
and apoptosis (23, 24). Inactivation of Notch signaling with γ-secretase inhibition or Notch1 knockdown inhibits transdifferentiation of glioma stem-like cells into endothelial cells (5, 25). The glycans, including N-glycan, O-fucose glycan, and O-glycan, regulate the activation of Notch signaling by manipulating Notch receptor–ligand interaction (26–28). Therefore, studying the role of N-glycans in the transdifferentiation of glioma stem-like cells into endothelial cells contributes to elucidation of the mechanisms of glioma vasculogenesis.

Here we evaluate the role and mechanism of β1,4GalTV in the transdifferentiation of glioma stem-like cells into endothelial cells. Our findings will provide a framework for a better understanding of the role of glycosylation in tumor development and might offer a new strategy to manipulate Notch signaling for therapeutic purposes.

Results

β1,4GalTV depletion inhibits transdifferentiation of glioma stem-like cells into endothelial cells in vitro

To determine whether β1,4GalTV regulates the transdifferentiation of glioma stem-like cells into endothelial cells, T698968 glioma stem-like cells were isolated from glioblastoma surgical biopsy specimens as described previously (29, 30). Under serum-free culture conditions, T698968 cells formed typical neurosphere structures (Fig. S1A). These neurospheres expressed the neural stem cell marker Nestin (Fig. S1B). Upon exposure to differentiation conditions, a mixture of cells expressed the markers of neurons and astrocytes (Fig. S1B). Thus, T698968 cells have self-renewal capability and multilineage differentiation capacity in vitro. Similarly, we isolated T109002 glioma stem-like cells from glioblastoma surgical biopsy specimens using the same method.

Next we constructed lentiviral shRNA vectors targeting human β1,4GalTV. RT-PCR results showed that β1,4GalTV shRNA reduced β1,4GalTV mRNA expression in T698968 cells and T109002 cells (Fig. 1, A–C). β1,4GalTV galactosylates the GlcNAcβ1–6Man arm of the highly branched N-glycan (31). Using a lectin blot with RCA-1 lectin, which interacts with Galβ1,4GlcNAc, a significant decrease in the binding of total glycoprotein with RCA-1 was observed in cells expressing β1,4GalTV shRNA compared with control cells (Fig. S1C).

To investigate the role of β1,4GalTV in the transdifferentiation process of glioma stem-like cells into endothelial cells in vitro, a tube formation assay was performed. β1,4GalTV knockdown inhibited tube formation and decreased the length of tubes in T698968 or T109002 cells (Fig. 1, D–F). CD31 is generally considered a specific marker for endothelial cells (5, 6). The RT-PCR assay showed that β1,4GalTV knockdown significantly decreased CD31 expression (Fig. S1, D and E). Consistent with this, immunofluorescent staining showed that β1,4GalTV depletion decreased CD31 expression in the tube formation assay (Fig. S1, F and G). Thus, β1,4GalTV could regulate the endothelial cell transdifferentiation of glioma stem-like cell in vitro.

Reduction of β1,4GalTV expression inhibits transdifferentiation of glioma stem-like cells into endothelial cells in vivo

We used an intracranial glioma model to evaluate the contribution of β1,4GalTV in the transdifferentiation of glioma stem-like cells into endothelial cells in vivo. Glioma stem-like cells marked with GFP were implanted into the frontal lobe of nude mice. β1,4GalTV knockdown reduced tumor burdens in nude mice (Fig. S2, A and B). Depletion of β1,4GalTV in glioma stem-like cells significantly increased the overall survival of tumor-bearing nude mice (Fig. 2, A–C). Thus, β1,4GalTV depletion inhibits gliomagenesis in vivo.

To examine whether β1,4GalTV regulates endothelial cell transdifferentiation of glioma stem-like cell in vivo, co-immunofluorescence analysis of GFP and the endothelial cell marker CD31 was performed in tumor xenografts formed by T698968 cells or T109002 cells marked with GFP. β1,4GalTV depletion significantly reduced the number of CD31+ endothelial cells co-expressing GFP in xenografts formed by both T698968 cells and T109002 cells (Fig. 2, D and E, and Fig. S2, C and D). Consistent with this, β1,4GalTV depletion significantly reduced the protein level of CD31 (Fig. 2F). β1,4GalTV knockdown inhibits transdifferentiation of glioma stem-like cells into endothelial cells in vivo.

β1,4GalTV V regulates the transdifferentiation of glioma stem-like cells into endothelial cells depending on its galactosylation activity

β1,4GalTV protein consists of a short NH2-terminal cytoplasmic domain, a stem region, and a catalytic domain that contains two conserved residues (Try268/Trp294), which are important for its galactosylation activity (32–34). To investigate the contribution of β1,4GalTV galactosylation activity in the transdifferentiation of glioma stem-like cells into endothelial cells, we constructed shRNA-resistant, hemagglutinin-tagged point mutant of β1,4GalTV (Y268G/W294G). Using a lectin blot assay, overexpression of wildtype β1,4GalTV, but not the Y268G/W294G mutant, could rescue the decreased Galβ1,4GlcNAc-glycosylated protein (43- to 130-kDa protein bands) by β1,4GalTV shRNA (Fig. 3A). The expression of wildtype β1,4GalTV, but not the Y268G/W294G mutant, could rescue gliomagenesis and mouse survival in the β1,4GalTV knockdown group (Fig. 3, B and C). Thus, β1,4GalTV regulates gliomagenesis depending on its galactosylation activity.

Co-immunofluorescence staining in tumor xenografts also showed that overexpression of wildtype β1,4GalTV, but not the β1,4GalTV mutant (Y268G/W294G), increased the number of CD31+ endothelial cells co-expressing GFP in the β1,4GalTV knockdown group (Fig. 3, D and E). Furthermore, a tube formation assay also showed that overexpression of wildtype β1,4GalTV reduced the inhibitory effect of β1,4GalTV depletion on tube formation (Fig. 3, F and G). Together, β1,4GalTV regulates the transdifferentiation of glioma stem-like cells into endothelial cells depending on its galactosylation activity.
Figure 1. β1,4GalT V depletion inhibits the transdifferentiation of glioma stem-like cells in vitro. A. RT-PCR analysis of β1,4GalT V mRNA expression in T698968 cells expressing control or β1,4GalT V shRNA. Actin mRNA expression served as a loading control. B. RT-PCR analysis of β1,4GalT V mRNA expression in T109002 cells expressing control or β1,4GalT V shRNA. Actin mRNA expression served as a loading control. C. The relative densities of β1,4GalT V PCR product levels in A and B were quantified using densitometry. Values are normalized to that of cells expressing control shRNA. Results are expressed as mean ± S.D. (n = 3; *, p < 0.05; **, p < 0.01; ***, p < 0.001). D and E, representative images of tube formation derived from T698968 (D) or T109002 (E) cells expressing control or β1,4GalT V shRNA. Scale bars = 20 μm. F, quantification of mean tube length in D and E. The results are expressed as mean ± S.E. (n = 3; *, p < 0.05; ***, p < 0.001).
β1,4GalT V regulates Notch signaling depending on its galactosylation activity

We next examined the mechanisms by which β1,4GalT V regulates the transdifferentiation of glioma stem-like cells into endothelial cells. It has been reported that the Notch signaling pathway regulates endothelial transdifferentiation of glioma stem-like cells (5, 25). Thus, we primarily examined whether β1,4GalT V regulates Notch signaling. Each activated Notch1 molecule is consumed to produce a Notch1 intracellular domain (NICD) (35). Western blotting using an antibody against the Notch1 intracellular domain showed that β1,4GalT V depletion significantly decreased the expression of full-length Notch1 and NICD protein (Fig. 4, A and B). β1,4GalT V depletion also significantly decreased the expression of NICD in the nucleus (Fig. 4C). Consistent with this, β1,4GalT V depletion inhibited the mRNA expression of a canonical Notch target gene, Hes-1, which could be rescued by wildtype β1,4GalT V other than the β1,4GalT V mutant (Y268G/W294G) (Fig. 4, D and E). Thus, β1,4GalT V regulates Notch signaling.

Next, we examined the mechanism of β1,4GalT V regulating Notch1 expression. β1,4GalT V knockdown slightly reduced the level of Notch1 mRNA (Fig. 4F). Previous studies have shown that Notch1 could be degraded by the lysosome (36). Thus, we examined the effect of β1,4GalT V knockdown on Notch1 lysosome-mediated degradation. We found no obvious

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**Figure 2. Reduction of β1,4GalT V expression inhibits gliomagenesis and transdifferentiation of glioma stem-like cells into the endothelium in vivo.**

A–C, Kaplan–Meier survival curves of nude mice injected with T698968 (A and B) or T109002 (C) cells expressing control or β1,4GalT V shRNA. D, confocal immunofluorescence analysis shows co-localization of the human endothelial cell marker CD31 and the tumor cell marker GFP in tumor xenografts formed by T698968 cells expressing GFP and control or β1,4GalT V shRNA. Scale bars = 10 μm. E, the relative vascular density derived from glioma stem-like cells was quantified. Results are expressed as mean ± S.E. (n = 3; *, p < 0.05; ***, p < 0.001). F, Western blot analysis of the endothelial cell marker CD31 in xenografts formed by T698968 cells expressing control or β1,4GalT V shRNA. GAPDH protein expression served as a loading control.
effect of the lysosomal inhibitor chloroquine on Notch 1 levels in control cells, whereas chloroquine significantly increased the level of Notch 1 protein in cells expressing H92521,4GalTV shRNA (Fig. 4G). Thus, H92521,4GalTV depletion might promote Notch1 lysosome-mediated degradation.

A previous report has shown that Notch1 is modified with N-glycan and that N-glycan regulates Notch1 stability (37). This finding motivated us to examine whether Notch could be H92521,4-galactosylated using RCA-1 lectin. Notch1 protein immunoprecipitated from T698968 cells reacted with the lectin RCA-1. Furthermore, H1,4GalTV depletion inhibited the H92521,4-galactosylation of Notch1. In addition, H92521,4GalTV could be immunoprecipitated with Notch1 (Fig. 4H). Thus, Notch might be a substrate of H1,4GalTV.

Glycosylation is required for protein processing and transport to the plasma membrane (38). Thus, we next examined the

Figure 3. H1,4GalTV regulates gliomagenesis and the transdifferentiation of glioma stem-like cells into endothelial cells depending on its galactosylation activity. A, total lysates of T698968 cells expressing LacZ shRNA + FLAG, H1,4GalTV shRNA3 + FLAG, or H1,4GalTV shRNA3 + GalTV, or H1,4GalTV shRNA3 + GalTV (Y268G/W294G) were analyzed with an RCA-1 lectin blot. The protein expression of GAPDH served as a loading control. B, nude mice were injected with T698968 cells expressing LacZ shRNA + FLAG, H1,4GalTV shRNA3 + FLAG, or H1,4GalTV shRNA3 + GalTV (Y268G/W294G). Four weeks later, photos were taken for the xenograft. Scale bars = 2 mm. C, Kaplan–Meier survival curves of nude mice injected with T698968 cells expressing LacZ shRNA + FLAG, H1,4GalTV shRNA3 + FLAG, or H1,4GalTV shRNA3 + GalTV (Y268G/W294G). D, immunofluorescence analysis shows co-localization of the human endothelial cell marker CD31 and the tumor cell marker GFP in tumor xenografts formed by T698968 cells expressing GFP and LacZ shRNA + FLAG, or H1,4GalTV shRNA3 + GalTV (Y268G/W294G). Scale bars = 10 μm. E, the relative vascular density derived from glioma stem-like cells was quantified. Results are expressed as mean ± S.E. (n = 3; *, p < 0.05; **, p < 0.01). F, representative images of tube formation derived from T698968 cells expressing LacZ shRNA + FLAG, or H1,4GalTV shRNA3 + GalTV (Y268G/W294G). Scale bars = 20 μm. G, quantification of mean tube length in F. The results are expressed as mean ± S.E. (n = 3; ***, p < 0.001).
Figure 4. \(\beta 1,4\)GalTV regulates the activity of Notch1 signaling. A, total lysates of T698968 cells expressing control or \(\beta 1,4\)GalTV shRNA3 were analyzed with Notch1 antibody by Western blotting. The protein expression of GAPDH served as a loading control. The relative densities of NICD protein levels in A were quantified using densitometry. Values are normalized to that of T698968 cells expressing LacZ shRNA. Results are expressed as mean ± S.D. (n = 3; *, p < 0.05). B, RT-PCR analysis of Hes-1 expression in T698968 cells expressing LacZ shRNA, \(\beta 1,4\)GalTV shRNA3, or \(\beta 1,4\)GalTV shRNA3 + GalTV (Y268G/W294G). C, Western blot analysis of nuclear NICD expression in T698968 cells expressing control or \(\beta 1,4\)GalTV shRNA3. Sp1 served as a nuclear marker. D, RT-PCR analysis of Hes-1 expression in T698968 cells expressing LacZ shRNA, \(\beta 1,4\)GalTV shRNA3, or \(\beta 1,4\)GalTV shRNA3 + GalTV (Y268G/W294G). E, the relative densities of Hes-1 PCR product levels were quantified using densitometry. Values are normalized to that of cells expressing LacZ shRNA + FLAG. Results are expressed as mean ± S.D. (n = 3; ***, p < 0.001). F, RT-PCR analysis of Notch1 expression in T698968 cells expressing control or \(\beta 1,4\)GalTV shRNA. Actin expression served as a loading control. The relative densities of Notch1 PCR product levels were quantified using densitometry. Values are normalized to that of cells expressing control shRNA. **, p < 0.01. G, Western blot analysis of Notch1 from lysates of T698968 cells expressing control or \(\beta 1,4\)GalTV shRNA in the absence or presence of the lysosome inhibitor chloroquine overnight. H, Notch1 proteins immunoprecipitated from T698968 cells expressing control or \(\beta 1,4\)GalTV shRNA were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was overlaid with Notch1 antibody to verify the immunoprecipitation efficiency, with lectin RCA-1 to confirm \(\beta 1,4\)-linked galactosylation, and with \(\beta 1,4\)GalTV antibody to examine the interaction between \(\beta 1,4\)GalTV and Notch1. I, trafficking of newly synthesized Notch1 to the cell surface in T698968 cells expressing control or \(\beta 1,4\)GalTV shRNA treated with His-tagged galectin-3 for 30 min were analyzed with Notch1 antibody by Western blotting. The protein expression of GAPDH served as a loading control. FL-Notch1, full-length Notch1.
The inhibitory effect of β1,4GalT V depletion on the transdifferentiation of glioma stem-like cells into endothelial cells could be rescued by ectopic expression of NICD

Next, we explored the role of Notch1 signaling in β1,4GalT V promotion of endothelial cell transdifferentiation of glioma stem-like cells. First we tested the effect of DAPT on the transdifferentiation of glioma stem-like cells. DAPT is a γ-secretase inhibitor that effectively inhibits Notch signaling (42). A tube formation assay showed that DAPT treatment decreased tube formation in vitro (Fig. 5A).

Next we used an intracranial glioma model to evaluate the contribution of Notch1 signaling during β1,4GalT V regulation of the transdifferentiation process from glioma stem-like cells in vivo. T698968 cells expressing β1,4GalT V shRNA3 and NICD were implanted into the frontal lobe of nude mice. Bioluminescent imaging (BLI) targeting GFP showed that NICD protein overexpression rescued the inhibitory effects of β1,4GalT regulation on gliomagenesis (Fig. 5B). Additionally, NICD protein overexpression reversed the effects of β1,4GalT V knockdown on tumor-bearing mouse survival (Fig. 5C).

Next we performed immunohistochemistry staining with tumor xenografts formed by β1,4GalT V knockdown T698968 cells overexpressed with NICD protein with the human CD31 antibody. Overexpression of NICD protein could rescue the inhibitory effect of β1,4GalT V knockdown on endothelial cell transdifferentiation of glioma stem-like cells (Fig. 5, D and E). Overexpression of NICD protein could reverse the inhibitory effect of β1,4GalT V knockdown on tube formation (Fig. 5, F and G). Taken together, these data demonstrate that β1,4GalT V regulates the transdifferentiation of glioma stem-like cells into endothelial cells through Notch1 signaling.

Nucleus NICD expression in human GBM correlates with β1,4GalT V protein level

To further investigate the critical role of β1,4GalT V in glioma malignancy, we performed immunohistochemistry with human brain tumor specimens classified according to the World Health Organization. The expression of β1,4GalT V protein increased, accompanied by tumor malignancy (Fig. 6, A and B). This result was consistent with the Repository of Molecular Brain Neoplasia Data (REMBRANDT) database of the NCI, National Institutes of Health, which showed a significant decrease in the probability of survival with elevated β1,4GalT V expression (Fig. 6C). To further confirm the correlation between Notch1 signaling activation and β1,4GalT V expression, immunohistochemistry was performed on human GBM cells. The immunohistochemistry assay showed a strong linear relationship between β1,4GalT V expression and nucleus NICD expression (Fig. 6D). These data strongly support that β1,4GalT V regulates Notch1 signaling.

Discussion

Here we report for the first time that β1,4GalT V can regulate the transdifferentiation of glioma stem-like cells into endothelial cells in vitro and in vivo. β1,4GalT V depletion could significantly improve the survival of tumor-bearing mice. Additionally, the REMBRANDT database indicated a significant decrease in the probability of survival with high β1,4GalT V expression. The expression of β1,4GalT V was correlated with glioma grade. Thus, β1,4GalT V might be a therapeutic target for GBM.

Tumor angiogenesis is fundamental to glioma development (43, 44). The regulation of glioma angiogenesis is a complex and intricately orchestrated process involving multiple interrelated signaling pathways, such as VEGF/VEGFR2 and HIF-1 (45–48). Increasing evidence has revealed that N-glycan plays a critical role in tumor angiogenesis (49, 50). Recent studies have also reported that tunicamycin, a widely known N-glycosylation inhibitor, inhibited angiogenesis in breast tumors (51). Therefore, studying the role of N-glycan in tumor angiogenesis might help to elucidate the mechanism of angiogenesis. β1,4GalT V regulates the transdifferentiation of glioma stem-like cells into endothelial cells depending on its galactosylation activity. To our knowledge, our study reports for the first time that glycosyltransferase regulates the transdifferentiation process from glioma stem-like cells into endothelial cells. Our findings might help to understand the role of N-glycan chains in tumor development.

Another interesting finding was that β1,4GalT V regulated Notch signaling. Results from previous studies have demonstrated that the Notch1 signaling pathway is critical for the transdifferentiation process from glioma stem-like cells into vascular endothelial cells (5, 25). Our results demonstrate that decreasing the expression of β1,4GalT V reduces Notch1 cleavage. Ectopic expression of the Notch1 intracellular domain could rescue the inhibitory effect of β1,4GalT V knockdown on transdifferentiation from glioma stem-like cells. Thus, we speculate that β1,4GalT V regulates the transdifferentiation process from glioma stem-like cells into endothelial cells through the Notch1 signaling pathway.

The extracellular domains of Notch receptors contain numerous potential sites for N-linked and O-linked glycosylation (28, 52, 53). Fringe is a β3 N-acetylgalcosaminyltransferase (β3GlcNAcT) that transfers GlcNAc to O-fucose in epidermal growth factor-factor-like repeats of Notch. Fringe regulates the
response of Notch to ligand (54, 55). The glycosyltransferase GnT-III activates Notch signaling through inhibition of Notch degradation (37). Our data suggest that Notch1 has /H9252 1,4-galactosylation. In addition, /H9252 1,4GalTV can be immunoprecipitated with Notch. Thus, these data suggest that Notch might be a substrate of /H9252 1,4GalTV. Furthermore, /H9252 1,4GalTV knockdown obviously inhibited the interaction between Notch1 and galectin-3. Galectin-3 treatment could rescue the inhibitory effect of /H9252 1,4GalTV knockdown on Notch1 expression. However, the mechanisms by which /H9252 1,4GalTV regulates Notch1 lysosome-mediated degradation need further exploration.

Gliomas develop as a result of stepwise accumulations of multiple genetic alterations that result in the activation of oncoproteins such as Ras/Raf/extracellular signal-regulated kinase and phosphatidylinositol 3-kinase/Akt signaling pathways (56). Our previous studies have shown that /H9252 1,4GalTV expression is activated by Ras/Raf/extracellular signal-regulated kinase and Akt signaling pathways (33). Thus, our finding that /H9252 1,4GalTV...
regulates the transdifferentiation of glioma stem-like cells into endothelial cells might provide cues for understanding the mechanisms of the Akt and Ras signaling pathways promoting glioma development.

Our findings expand the understanding of the mechanism involved in tumor angiogenesis and provide a novel strategy for glioma therapy. The molecular mechanism of β1,4GalTV regulating Notch signaling should be further explored.

**Experimental procedures**

**Cell culture**

The tumor sample studies were approved by the Research Ethics Committee of Fudan University, and informed consent was obtained from all brain tumor patients contributing tumor specimens. T698968 cells and T109002 cells were obtained from primary human brain tumor patient specimens. Briefly, tumors were digested with collagenase (type V, Sigma) and filtered with a 70-μm cell strainer to remove tissue pieces. Cells were then cultured under non-adherent conditions in neural stem cell culture medium composed of Dulbecco’s modified Eagle’s medium (Gibco) and Ham’s F-12 medium supplemented with serum-free B27 (Gibco), 50 mM Hepes, 2 μg/ml heparin (Sigma), 20 ng/ml epidermal growth factor (Chemicon), and 20 ng/ml FGF-2 (Chemicon) in a humidified CO₂ incubator (5% CO₂, 95% air).

**Lentivirus production and cell infection**

The pLL3.7 vector containing a GFP expression cassette was a gift from Prof. V. Parijs (Massachusetts Institute of Technology, Cambridge, MA). Hairpin sequences used for the β1,4GalTV shRNA were as follows: β1,4GalTV (human) shRNA1, forward 5'-TGATGACGACCTCTGGAACATTCAAGAGATGTTCCAGAGGTCGTCATCTTTTTTC-3' and reverse 5'-TCGAGAAAAAAGATGACGACCTCTGGAACATCTTTGATGGTGTCGTATGTGTCTT-3'; β1,4GalTV (human) shRNA2, forward 5'-TGACATCACATACGACGCCTTTTCAAGAGAAAGGCGTCGTATGTGATGGTTTTTTTC-3' and reverse 5'-TCGAGAAAAAAACATCACAATCAGCCCTTTTCAAGAGAAAGGCGTCGTATGTGATGGTTTTTTTC-3'; β1,4GalTV (human) shRNA3, forward 5'-TCGGAGTGAGTGGCTTAACATTCAAGAGATGTTAAGCCACTCCA-3' and reverse 5'-TCGAGAAAAAAACGAGTGAGTGGCTTAACATTCAAGAGATGTTAAGCCACTCCA-3'. The β1,4GalTV shRNA sequences were cloned into the pLL3.7 vector to generate a lentiviral expression vector. β1,4GalTV (human) was cloned into the LV-FLAG vector using BamH1 and AgeI restriction enzymes. The β1,4GalTV (human) primers were as follows: forward 5'-GGGTGGATCCATGTACCCATACGATGTTCCAGATTA-3' and reverse 5'-CGGAAGGTCGTATGTGATGGTTTTTTTC-3'.
CGTACTCGTTCACCCT-3’. NICD was subcloned into the LV-FLAG vector using BamHI and AgeI restriction enzymes. The NICD primers were as follows: forward 5’-CGCATGGATCTCATGCCGCGAGCATGCGCAG-3’ and reverse 5’-AGGCCACCGGTGCTTGAGGGCCCTCGGAAT-3’. Lentivirus production was done by transfection of 293T cells using calcium phosphate transfection. Supernatants were collected 72 h after transfection and filtered; the viral titers were then determined by FACS 48 h post-transduction. Subconfluent cells were infected with the lentivirus at a multiplicity of infection of 5 in the presence of 8 μg/ml Polybrene (Sigma-Aldrich).

**Tube formation assay**

The *in vitro* tube formation assay was performed as described previously (57). In brief, 12 μl of tail collagen was dropped onto glass coverslips on 12-well plates and allowed to polymerize for 1 h at 37 °C. Cells (1 × 10⁶) were then suspended in 2 ml of endothelial basal medium (Gibco) containing 2% fetal bovine serum and incubated in a humidified CO₂ incubator (5% CO₂, 95% air) for 7 days.

Data were photographically recorded every day. Images were acquired using Motic Microscopy connected to a computer with the online image acquisition software WinFast PVR2. For quantification of tube lengths, images were exported to Image-Pro Plus software.

**Immunoblot analysis**

The Western blot assay was performed as described previously (33). The following primary antibodies were used: mouse monoclonal anti-Notch1 (BD Pharmingen, catalog no. 552466), rabbit polyclonal anti-FLAG (Sigma, catalog no. F7425), rabbit polyclonal anti-galectin-3 (Abcam, catalog no. 31707), and rabbit polyclonal anti-β1,4GalIT (Santa Cruz Biotechnology, catalog no. sc-22289). Horseradish peroxidase (HRP)–conjugated secondary antibodies were as follows: goat anti-mouse (Santa Cruz Biotechnology, catalog no. sc-2005) and goat anti-rabbit (Santa Cruz Biotechnology, catalog no. sc-2004). Relative protein levels were quantified by scanning densitometry. The gray value of the protein level was measured with National Institutes of Health ImageJ Software.

Lectin blots were also performed as described previously (33). The primary antibody was biotinylated lectin *Ricinus communis* agglutinin I (RCA-1) (Vector, catalog no. B-1085). The secondary antibody was HRP conjugated with streptavidin (Southern Biotech, catalog no. 7100-05).

**Immunofluorescence**

Immunofluorescence assays were performed on cells and frozen sections following protocols described previously (58). The following primary antibodies were used: mouse monoclonal anti-Nestin (Millipore, catalog no. MAB5326), rabbit polyclonal anti-GFAP (glial fibrillary acidic protein) (Millipore, catalog no. AB5804), rabbit polyclonal anti-β-tubulin III (Sigma, catalog no. T2200), goat polyclonal anti-CD31 (Santa Cruz Biotechnology, catalog no. sc-1506), and goat polyclonal anti-Notch1 (Santa Cruz Biotechnology, catalog no. sc6014). The secondary antibodies used were as follows: Alexa Fluor 594 anti-mouse IgG, Alexa Fluor 594 anti-rabbit IgG, and Alexa Fluor 594 anti-goat IgG. Nuclei were stained with Hoechst (Sigma, catalog no. 33258). The images were obtained by confocal laser-scanning microscopy (Leica TCS SP5), and the obtained images were processed with LAS-AF-Lite software.

**Tumor formation assay**

For intracranial xenografts, 4-week-old male nude mice were intracranially injected with 1.0 × 10⁵ T698968 cells or T109002 cells into the right frontal lobes under the Fudan University Animal Care Committee protocol. Four weeks later, bioluminescence imaging was performed on nude mice to measure tumor size. All mice were maintained until development of neurologic signs, sacrificed, and perfused with 4% paraformaldehyde.

**Immunohistochemistry**

Human brain tumor specimens of different grades were provided by Zhongshan Hospital of Fudan University and Provincal Hospital affiliated with Shandong University in accordance with the appropriate institutional review boards. Immunohistochemistry studies were performed on paraffin-embedded primary human glioblastoma sections and mouse intracranial glioblastoma xenograft sections following a protocol described previously (59). The following primary antibodies were used: mouse monoclonal anti-human-CD31 (BD Pharmingen, catalog no. 550389) and rabbit polyclonal anti-β1,4GalITV (Santa Cruz Biotechnology, catalog no. sc-22289). Secondary antibodies conjugated with HRP were as follows: goat anti-mouse (Santa Cruz Biotechnology, catalog no. sc-2005) and goat anti-rabbit (Santa Cruz Biotechnology, catalog no. sc-2004). Tris-EDTA buffer (pH 9.0) was used for antigen retrieval in all cases. Images were acquired using Motic Microscopy connected to a computer with the online image acquisition software WinFast PVR2. For quantification, images were exported to Image-Pro Plus software.

**RT-PCR**

Total RNA (1 μg) extracted was used as a template for complementary DNA synthesis with a TaKaRa RNA PCR kit and specific primers as follows: β1,4GalITV (human), forward 5’-GCTGCTGTACTCTGTATGTGGTGA-3’ and reverse 5’-GCCTGGCATCTGGCACCACATCC-3’; CD31 (human), forward 5’-ATTGCAAGTTTATCATCGAGGATG-3’ and reverse 5’-CTCGTGGTTGAGGGTTGAAGTG-3’. Amplification was carried out for 22–27 cycles under saturation (each at 94 °C, 45 s; 60 °C, 45 s; and 72 °C, 1 min) in a 50-μl reaction mixture containing 2 μl of each complementary DNA, 0.2 μM each primer, 0.2 mM dNTP, and 2.5 units of DNA polymerase (Sino Develop). After amplification, 10 μl of each reaction mixture was analyzed by 1%–2.5% agarose gel electrophoresis, and the bands were then visualized by ethidium bromide staining. Images were acquired by Gel Imaging (Xia Ri Technology). System levels of mRNA were quantified with National Institutes of Health ImageJ software.

**Pulse–chase analysis**

Cells were incubated with Dulbecco’s modified Eagle’s medium/F12 medium depleted of methionine for 1 h at 37 °C. Pulse
labeling was performed with 1 mM AHA (1-azidohomoalanine, a structural analog of methionine) for 60 min as described previously (60). Cells were returned to the above culture conditions containing methionine for 0, 15, 30, 45, 60, or 75 min. Then cell surface protein was eluted using a cell surface protein isolation kit (Pierce, 89881). AHA-labeled protein on the cell surface was purified by immunoprecipitation. Immunoprecipitates were subjected to SDS-PAGE, and AHA-labeled Notch1 was examined.

Statistical analysis

All in vitro experiments were repeated at least three times. All numerical data are expressed as mean ± S.D. or mean ± S.E. as indicated. Data were analyzed using Student’s t test or one-way analysis of variance. Survival curves were generated using Kaplan–Meier methods and compared using a log-rank test. Kendall correlation analysis was used to analyze the correlation between nucleus NICD protein expression and β1,4GalT V expression.

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