Fibulin-3 Promotes Glioma Growth and Resistance through a Novel Paracrine Regulation of Notch Signaling

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Abstract
Malignant gliomas are highly invasive and chemoresistant brain tumors with extremely poor prognosis. Targeting of the soluble factors that trigger invasion and resistance, therefore, could have a significant impact against the infiltrative glioma cells that are a major source of recurrence. Fibulin-3 is a matrix protein that is absent in normal brain but upregulated in gliomas and promotes tumor invasion by unknown mechanisms. Here, we show that fibulin-3 is a novel soluble activator of Notch signaling that antagonizes DLL3, an autocrine inhibitor of Notch, and promotes tumor cell survival and invasion in a Notch-dependent manner. Using a strategy for inducible knockdown, we found that controlled downregulation of fibulin-3 reduced Notch signaling and led to increased apoptosis, reduced self-renewal of glioblastoma-initiating cells, and impaired growth and dispersion of intracranial tumors. In addition, fibulin-3 expression correlated with expression levels of Notch-dependent genes and was a marker of Notch activation in patient-derived glioma samples. These findings underscore a major role for the tumor extracellular matrix in regulating glioma invasion and resistance to apoptosis via activation of the key Notch pathway. More importantly, this work describes a noncanonical, soluble activator of Notch in a cancer model and shows how Notch signaling can be reduced by targeting tumor-specific accessible molecules in the tumor microenvironment. Cancer Res; 72(15); 3873–85. ©2012 AACR.

Introduction
Malignant gliomas are one of the most common primary brain tumors and one of the types of cancer with worst prognosis (1, 2). Despite significant advances in neurosurgery and chemoradiotherapy, gliomas remain highly resistant to conventional treatments and improvements in patient outcome have been modest (3). A major challenge for glioma therapy is the typical scattering of invasive tumor cells in the brain, escaping resection and leading to recurrence (4). Cumulative evidence suggests that these invasive cells are highly resistant to cytotoxic therapies (5, 6) and that their dispersion may be triggered, in part, by these treatments (7, 8). Improved targeting of the mechanisms that promote glioma invasion and facilitate chemoresistance is therefore critical to increase the long-term efficacy of current therapeutic approaches.

The discovery and characterization of glioma-initiating stem-like cells (GICs) that share properties with neural stem cells (9) has brought attention to a number of signaling pathways in glioma that are also involved in neural development, such as Hedgehog, Wnt, and Notch (10, 11). These pathways are necessary for the maintenance of GICs (12, 13) and critical for glioma initiation, self-renewal, and progression through stages of malignancy (11, 14), all of which makes them appealing pharmacologic targets. While Hedgehog and Wnt are activated by soluble factors, Notch activation is usually a juxtacrine signaling mechanism that mediates close cell-to-cell signaling (15), which becomes less likely as tumor cells scatter. A few soluble, noncanonical activators of Notch signaling have been described in mammals [e.g., MAGP-1/2, CCN3, and YB-1 (ref. 15)] but their relationship with Notch signaling in cancer progression has not been elucidated.

The fibulins are a family of secreted proteins that associate to the extracellular matrix (ECM) scaffold, forming anchoring structures that can regulate cell proliferation and migration (16, 17). Several fibulins have been associated with the development of solid tumors such as ovarian, breast, and colorectal cancers (16, 17), but their role in cancer initiation and progression has been difficult to define (16–18). Fibulin-3, also known as EFEMP1, is a protein of restricted expression in the body, predominantly localized in the ECM of elastic tissues (19). This protein is absent from normal brain (19, 20) and is downregulated in several types of solid tumors (21, 22). Surprisingly, fibulin-3 is highly upregulated in gliomas, where it promotes tumor growth and invasion (22). Recent results suggest that fibulin-3 is also upregulated in some highly
metastatic tumors, where it correlates with the progression of these tumors toward the invasive phenotype (23, 24). The molecular mechanisms of this protein in cancer are still essentially unknown.

Here, we report that fibulin-3 is a novel soluble activator of Notch signaling, acting through an unconventional mechanism that reduces cis-inhibition of Notch. Our results show that fibulin-3 regulates the Notch pathway in glioma and promotes resistance to apoptosis as well as tumor growth and invasion in a Notch-dependent manner. This is the first demonstration of a secreted matrix molecule that regulates the Notch pathway in cancer to promote tumor progression.

Materials and Methods

Cells and tissue specimens

The human glioblastoma cell lines U87 and U251 and the rat glioblastoma cell line CNS1 were cultured as previously described (22) and authenticated using the Cell Check service provided by the Research Animal Diagnostic Laboratory (RADIL, Columbia, MO). Primary glioblastoma-derived initiating cells (GiCs G2, G8, G34, and G146) were kindly provided by Drs. E. Chiocca and I. Nakano of the Department of Neurological Surgery, The Ohio State University (OSU; Columbus, OH), and cultured as floating neurospheres in DMEM: Ham’s F-12 supplemented with 2 μmol/L glutamine, 20 ng/mL EGF, 20 ng/mL basic fibroblast growth factor (bFGF), 2 μg/mL grade-1A heparin, and B27 supplement (Invitrogen). These cells were validated for self-renewal, ability to form tumors in low numbers, and multilineage differentiation. Tissue microarrays containing multiple replicate cores of formalin-fixed, paraffin-embedded glioma specimens were purchased from US Biomax, yielding 65 independent samples.

DNA constructs, siRNAs, and lentivirus

A clone containing the full-length coding sequence of fibulin-3 was previously described (22). From this clone, the variants fib-3ΔA and fib-3ΔC were generated by deleting the sequences Glu19–Ser106 and Cys379–Phe493, respectively. Full-length DLL3 cDNA (25) was subcloned into pcDNA4.1-V5/His for expression and epitope tagging. The following constructs have already been described (26, 27) and were used without modifications: full-length rat Notch-1 (pBOS-rNotch1), full-length rat Jagged-1 (pBOS-SN3T), constitutively active mouse Notch-1 intracellular domain (pSG5-FLAG-NICD), and a Notch reporter construct carrying firefly luciferase under control of 4xCBF1 binding elements (pGL2Pro-CBF1-Luc). Comparison of N-terminal sequences of fibulin-3 and Notch ligands was carried out using ClustalW software (Supplementary Fig. S1).

siRNA oligonucleotides against fibulin-3 and Notch-1 were purchased from Qiagen and validated at the mRNA and protein levels (Supplementary Fig. S2).

The lentiviral vector pLVET-tTR-KRAB-eGFP used for doxycycline-dependent short hairpin RNA (shRNA) expression has been described (28). shRNAs against fibulin-3 or a nontargeting control sequence (GenScript) were cloned into the shuttle vector pLVTHM and then subcloned into the lentiviral backbone. After stable transduction, tumor cells were treated with 20 μg/mL doxycycline for 48 hours to induce shRNA and eGFP expression. Inducible fibulin-3 knockdown was confirmed in 3 independent glioma cell lines (Supplementary Fig. S3).

Immunohistochemistry

Human tissue sections (5 μm) were deparaffinized and processed for immunohistochemistry with antibodies against fibulin-3 and Hes1 (listed in Supplementary Table S1). Scoring was conducted blindly by a pathologist (G.J. Nuovo), using a scale from 0 (absent staining) to 100 (whole visual field stained; ref. 29). Frozen mouse brains were coronally sectioned at 20 μm and every fourth section stained with 0.1% w/v cresyl violet (22). Imaging software (ImageJ v.1.45) was used to measure maximum coronal area and distance from the geometric center of the tumor to the most distant border. Sections selected for immunohistochemistry were probed with an antibody against the proliferation marker Ki67 (22) or processed for terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining (ApoTag Red Kit, Millipore).

Biochemical assays

Cells were recovered from culture, lysed, and processed for Western blotting using standard protocols (ref. 22; antibodies are listed in Supplementary Table S1). To analyze protein phosphorylation, cells were incubated in serum-free media overnight before treatments. In vitro effects of fibulin-3 were analyzed by incubating glioma cells with purified fibulin-3 (100 ng/mL) for 2 hours. Short-time incubations (15 minutes) were also carried out to compare activity of fibulin-3 against a canonical EGFR receptor (EGFR) ligand (EGF, 5 ng/mL). For semiquantitative real-time PCR (RT-PCR), cells or tissue samples were processed using TRizol reagent (Invitrogen) and total RNA was purified by ethanol precipitation. For Notch reporter assays, cells were transfected with the Notch reporter construct and Renilla luciferase as loading control. Reporter cells were exposed to purified fibulin-3 for 8 hours or cotransfected with different constructs and processed after 24 hours to quantify luciferase activity.

Migration and invasion assays

Cell migration was quantified with a conventional assay in culture inserts (Transwell, 8 μm pore size), using bovine fibronectin (5 μg/mL) as chemoattractant. Cells (5,000 cells/well) were allowed to migrate for 16 hours and subsequently fixed, stained, and counted (30). Invasion of cells out of spheroids implanted in cultured brain slices was carried out as described (30) and total dispersion quantified by fluorescence microscopy. The γ-secretase inhibitor DAPT (25 μmol/L, Tocris) was added to the cells 2 hours before seeding and maintained in the medium during these experiments. Transfection with cDNAs or siRNAs was carried out 48 hours before preparing cell spheroids to deposit on brain slices.

Cell viability and self-renewal

Cell viability was monitored using a standard redox assay (Promega CellTiter Kit). Cells treated with serum depletion or temozolomide (Tocris) were labeled with propidium iodide...
(PI)/Annexin-V following standard protocols and analyzed using a FACSCalibur flow cytometer (Becton Dickinson). To measure apoptosis/necrosis in multwell plates, cells were labeled as before and quantified by fluorescence microscopy. To evaluate GIC self-renewal, cells were dissociated, plated in serial dilutions as described (31), and new spheroids quantified after 12 to 14 days.

**Animal studies**

All studies involving animals were approved by the Institutional Animal Care and Use Committee at OSU. Glioma cells were resuspended at $2.5 \times 10^6$ cells/$\mu$L in Hanks’ buffered saline solution supplemented with 0.1% w/v glucose. The cell suspension (2 $\mu$L) was injected into the right striatum of 8-week-old nude (nu/nu) mice following standard protocols. Induction of fibulin-3 shRNA was achieved with 1 mg/mL doxycycline in the drinking water, starting 3 days after tumor implantation. Animals were euthanized and tumors harvested for histologic analysis 20 days after implantation. For survival studies, animals were kept until they reached physiologic criteria for early removal or euthanasia.

**Statistics**

All experiments in vitro were repeated at least in triplicate with 3 independent replicates (8 replicates for brain slice invasion assays). Animal studies were conducted with $N = 5$ (histology) or $N = 10$ (survival) animals per experimental condition. Results were analyzed by one-factor or multifactorial ANOVA, followed by the Bonferroni posthoc test. Immunohistochemical scoring was analyzed using Spearman non-parametric rank correlation. Survival curves were compared by log-rank test.

**Results**

**Fibulin-3 is a paracrine activator of Notch signaling**

Fibulin-3 binds to large ECM proteins with lower affinity than other members of this family (19), therefore being a largely soluble factor (22). Other than its binding to ECM proteins, the molecular mechanisms triggered by fibulin-3 are almost completely unknown.

We first investigated whether fibulin-3 could activate the EGFR pathway in glioma cells because this mechanism has been described in pancreatic carcinoma cells (32), although opposite results have been shown in nasopharyngeal carcinomas (33). We used a conventional glioma cell line (U87) with moderate expression of endogenous fibulin-3 (22) and well characterized EGFR signaling. Short-time exposure (15 minutes) of these cells to purified fibulin-3 failed to activate EGFR, mitogen–activated protein kinase (MAPK), or Akt (Fig. 1A); similar results were observed with longer exposure (2 hours) or after transfection of the cells with fibulin-3 cDNA. This was not caused by lack of EGFR activity because the pathway was triggered by the canonical ligand, EGF, incubated for the same amounts of time (Fig. 1A). The same results were obtained with the glioma cell line U251 (not shown), indicating that this mechanism was not a primary target of fibulin-3 in glioma cells.

To identify other potential mechanisms triggered by fibulin-3 we analyzed its sequence and identified a considerable conservation of a Delta-Serrate-Lag motif, characteristic of the extracellular domain of Notch ligands (Supplementary Fig. S1). Accordingly, fibulin-3 promoted Notch-1 cleavage and upregulation of the active Notch-1 intracellular domain (NICD; Fig. 1A), an effect that has never been described for the members of the fibulin family.

To validate this finding, we generated reporter HEK293 cells, which do not express fibulin-3, expressing full-length Notch-1 and an RBPjK-dependent Notch-responsive luciferase reporter (26). These cells were cocultured in equal proportion with HEK293 cells expressing fibulin-3 or the canonical Notch ligand, Jagged-1. The luciferase reporter was activated in both cases, showing that Notch signaling was activated by fibulin-3 from neighboring cells (Fig. 1B). Moreover, when the reporter cells were cultured in the conditioned medium of the ligand-expressing HEK293 cells, only the medium from fibulin-3-secreting cells activated the reporter whereas the medium from Jagged-expressing cells failed to do so (Fig. 1B), confirming the role of fibulin-3 as a paracrine, rather than juxtacrine, Notch activator. Exposure of reporter HEK293 cells to affinity-purified fibulin-3 yielded the same results (Fig. 1B).

To further verify these findings, we used rat glioblastoma CNS1 cells, which reproduce the phenotype of highly invasive gliomas and share multiple properties with neural progenitors including high endogenous expression of Notch-1. Transfection of these cells with the Notch-responsive reporter confirmed that purified fibulin-3 was sufficient to activate the reporter (Fig. 1C). Moreover, this activation was abolished by preincubation of the cells with the $\gamma$-secretase inhibitor DAPT or by transfection with siRNAs against Notch-1, suggesting that this was the predominant Notch receptor mediating the effect of fibulin-3. Exposure of CNS1 cells to fibulin-3 confirmed activation of endogenous Notch-1 in a $\gamma$-secretase–dependent manner (Fig. 1D).

Finally, to determine which domain(s) of fibulin-3 were required for Notch activation we tested deletion constructs of fibulin-3 lacking either the N-terminal domain (fib-3NΔ) containing the DSL-like motif or the C-terminal domain (fib-3CΔ) that contains a domain common to all fibulins. CNS1 cells expressing fib-3NΔ failed to show activation of the Notch-dependent reporter (Fig. 1E) or increase of endogenous NICD (Fig. 1F), whereas the effects of fib-3ΔC were essentially indistinguishable from those of full-length fibulin-3, suggesting that the DSL-like motif was critical for Notch pathway activation.

**Fibulin-3 antagonizes the cis-inhibitor of Notch, DLL3**

We next investigated whether the effects of fibulin-3 were a result of direct binding to Notch-1 but could not detect coprecipitation of these 2 proteins. Similarly, we were unable to detect any effect of fibulin-3 on the activity of the proteases ADAM10/ADAM17 involved in Notch activation (not shown).

Therefore, we focused on a possible effect of fibulin-3 on the mechanism of cis-inhibition that regulates Notch activity. Notch ligands of the Delta family are known to increase Notch activity when expressed in a neighboring cell (trans-activation) but inhibit Notch when expressed in the same cell as Notch.
The Notch ligand DLL3 is unique in that it acts as a cis-inhibitor of Notch receptors expressed in the same cell but lacks trans-activating activity (25). Using CNS1 glioma cells, we observed that transient upregulation or knockdown of fibulin-3 resulted in opposite changes in the expression of DLL1 and DLL3 (Fig. 2A), suggesting that fibulin-3 mechanisms could involve effects on these proteins. In addition, purified fibulin-3 (not shown) as well as endogenous fibulin-3 consistently coprecipitated with DLL3 expressed in glioma cells (Fig. 2B).

To confirm whether fibulin-3 was competing with DLL3 we expressed both proteins in CNS1 cells carrying the Notch-responsive reporter (Fig. 2C). Overexpression of DLL3 did not reduce baseline Notch activity, but significantly inhibited Notch activation mediated by fibulin-3 or the canonical ligand Jagged-1. Together, these results suggested that the Notch-activating effect of fibulin-3 was likely mediated by down-regulating DLL3 or blocking the inhibitory effect of DLL3 over Notch.

Fibulin-3 regulates Notch activity and correlates with Notch activation in glioma

We next investigated whether the expression of fibulin-3 in gliomas effectively correlated with Notch activity. Overexpression of fibulin-3 in glioma cells resulted in significant upregulation of Notch-dependent genes such as Hes1 (Fig. 3A) and Hes5 (Fig. 3B), whereas siRNA-mediated knockdown of fibulin-3 downregulated both genes. More importantly, the effects of fibulin-3 downregulation were reproduced using GICs, suggesting that fibulin-3 levels can modulate Notch activity in this key population of tumor cells.

to determine whether the correlation between fibulin-3 levels and Notch activity was maintained in patient-derived specimens, we carried out double immunohistochemistry for...
Fibulin-3 Activates Notch Signaling in Gliomas

Fibulin-3 and Hes1 in 13 controls and 52 glioma specimens. Results showed that fibulin-3 and Hes1 were very low or absent in normal tissue (Fig 3C) but were coexpressed in tumor cells (not shown) and their expression increased with tumor grade (Fig. 3C). There was a significant positive correlation between fibulin-3 and Hes1 expression (Fig. 3D) except in a small subset of anaplastic oligodendrogliomas that had low fibulin-3 expression but high levels of Hes1. Interestingly, overexpression of NICD or knockdown of Notch-1 in glioma cells did not affect fibulin-3 expression (Supplementary Fig. S4), suggesting that fibulin-3 is not a Notch target. Therefore, the observed correlation in gliomas was most likely due to regulation of Notch signaling by fibulin-3. Taken together, these results underscored fibulin-3 as a marker of Notch activity and potential regulator of this pathway in glioma.

The proinvasive effect of fibulin-3 in gliomas is Notch-dependent

In a previous study, we showed that fibulin-3 promotes glioma invasion (22) but did not identify the underlying molecular mechanism. Therefore, we next asked whether the proinvasive role of fibulin-3 in gliomas would be mediated by Notch signaling.

To test this hypothesis we first analyzed cell migration in a Transwell assay using U251 and CNS1 cells. Fibulin-3 overexpression increased total cell migration, as expected, but this effect was abolished by DAPT (Fig. 4A). Because DAPT did not affect baseline migration, we concluded that Notch signaling was not necessary for basal cell motility but was required to mediate the promigratory effect of fibulin-3.

To further determine whether Notch was necessary for the proinvasive role of fibulin-3 we analyzed cell invasion through neural ECM, using glioma spheroids implanted in cultured brain slices. This is an extensively validated model (22, 30, 34) to study glioma cell invasion under conditions that accurately reproduce the natural barriers to cell motility in the brain. Using highly invasive CNS1 cells, we confirmed that the proinvasive effect of fibulin-3 could be suppressed by DAPT (Fig. 4B) or by transfection of the cells with Notch-1 siRNA (Fig. 4C).

We finally investigated the proinvasive effects of fibulin-3 on GICs, which are thought to be a key infiltrative population in gliomas (6). Knockdown of Notch-1 reduced GIC invasion through neural ECM and completely abolished the proinvasive effect of fibulin-3 (Fig. 4D). Together, these results support a novel role for Notch signaling as a proinvasive pathway in gliomas and the major underlying mechanism for the invasive effects of fibulin-3.

Fibulin-3 promotes glioma cell survival in a Notch-dependent manner

The Notch pathway is critically required for survival of glioma cells (35). Given our results showing the regulation of Notch signaling by fibulin-3, we next asked whether this ECM protein could regulate glioma cell viability, making it a potential chemosensitizing target.

To test this hypothesis, U251 and CNS1 cells were transfected with control or fibulin-3–specific siRNAs and subjected to serum starvation as a simple proapoptotic condition.

Figure 2. Fibulin-3 antagonizes the effect of DLL3. A, CNS1 cells were transfected with fibulin-3 cDNA, fibulin-3 siRNA, or their respective controls and processed for qRT-PCR after 24 hours. Overexpression or knockdown of fibulin-3 resulted in the opposite expression of DLL1 and DLL3 (**, P < 0.01; ***, P < 0.001, one-way ANOVA). B, CNS1 cells expressing endogenous fibulin-3 and V5-tagged DLL3 (DLL3-V5) were lysed and subjected to coimmunoprecipitation (IP) with anti-fibulin-3 or anti-V5 antibodies. Western blotting (WB) revealed a direct association between fibulin-3 and DLL3 (arrows). Asterisks indicate antibody bands. C, CNS1 cells stably expressing the Notch-responsive reporter were transfected with combinations of plasmids carrying Notch-1, DLL3, fibulin-3 (fib-3), or Jagged-1 (Jag-1) and analyzed after 24 hours. DLL3 did not affect the baseline reporter activity, but significantly blocked the enhancing effects of fibulin-3 and Jagged-1 (differences from control at *, P < 0.05; ***, P < 0.001, one-way ANOVA).
Transient knockdown of fibulin-3 was sufficient to increase the stress sensitivity of glioma cells, resulting in higher percentages of apoptotic (Annexin-V-positive) and necrotic (PI-positive) cells (Fig. 5A) as well as overall reduced viability in the cultures (Supplementary Fig. S5). Biochemical analysis of starved CNS1 cells showed that fibulin-3 knockdown increased the
expression of cleaved caspase-3, a typical marker of apoptosis (Fig. 5B). However, when fibulin-3 siRNA was cotransfected with increasing amounts of the NICD fragment, the upregulation of cleaved caspase-3 was significantly reduced upon starvation (Fig. 5C). This result suggested that the antiapoptotic effect of fibulin-3 was Notch-dependent and could be rescued by independent activation of Notch signaling.

To confirm whether fibulin-3 downregulation could be used as a chemosensitizing strategy, U251 cells were subjected to fibulin-3 knockdown followed by brief treatment with temozolomide, which is the standard-of-care cytotoxic agent for malignant gliomas. Downregulation of fibulin-3 significantly reduced cell viability in presence of temozolomide (Fig. 5D) and increased the percentages of apoptotic and necrotic cells in a time- and dose-dependent manner (Fig. 5E). This underscored the potential relevance of fibulin-3 downregulation for chemosensitizing strategies in gliomas.

**Downregulation of fibulin-3 reduces glioma stem cell viability and self-renewal**

We next attempted to generate glioma cells with stable knockdown of fibulin-3 but these cells showed deficient growth and cell clumping (Supplementary Fig. S5), possibly due to sustained negative effects on Notch signaling. Therefore, we used a lentiviral system for doxycycline-inducible expression of shRNAs (described in Materials and Methods and Supplementary Fig. S3). Lentiviruses carrying inducible shRNAs were used for stable transduction of glioma cells (U251 and CNS1) and GICs (G8 and G34).
Figure 5. Downregulation of fibulin-3 promotes apoptosis and is rescued by Notch-1. A, CNS1 and U251 cells transfected with fibulin-3 or control siRNAs were cultured for 24 hours in normal (FBS 10%) or serum-depleted (FBS 0.1%) conditions and analyzed by flow cytometry. There was a significant increase in the percentages of early apoptotic (Annexin-V-positive) and necrotic (PI-positive) cells following fibulin-3 knockdown and serum starvation (differences from control at *; P < 0.05; **; P < 0.001; 2-way ANOVA for each cell type). B, Western blotting of CNS1 cells showed increase of cleaved caspase-3 following fibulin-3 knockdown. C, CNS1 cells transfected with fibulin-3 siRNA and increasing amounts of NICD were cultured in serum deprivation. NICD reduced the increase of cleaved caspase caused by fibulin-3 downregulation. Numbers show the attenuation in increase of cleaved caspase (optical density fib-3 siRNA/control siRNA) as NICD increases. D and E, U251 cells were transfected with fibulin-3 or control siRNAs and treated with temozolomide (TMZ). D, viability of control cells without temozolomide was calculated as 100% for each day. E, in parallel wells, cells were labeled with Annexin-V/PI and counted each day. Results show increased chemosensitivity following fibulin-3 knockdown. Results in D and E, significant differences between fibulin-3 and control siRNA for each condition at *; P < 0.05; **, P < 0.01; ***; P < 0.001; multifactorial ANOVA.
Cultures transduced with shRNAs and maintained in absence of doxycycline showed similar growth curves (Fig. 6A). In contrast, induction of fibulin-3 shRNA caused a significant loss of viability in conventional glioma cells and GICs (Fig. 6A). We investigated the effect in GICs in further detail, asking whether fibulin-3 knockdown would affect the self-renewal ability of these cells. G8 and G34 GICs cultured in limiting dilutions after induction of fibulin-3 shRNA showed significantly reduced formation of tumor spheres, an effect that was more noticeable when the cells were processed for a second round of sphere formation (Fig. 6B). Visual inspection of the tumor spheres showed in many cases the formation of partially dissociated cell aggregates (Fig. 6C). These results strongly supported the prosurvival role of fibulin-3 in glioma cells.

**Downregulation of fibulin-3 reduces tumor growth and increases overall survival**

Finally, we determined whether controlled downregulation of fibulin-3 would affect tumor development in vivo. We implanted CNS1, G8, and G34 cells intracranially and induced shRNA expression after tumor implantation. The resulting gliomas showed efficient long-term downregulation of fibulin-3 and Notch-regulated genes in the tumor tissue (Supplementary Fig. S6). Histologic (Fig. 7A) and morphometric (Fig. 7B) analysis showed a significant reduction in size and dispersion of tumors with fibulin-3 knockdown compared with controls. This was not due to defects in cell proliferation because the proportion of Ki67-positive cells in fibulin-3-deficient tumors was not significantly different from controls (Supplementary Fig. S6). However, fibulin-3 knockdown resulted in a significant increase in the percentage of apoptotic cells (Supplementary Fig. S6), explaining the reduced growth. In agreement with these results, animals bearing tumors with controlled downregulation of fibulin-3 showed significantly extended survival compared with controls (Fig. 7C).

Our results from animal models agreed with data from human glioblastomas queried from the databases The Cancer Genome Atlas (TCGA) and Repository for Molecular Brain Neoplasia Data (REMBRANDT). Fibulin-3 was highly upregulated in the vast majority of samples from those databases (Supplementary Fig. S7). Nevertheless, survival analysis showed that the small subpopulation of patients with downregulated fibulin-3 had significantly longer overall survival (Fig. 7D). Furthermore, meta-analysis of high-grade gliomas grouped by transcriptional profile (Gene Expression Omnibus data set GSE4271; ref. 36) showed that fibulin-3 levels were always higher in gliomas identified as "mesenchymal" (Supplementary Fig. S7), which have poor overall survival compared with other molecular subtypes. Together, these results underscored the potential relevance of fibulin-3 for high-grade glioma classification and prognosis.

**Discussion**

Malignant gliomas are highly resistant to conventional radio- and chemotherapy, which, coupled to their invasive ability, facilitates tumor recurrence. Novel pharmacologic and molecular strategies are focused on targeting signaling...

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**Figure 6.** Controlled downregulation of fibulin-3 reduces tumor cell viability and self-renewal. A, CNS1 and G8 cells transduced with inducible fibulin-3 or control shRNAs were treated with doxycycline (Dox, 20 μg/mL) for 48 hours and analyzed for viability. Continuous fibulin-3 knockdown reduced viability in both cell types (*, P < 0.05; **, P < 0.01; 2-way ANOVA). B, GICs (G8 and G34) transduced with inducible shRNAs were dissociated, cultured at limiting dilutions (1–10 cells/well) in presence of doxycycline, and quantified for formation of neurospheres. Spheres from individual wells were dissociated and plated for a second passage. Fibulin-3 knockdown caused a significant loss of self-renewal (*, P < 0.05; **, P < 0.001; 2-way ANOVA for repeated measures). C, representative images of G8 cells after 2 weeks in presence of doxycycline. Fibulin-3 knockdown resulted in aberrant formation of neurospheres (arrows).
pathways to increase apoptotic sensitivity (37, 38) and improve the efficacy of adjuvant therapies. Importantly, accumulated evidence suggests that an invasive cellular phenotype is associated with increased chemoresistance (4, 6). Therefore, targeting proinvasive factors in glioma could contribute not only to reducing tumor dispersal but also to increasing the sensitivity of already dispersed malignant cells (37, 39).

Antinvasive treatments for glioma have targeted several potential candidates, including cytoskeletal proteins, cell-surface adhesion receptors, secreted proteases, and soluble paracrine/autocrine factors (40). However, there has been little direct evidence of how targeting these proinvasive factors could have a direct impact on tumor cell survival. Our results provide evidence of a molecule that is specifically expressed in...
Notch signaling may be activated by additional mechanisms, correlating better with tumor grade (10, 46), suggesting that downstream genes regulated by Notch (Hes1, Hes2, and Hey1) dysregulated in cancers (43), including gliomas (10). Notch in mammalian cells include the proteins MAGP-1/2, CCN3, and YB-1 (15). Of these, CCN3 and YB-1 have been shown to promote tumor growth (47, 48) but it has not been elucidated if they achieve this effect through Notch activation. In contrast, our results are the first to show a solubule factor in a cancer model that activates the Notch pathway and likely achieves its protumoral effects in gliomas by reducing cis-inhibition of Notch and increasing Notch signaling in tumor cells.

A possible mechanism of fibulin-3 as antagonist of DLL3 is particularly interesting because the role of this Notch ligand in cancer is poorly, if at all, defined. Current evidence indicates that DLL3 does not trans-activate Notch but inhibits this pathway when expressed in the same cell as the Notch receptor (25). It has been proposed that this could be partly due to the formation of a Notch–DLL3 complex retained in the endoplasmic reticulum (49), although the localization of DLL3 is still subject of debate (25) and has not been analyzed in cancer cells. Fibulin-3 could bind to and inhibit DLL3 intracellularly, or trigger DLL3 downregulation by additional mechanisms to activate Notch signaling.

Interestingly, DLL3 is a signature gene of high-grade gliomas defined as “proneural” by their transcriptional profile (36). These tumors are characterized by expression of typical markers of neural differentiation and have better prognosis than other molecularly defined subtypes. In contrast, both fibulin-3 and its close homologue fibulin-4 are expressed at much higher levels in mesenchymal gliomas than proneural tumors (Supplementary Fig. S7). The mesenchymal subtype can be clearly differentiated from proneural gliomas by its molecular signature and is characterized by increased angiogenesis and poorer prognosis. It is tempting to speculate whether increased expression of fibulins may upregulate Notch signaling in the tumor or its microenvironment (e.g., blood vessels) and contribute to tumor progression toward the more aggressive mesenchymal phenotype, which is also the predominant phenotype upon recurrence (36).

In conclusion, our results highlight fibulin-3 as an upstream marker of Notch signaling in gliomas and a novel Notch activator increasing tumor cell self-renewal, chemoresistance, invasion, and tumor growth. This evidence of Notch regulation by the tumor microenvironment opens a way to potential strategies for targeting this pathway more efficiently. Targeting fibulin-3 as part of anti-Notch strategies could restrict anti-invasive and chemosensitizing effects to the tumor cells without the pleiotropic effects of current pharmacologic inhibitors.
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