Sprouty2 Drives Drug Resistance and Proliferation in Glioblastoma

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Abstract

Glioblastoma multiforme (GBM) is notoriously resistant to therapy, and the development of a durable cure will require the identification of broadly relevant regulators of GBM cell tumorigenicity and survival. Here, we identify Sprouty2 (SPRY2), a known regulator of receptor tyrosine kinases (RTK), as one such regulator. SPRY2 knockdown reduced proliferation and anchorage-independent growth in GBM cells and slowed xenograft tumor growth in mice. SPRY2 knockdown also promoted cell death in response to coinhibition of the epidermal growth factor receptor (EGFR) and the c-MET receptor in GBM cells, an effect that involved regulation of the ability of the p38 mitogen-activated protein kinase (MAPK) to drive cell death in response to inhibitors. Analysis of data from clinical tumor specimens further demonstrated that SPRY2 protein is definitively expressed in GBM tissue, that SPRY2 expression is elevated in GBM tumors expressing EGFR variant III (EGFRvIII), and that elevated SPRY2 mRNA expression portends reduced GBM patient survival. Overall, these results identify SPRY2 and the pathways it regulates as novel candidate biomarkers and therapeutic targets in GBM.

Implications: SPRY2, counter to its roles in other cancer settings, promotes glioma cell and tumor growth and cellular resistance to targeted inhibitors of oncogenic RTKs, thus making SPRY2 and the cell signaling processes it regulates potential novel therapeutic targets in glioma.

Introduction

Glioblastoma multiforme (GBM) is the most common adult malignancy of the brain (1). Standard treatment includes surgical resection, radiotherapy, and chemotherapy, resulting in an average survival of just 12 to 15 months (2). Targeted approaches based on the molecular pathogenesis of GBM have shown some promise in clinical trials but have thus far failed to demonstrate significant benefit over traditional treatment approaches (3, 4). Significant challenges limiting the effectiveness of available treatments are the diffuse spreading of GBM tumors and heterogeneity in the expression of oncogenic proteins among GBM tumor cells (5, 6). The identification of broadly relevant regulators of tumorigenicity and therapeutic resistance is therefore necessary to improve patient outcomes. Here, we identify Sprouty2 (SPRY2) as one such protein.

SPRY2 is a regulator of receptor tyrosine kinase (RTK) signaling whose most well-characterized role is regulation of extracellular signal–regulated kinase (ERK) activity (7) and whose expression is promoted by ERK activity (8, 9). Studies in different systems have demonstrated varied, and sometimes conflicting, roles for SPRY2. SPRY2 inhibits ERK activity downstream of several RTKs through regulation of RAS, by preventing growth factor receptor–bound protein 2 (GRB2)-son of sevenless (SOS) binding (10) or by preventing RAS activation downstream of GRB2-SOS (11), or through regulation of RAF (7), depending on the cellular context. In contrast, SPRY2 has also been reported to potentiate epidermal growth factor (EGF)–induced ERK activation by interfering with CBL-mediated EGF receptor (EGFR) downregulation (12). Downstream of the fibroblast growth factor receptor, SPRY2 specifically inhibits ERK activation without effect on p38 or JUN N-terminal kinase (JNK) activation (7), two other members of the mitogen-activated protein kinase (MAPK) family. However, results showing that interferon-stimulated p38 phosphorylation is enhanced in SPRY1/SPRY2/SPRY4 knockout murine embryonic fibroblasts (13) could indicate an ability for SPRY2 (or other Sprouty family members) to regulate other MAPKs more broadly.

SPRY2 expression has been reported to be reduced in cancers of the breast, prostate, lung, and liver compared with normal tissues, and SPRY2 expression can suppress the proliferative and...
tumorigenic capacity of hepatocellular carcinoma and non–small cell lung cancer cell lines (14–16). These and other findings have led some investigators to propose SPRY2 as a tumor suppressor. However, as with its signaling regulatory functions, SPRY2 expression patterns and SPRY2–mediated control of key phenotypes also appear to be context dependent. In colon cancer cells, for example, SPRY2 expression appears to be elevated compared with normal tissue and to promote metastasis (17). In terms of regulating response to therapy, SPRY2 expression promotes response to EGFR inhibition in colon cancer cells (18) but drives resistance to EGFR inhibition in non–small cell lung cancer cell lines (19). In GBM, little has been done to investigate the functional role of SPRY2. There has been one report of SPRY2 downregulation via microRNA-21 expression in high-grade gliomas (20). Based on data shown here, however, SPRY2 protein is expressed at functionally meaningful levels in glioblastoma primary tumor samples and cell lines.

Here, we investigated the function and expression of SPRY2 in GBM cell lines, tumor xenografts, and human tumor samples. In a panel of GBM cell lines, SPRY2 knockdown reduced proliferation, antagonized colony formation in soft agar, and potentiated response to coinhibition of EGFR and c-MET (the receptor for hepatocyte growth factor). In some cell lines, SPRY2 control of cell death response to inhibitors involved regulation of the ability of p38, which was phosphorylated in response to EGFR and c-MET coinhibition, to promote cell death. In a mouse tumor xenograft model, SPRY2 knockdown significantly impaired tumor growth, confirming the relevance of SPRY2 in vivo. Analysis of gene expression from primary human tumor samples and rat tumor allografts revealed that SPRY2 mRNA was upregulated in EGFR variant III (EGFRvIII)–positive tumors compared with EGFRvIII-negative tumors. This is of interest because EGFRvIII is present in 41% to 67% of GBMs with EGFR amplification (21–23), promotes an invasive and proliferative GBM cell phenotype (24, 25), and is associated with especially poor prognosis for patients with EGFR amplification (26). Immunohistochemical analyses confirmed SPRY2 protein expression in human GBMs with or without EGFRvIII expression, with a tendency for greater SPRY2 expression in EGFRvIII–positive tumors. Analysis of SPRY2 protein from patient-derived xenograft and glioma stem cell (GSC) samples demonstrated SPRY2 expression levels at least as high as those found in GBM cell lines where SPRY2 knockdown produced substantial phenotypic effects. Through an analysis of The Cancer Genome Atlas (TCGA) GBM dataset, we further produced substantial phenotypic effects. Through an analysis of The Cancer Genome Atlas (TCGA) GBM dataset, we further

Materials and Methods

Cell culture

U87MG cells expressing EGFRvIII or kinase-dead EGFRvIII (27, 28) and parental U87MG cells were provided by Dr. Frank Furnari [University California, San Diego (UCSD), La Jolla, CA]. U251 and SF188 cells were gifts from Dr. Gary Kuo and Craig Thompson (University of Pennsylvania, Philadelphia, PA), respectively. LN18 and U118MG cells were obtained from the American Type Culture Collection. All cells were maintained in DMEM supplemented with 10% fetal bovine serum, 1 mmol/L l-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies).

Knockdown of SPRY2, MKP-1, and MKP-5

Oligonucleotides encoding hairpins targeting nucleotides 2061–2079 (main sequence used; shRNA #1) or 1195–1213 (shRNA #2) of human SPRY2, nucleotides 2041–2059 of human MKP-1, or nucleotides 935–953 of human MKP-5 were purchased from IDT and inserted into pSicoR.puro (Dr. Tyler Jacks, MIT, Cambridge, MA; ref. 29). A control shRNA was created using a hairpin that does not target a known human mRNA. Lentivirus was produced by calcium phosphate–mediated transfection of 293FT cells (Life Technologies) with the pSicoR.puro plasmid as well as the pCMV-VSVg, pMDL-gp-RRE, and pRSV-Rev plasmids (Dr. Marilyn Farquhar, UCSF). Virus-containing supernatant was filtered and target cells were selected in 1 to 2 μg/mL puromycin (Sigma).

Retroviral protein expression

Y55F SPRY2 cDNA (Dr. Dafna Bar-Sagi, New York University, New York, NY) and MKK3 cDNA (Dr. Margaret Chou, University of Pennsylvania) were subcloned into pBABE.hygro. For all constructs, retrovirus was produced by calcium phosphate–mediated transfection of amphotropic Phoenix cells (Dr. Gary Nolan, Stanford University, Stanford, CA). Virus-containing supernatant was filtered, and target cells were selected in 150 μg/mL hygromycin B (Sigma) or 2 μg/mL puromycin (Sigma).

Mouse tumor xenografts

Eight female NIH-III mice (Charles River) were subcutaneously injected in each flank with 2 million control (left side) or SPRY2–depleted (right side) U87MG-L cells. Tumors were measured with calipers starting 7 days after cell injection (when measurable tumors had formed) and every 2 to 3 days thereafter. Tumor volumes were calculated as π/6 × A × B², where A and B are the larger and smaller tumor diameters, respectively. After final caliper measurements were made, animals were sacrificed and dissected tumors from the 6 animals bearing measurable control and SPRY2 knockdown tumors were weighed. Note that in 2 of the 8 animals injected with cells, SPRY2 knockdown cells failed to form apparent tumors; no tumors were dissected from these animals. All experiments were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and performed in accordance with NIH guidelines.

Western blotting

Whole-cell lysates were prepared and Western blotting was performed as described previously (19). Details, including information on antibodies used, are provided in Supplementary Methods.

Flow cytometry

Floating and adherent cells were pooled and stained with ToPro3 (Life Technologies). Cells were analyzed using a Becton Dickinson FACSCalibur cytometer.

Proliferation measurements

To measure cellular proliferation, cells were seeded at 50,000 cells per well in 6-well plates and counted with a hemocytometer 5 days later.
Inhibitors
Gefitinib, U0126, SB203580, SP600125 (all from LC Laboratories), and PHA665752 (Santa Cruz Biotechnology) were reconstituted in DMSO and added to cells in complete media.

Quantitative real-time PCR
RNA was extracted from cells using the RNeasy kit (Qiagen) with on-column Dnase I digestion. Equal amounts of RNA were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). PCR was performed using SYBR Green PCR Master Mix (Life Technologies) on an Applied Biosystems 7300 Real-Time PCR System. Relative amounts of mRNA were determined using the comparative C_t method.

Anchorage-independent growth assay
Five thousand cells were seeded per 35-mm dish in 0.3% low melting temperature agarose (Lonza) on top of a bottom layer of 0.6% agarose. Media was replaced every 3 days with or without inhibitors. At 3 weeks, plates were stained with 0.1% crystal violet (Sigma) and colonies were counted.

Immunohistochemistry
GBM sections were obtained from samples banked through the University of Pennsylvania Brain Tumor Tissue Bank, with the EGFRvIII status of individual tumors determined by PCR. Immunohistochemistry of formalin-fixed and paraffin-embedded tissues was performed on a Leica Bond instrument using the Bond Polymer Refine Detection System using the SPRY2 antibody. Heat-induced epitope retrieval was required for SPRY2 and done with 10 minutes with ER1 solution (Leica Microsystems).

Analysis of TCGA GBM dataset
Methods for preprocessing of TCGA exon-array data and analysis to determine EGFRvIII status are described in Supplementary Methods. A two-sided Student t test was applied to determine whether SPRY2 expression varied among GBM subtypes and normal brain samples using TCGA exon-array data. A student t test was also used to determine whether SPRY2 was differentially expressed between EGFRvIII-positive and EGFRvIII-negative samples based on TCGA RNA-seq samples. The upper quartile normalized RSEM (30) count estimates were base-10 log transformed before the t test. The R package “survival” was used to analyze the TCGA survival data (31). The log rank test was applied to test for differences between the survival curves.

Statistical analysis
Data were analyzed by a Student t test, and differences with P < 0.05 were considered statistically significant.

Accession numbers
Microarray data presented in this study were deposited in the National Center for Biotechnology Information's GEO database (GSE51062, GSE51147).

Results
SPRY2 knockdown slows cellular proliferation, reduces anchorage-independent growth, and enhances cellular sensitivity to EGFR and c-MET coinhibition
Using a set of common GBM cell lines, including U251, U118MG, and U87MG cells as well as U87MG cells engineered with "low" (U87MG-L, ∼1 x 10^5 EGFR/cell) or "high" (U87MG-H, ∼2 x 10^5 EGFR/cell) ectopic EGFRvIII expression or high expression of a control dead kinase EGFRvIII (U87MG-DK; K721M; ref. 27), we sought to investigate the effects of SPRY2 expression on several cellular phenotypes. SPRY2 was depleted by stable shRNA expression in the cell lines, with knockdown validated by Western blot analysis (Supplementary Fig. S1). SPRY2 knockdown reduced proliferation rates in U87MG-DK, U87MG-L, U251, and U118MG cells grown on tissue culture plastic (Fig. 1A). Colony formation in soft agar was also reduced by >50% in U87MG-L, U87MG-H, U251, and U118MG cells with SPRY2 knockdown compared with controls (Fig. 1B). A second independent shRNA expressed in a subset of the cell lines produced similar effects on proliferation and anchorage-independent growth (Supplementary Fig. S2A and S2B). Reductions in colony formation were also observed in a panel of EGFRvIII-expressing cell lines transiently transfected with SPRY2-targeting shRNA (Supplementary Fig. S2C). Note that colony formation data are not available for U87MG-DK cells because that cell line does not form colonies in soft agar.

Despite frequent EGFR overexpression and mutation in GBM, EGFR kinase inhibitors are not clinically effective (22, 32–34). GBM cell lines are also generally resistant to EGFR kinase inhibitors, but coinhibition of c-MET can augment response (22, 27, 35). We tested whether SPRY2 knockdown affected cellular response to EGFR and c-MET coinhibition using the EGFR inhibitor gefitinib and the c-MET inhibitor PHA665752. In multiple cell lines, cell death in response to gefitinib and PHA665752 increased significantly with SPRY2 knockdown (Fig. 1C). In one of the cell lines from Fig. 1C, we probed for markers of apoptosis and found that SPRY2 knockdown also promoted cleavage of poly-ADP ribose polymerase (PARP) in response to EGFR and c-MET coinhibition (Fig. 1D). The same general effect on response to EGFR and c-MET inhibitors was observed in parental U87MG cells with stable SPRY2 knockdown and U87MG-DK cells with transient SPRY2 knockdown (Supplementary Fig. S3A and S3B). Because the expression of functional phosphatase and tensin homolog (PTEN) has been shown to regulate GBM cell response to EGFR inhibition (22, 36), we also investigated whether PTEN status influenced the effects of SPRY2 knockdown. Ectopic expression of wild-type PTEN in U87MG cells, which do not express PTEN, did not reverse the cellular sensitivity to gefitinib and PHA665752 observed with SPRY2 knockdown (Supplementary Fig. S3C). In two cell lines that express wild-type PTEN, LN18 and SF188, SPRY2 knockdown also increased death response to the inhibitors (Supplementary Fig. S3D). Consistent with the effects of SPRY2 knockdown, expression of dominant-negative Y55F SPRY2 in U87MG-DK cells also enhanced cellular sensitivity to EGFR and c-MET coinhibition (Fig. 1E). This effect also repeated in multiple cell lines expressing the second nonoverlapping SPRY2-targeting shRNA (Supplementary Fig. S3E). Enhanced response to EGFR and c-MET inhibitors due to SPRY2 knockdown was also observed in a GSC cell line maintained in nonadherent sphere culture conditions (Supplementary Fig. S4).

SPRY2 controls p38’s ability to regulate anchorage-independent growth and response to inhibitors
In multiple cellular settings, SPRY2 regulates signaling through the ERK pathway (7, 10, 11). In the panel of U87MG cells, SPRY2 knockdown produced a small increase in basal ERK phosphorylation in U87MG-DK cells, but did not affect ERK phosphorylation in
cells expressing active EGFRvIII (Fig. 2A), where SPRY2 knockdown also produced phenotypic effects. Although SPRY2 has been reported to specifically inhibit ERK among the MAPK pathways (7), a recent study found enhanced p38 phosphorylation in SPRY1/SPRY2/SPRY4 knockout murine embryonic fibroblasts (13). We thus hypothesized that JNK and/or p38 MAPKs could be affected by SPRY2 knockdown. Indeed, basal JNK and p38 phosphorylation were increased in U87MG cells with SPRY2 knockdown compared with controls (Fig. 2A). We did not generally observe similar basal increases in p38 or JNK phosphorylation in other GBM cell lines with SPRY2 knockdown (with the exception of p38 in U118MG), but we did observe clearly increased p38 and JNK phosphorylation in U87MG-DK, U87MG-L, U118MG cells expressing DK EGFRvIII and transduced with an empty vector (EV) or Y55F SPRY2 were probed by Western blot analysis using antibodies against the indicated proteins. Cells were also treated with DMSO or G+P (10 μmol/L gefitinib + 5 μmol/L PHA665752) for 48 hours prior to flow cytometry analysis for ToPro3 permeability. Throughout the panels, data are represented as the average of three independent experiments ± SEM; asterisks, P < 0.05.

Figure 1. SPRY2 knockdown reduces cellular proliferation and anchorage-independent growth and enhances cellular sensitivity to EGFR and c-MET coinhibition. A, cellular proliferation and (B) colony formation in soft agar were measured with expression of control or SPRY2-targeting shRNA (shRNA #1) in a panel of GBM cell lines, including U87MG cells, expressing dead kinase (DK), low (L), or high (H) EGFRvIII. C, the indicated cell lines expressing control or SPRY2-targeting shRNA (shRNA #1) were treated with DMSO or a combination of gefitinib and PHA665752 (G+P; 10 μmol/L gefitinib + 5 μmol/L PHA665752 for U87MG-DK, U87MG-L, U118MG; 10 μmol/L gefitinib + 2 μmol/L PHA665752 for U251) for 48 hours prior to flow cytometry analysis for ToPro3 permeability. D, U87MG cells expressing DK EGFRvIII and control or SPRY2-targeting shRNA (shRNA #1) were treated with DMSO or G+P (20 μmol/L gefitinib + 2 μmol/L PHA665752) for 72 hours. Whole-cell lysates were probed by Western blot analysis using antibodies against the indicated proteins. E, whole-cell lysates of U87MG cells expressing DK EGFRvIII and transduced with an empty vector (EV) or Y55F SPRY2 were probed by Western blot analysis using antibodies against the indicated proteins. Cells were also treated with DMSO or G+P (10 μmol/L gefitinib + 5 μmol/L PHA665752) for 48 hours prior to flow cytometry analysis for ToPro3 permeability. Throughout the panels, data are represented as the average of three independent experiments ± SEM; asterisks, P < 0.05.
SPRY2 knockdown inhibits tumor xenograft growth

To determine the functional role of SPRY2 in an in vivo model, female NIH-III mice were injected subcutaneously with U87MG-L cells expressing control or SPRY2-targeting shRNA. Tumors arising from SPRY2 knockdown cells were significantly smaller than those arising from control cells throughout the course of the experiment (Fig. 4). Strikingly, SPRY2 knockdown cells failed to give rise to discernible tumors in 2 of 8 mice. At 16 days after injection, the average volume and weight of tumors from SPRY2 knockdown cells was approximately one-quarter of that for control tumors.

SPRY2 protein is expressed in human GBM, and elevated SPRY2 mRNA is present in EGFRvIII-positive tumors

To confirm the presence of SPRY2 protein in human tumors, we analyzed tissue from banked GBMs expressing or lacking EGFRvIII by immunohistochemical staining. To validate our antibody for immunohistochemistry, SPRY2 protein expression was first probed in sections of kidney and cerebellum (Supplementary Fig. S7). In GBM samples, all EGFRvIII-positive and -negative tumors analyzed showed the presence of SPRY2 protein. In some cases, SPRY2 staining was strong throughout the tumor section, while in others SPRY2 staining was localized in patches. In areas of SPRY2 expression, staining patterns included moderate focal, moderate granular, and strong diffuse staining (Fig. 5A). A semi-quantitative H-score analysis revealed a tendency for more intense SPRY2 staining in sections of EGFRvIII-positive GBM (Supplementary Table S1), in qualitative agreement with the results of the transcript analysis discussed below. To further assess SPRY2 protein expression in samples that should correspond well with what would be observed in the tumor environment, we probed for SPRY2 by Western blot analysis in samples of patient-derived xenografts, GSC cell lines, and a neurosphere culture (samples kindly provided by Dr. Frank Furnari, UCSF; Fig. 5B). SPRY2 expression in those samples was comparable to or greater than that observed in representative GBM cell lines where we observed phenotypic effects of SPRY2 knockdown or to that observed in a lung carcinoma cell line (H1666) where we previously documented effects of SPRY2 knockdown (19).

Given that EGFRvIII-positive tumors tend to be especially aggressive and that SPRY2 appears to promote proliferation and resistance to therapy, we went on to analyze EGFRvIII-dependent gene signatures in primary human GBM tumors and orthotopic rat tumor allografts to probe for any evidence that SPRY2 mRNA might be upregulated in EGFvIII-positive tumors. We first examined gene expression in 52 primary GBM tumors, which were stratified on the basis of the presence or absence of EGFRvIII expression using EGFRvIII-specific RT-PCR (41). Extracted RNA samples from these tumors were subjected to microarray analysis using HG-U133 2.0 Plus Affymetrix gene chips. This analysis revealed 355 gene probes whose expression was significantly increased (P < 0.05) by at least 1.5-fold in EGFRvIII-positive tumors compared with EGFRvIII-negative tumors. For the analysis in rats, 12 animals received intracranial injections of 9L rat gliosarcoma cells expressing EGFRvIII or an empty vector control. Three weeks after implantation, tumors were analyzed for gene expression profiling using the RatRef-1 Illumina chip array. This analysis revealed 1498 gene probes that were increased (P < 0.05) by at least 1.5-fold in EGFvIII-expressing tumors compared with empty vector control tumors. These gene probes were aligned against the gene probes upregulated in EGFvIII-positive human tumors. SPRY2 was one of nine upregulated genes that were shared by human EGFvIII-expressing GBMs and rat 9L EGFvIII-expressing tumors (Supplementary Table S2), suggesting the possibility that SPRY2 expression may be elevated in EGFvIII-positive GBM.

On the basis of the rat and human tumor transcript analysis, we proceeded to analyze SPRY2 expression patterns in a panel of GBM cell lines. Consistent with the documented ability of ERK to regulate SPRY2 expression (8, 9), we observed a positive correlation between SPRY2 expression and ERK phosphorylation that was supported by results of pharmacologic or genetic perturbations (Supplementary Fig. S8A–S8D). Among cell lines...
Figure 2. p38 and JNK phosphorylation are basally increased in some cells with SPRY2 knockdown and are uniformly increased in cells cotreated with EGFR and c-MET inhibitors, and SPRY2 regulates the ability of p38 to promote cell death and antagonize proliferation. A, whole-cell lysates from U87MG cells expressing dead kinase (DK), low (L), or high (H) EGFRvIII and control shRNA (−) or SPRY2-targeting shRNA (+; shRNA #1) were probed by Western blot analysis using antibodies against the indicated proteins, and blots were analyzed by densitometry. (Continued on the following page.)
engineered to express EGFRvIII, we also observed good correlation between SPRY2 expression and ERK phosphorylation, but we did not observe a consistent trend between EGFRvIII and SPRY2 expression (Supplementary Fig. S8E). The apparent lack of SPRY2/EGFRvIII expression correlation may arise because of the limited number of isogenic cell backgrounds tested or because the method used to drive EGFRvIII expression in cell lines differs drastically from the way that EGFRvIII expression is regulated in GBM (42). Whatever the explanation for the discrepancy, the results in this section provide overall support for the notion that SPRY2 expression correlates with functionally relevant levels in tumors with or without EGFRvIII expression. In the future, a more detailed analysis of potential SPRY2 protein expression differences between EGFR-negative and EGFRvIII-positive settings should be undertaken using tumor tissues.

Analysis of TCGA data reveals that elevated SPRY2 expression correlates with EGFRvIII expression, the classical GBM subtype, and reduced patient survival

We probed the TCGA GBM dataset to ask if SPRY2 expression correlates with EGFRvIII expression in a larger set of patient samples and to determine whether SPRY2 expression correlates with previously defined GBM tumor subtypes and patient survival. TCGA samples were classified by EGFRvIII expression status based on RNA-seq data, which was available for 161 samples. Forty-one of 161 samples (25%) were EGFRvIII-positive. SPRY2 expression was increased in EGFRvIII-positive samples compared with EGFRvIII-negative samples (P = 1.00 × 10^-5), with a fold difference of 1.49 (Fig. 6A), consistent with the results in Supplementary Table S2. On the basis of the definitions of four clinically relevant GBM subtypes determined by gene signatures...
and using TCGA GBM exon-array data (173 "core" representative samples), we further found that SPRY2 expression was lower in the proneural subtype than the other three subtypes ($P = 8.06 \times 10^{-5}$ and fold difference of 0.60; Fig. 6B).

SPRY2 expression was highest in the classical subtype ($P < 2.20 \times 10^{-14}$ and fold difference of 1.58 for comparison with other subtypes), which exhibits a high rate of EGFR amplification and mutation.

We also determined the relationship between SPRY2 expression and patient survival (Fig. 6C), using median SPRY2 expression as a cutoff between low and high expression. Across all patients, low SPRY2 expression was associated with reduced mortality compared with high SPRY2 expression ($P = 0.0123$), with median survival of 631 or 451 days for low or high SPRY2 expression, respectively. Because age is an established important prognostic factor in GBM (44, 45), we further looked at the effect of SPRY2 expression in patients stratified by age. Parsing patients into age groups using the median age (59 years) as a cutoff, we found that the effect of SPRY2 expression differences was more pronounced in the younger patient cohort ($P = 0.000416$), with median survival of 631 or 451 days for low or high SPRY2 expression, respectively, and that there was no effect of SPRY2 expression on survival in the older cohort. The difference in survival was even further increased using an age cutoff of 40 yrs ($P = 0.00316$), with median survival of 1024 or 538 days for low or high SPRY2 expression, respectively (Supplementary Fig. S9).

The larger difference in survival for patients 40 years of age and younger may be observed because younger patients survive longer overall, allowing for a greater time to observe the effects of differential SPRY2 expression.

Our findings that SPRY2 expression is highest in the classical subtype and that elevated SPRY2 expression portends reduced patient survival need not be viewed as in conflict with the finding by Verhaak and colleagues (43) that patients in the classical subtype responded best to "more intensive treatment," defined by the authors as "concurrent chemo- and radiotherapy or more than three subsequent cycles of chemotherapy," versus patients undergoing nonconcurrent therapies or short regimens. Our analysis does not parse patients by therapeutic approach. Of course, the analysis of Verhaak and colleagues is fully consistent with our findings in that overall median survival was highest for the proneural subtype, where we found the lowest SPRY2 expression.
Discussion

The basic findings of our study are summarized schematically in Fig. 7. In GBM cell lines with or without EGFRvIII expression, we found that SPRY2 surprisingly acted as a driver of GBM cell proliferation, anchorage-independent growth, and resistance to inhibition of RTKs that promote GBM survival. SPRY2 also promoted the growth of subcutaneous tumor xenografts, demonstrating the relevance of SPRY2 in vivo. In at least some cell lines, SPRY2 exerted control over phenotypes by regulating the ability of p38 to influence cell outcomes. In one cell line context, U87MG cells, SPRY2 control over p38 and JNK phosphorylation occurred at least, in part, through regulation of MKP1 and MKP5 expression, which altered baseline p38 and JNK phosphorylation. However, the ability of SPRY2 to control the effects of p38 activity on cell phenotypes in cell lines where baseline p38 or JNK phosphorylation levels were not altered by SPRY2 knockdown suggests that the ability of SPRY2 to regulate GBM cell phenotypes is likely to be more complex than we have uncovered thus far. Through analysis of human GBM samples, rat tumor allografts, and lysates from patient-derived xenografts and GSCs, we determined that SPRY2 protein is definitively expressed in human GBM with or without EGFRvIII expression and that SPRY2 expression is elevated in tumors expressing EGFRvIII, which tend to be especially aggressive. Moreover, elevated SPRY2 transcript expression is associated with reduced patient survival and the classical GBM subtype. Importantly, the somewhat modest elevation of SPRY2 expression in EGFRvIII-positive tumors should not be misinterpreted to suggest that SPRY2 control of phenotypes is relegated to the EGFRvIII-positive setting. Indeed, our data suggest that SPRY2 exerts important control over cell fate decisions in GBM cells with or without EGFRvIII expression. In aggregate, our findings point to SPRY2 and key pathways it regulates as potential therapeutic targets and biomarkers in GBM.

The finding that SPRY2 promotes growth and resistance to inhibition of RTKs in the GBM cell context was initially surprising given SPRY2’s purported role as a tumor suppressor in hepatocellular carcinoma, lung cancer, breast cancer, and prostate cancer (14–16). Although SPRY2 appears to negatively regulate tumor growth in those settings, an oncogenic role for SPRY2 has recently been demonstrated in colon cancer, where elevated SPRY2 expression is a marker of poor prognosis (17, 46). Because the net outcome of SPRY2 expression and its mechanism of action appear highly cell context-dependent, additional work is clearly needed to clarify the determinants of SPRY2’s function in different contexts and to develop strategies to interrupt SPRY2’s functions in GBM and other cancers where it may promote growth and resistance to therapy.

Another question raised by our results is how SPRY2 expression might be promoted in EGFRvIII-positive tumors, as suggested by our analyses of human and rat tumor transcripts and our analysis of human GBM sections by immunohistochemistry. Our data show that SPRY2 protein expression correlates well with ERK phosphorylation across many GBM cell lines and with multiple genetic and pharmacologic perturbations. It has been suggested, however, that EGFRvIII-driven tumors do not activate canonical EGFR downstream signaling pathways such as ERK, STAT3, and AKT (47). It is possible that tumor microenvironment features altered by EGFRvIII expression allow for increased ERK activation, or that an alternative pathway not explored here promotes SPRY2 expression in GBM. If it is true that ERK regulates SPRY2 expression in vivo, MEK inhibition could be a useful approach to downregulate SPRY2 expression in GBM. Posttranscriptional regulation of SPRY2 by miR-21 (20, 48) could also potentially complicate the relationship between SPRY2 transcript and protein levels in some settings. Whether or not such regulation occurs in vivo as a means to suppress SPRY2 expression in gliomas relative to normal tissue, as suggested by one study (20), our aggregate...
results are clear that meaningful levels of SPRY2 protein are expressed in tumors with or without EGFRvIII expression.

Although the best-studied role of SPRY2 is regulation of the ERK pathway, our results suggest an important ability of SPRY2 to regulate GBM cell phenotypes by regulating p38 and JNK activities and/or the effects of those activities on cellular behaviors. We interpret the results of Fig. 2 to indicate that JNK activity is required for normal GBM cell proliferation and anchorage-independent growth, but that p38 activity antagonizes anchorage-independence growth and promotes cellular response to EGFR and c-MET co-inhibition. Because p38 inhibition mitigated the effects of SPRY2 knockdown on death response to EGFR and c-MET co-inhibition even in a cell line where SPRY2 knockdown did not generate basal increases in p38 phosphorylation (U251) p38 activation may only be a necessary step along the path leading to apoptosis. SPRY2’s absence (or reduced expression) may potentiate this effect of p38 activity through specific mechanisms yet to be identified. In that sense, our findings of altered MKP1 and MKP5 expression with SPRY2 knockdown in U87MG cell lines may not represent a broadly relevant mechanism for regulating the impact of p38 activity in glioma cell lines and tumors.

Looking forward, SPRY2 and the pathways it regulates should be assessed for their value as therapeutic targets or as prognostic markers for response to therapy in GBM. Although it is not presently possible to target SPRY2 with small-molecule inhibitors, it may eventually be possible to target SPRY2 through RNAi-based therapies, whose delivery to the brain may be enabled through nanocarrier delivery methodologies that are in ongoing development. Through the more thorough dissection of the signaling pathways that are broadly regulated by SPRY2 to enable its ability to govern glioma cell and tumor phenotypes, it may also eventually be possible to identify druggable targets whose pharmacologic inhibition phenocopies the effects of SPRY2 depletion.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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