Chromatin Remodeling Factor BRG1 Regulates Stemness and Chemosensitivity of Glioma Initiating Cells

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Abstract

Glioblastoma multiforme (GBM) is a highly aggressive and malignant brain tumor that is refractory to existing therapeutic regimens, which reflects the presence of stem-like cells, termed glioma-initiating cells (GICs). The complex interactions between different signaling pathways and epigenetic regulation of key genes may be critical in the maintaining GICs in their stem-like state. Although several signaling pathways have been identified as being dysregulated in GBM, the prognosis of GBM patients remains miserable despite improvements in targeted therapies. In this report, we identified that BRG1, the catalytic subunit of the SWI/SNF chromatin remodeling complex, plays a fundamental role in maintaining GICs in their stem-like state. In addition, we identified a novel mechanism by which BRG1 regulates glycolysis genes critical for GICs. BRG1 downregulates the expression of TXNIP, a negative regulator of glycolysis. BRG1 knockdown also triggered the STAT3 pathway, which led to TXNIP activation. We further identified that TXNIP is an STAT3-regulated gene. Moreover, BRG1 suppressed the expression of interferon-stimulated genes, which are negatively regulated by STAT3 and regulate tumorigenesis. We further demonstrate that BRG1 plays a critical role in the drug resistance of GICs and in GIC-induced tumorigenesis. By genetic and pharmacological means, we found that inhibiting BRG1 can sensitize GICs to chemotherapeutic drugs, temozolomide and carmustine. Our studies suggest that BRG1 may be a novel therapeutic target in GBM. The identification of the critical role that BRG1 plays in GIC stemness and chemosensitivity will inform the development of better targeted therapies in GBM and possibly other cancers.

Keywords

BRG1; STAT3; Glioblastoma; Gene expression; Tumorigenesis

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INTRODUCTION

Glioblastoma multiforme (GBM) is a highly aggressive and malignant brain tumor that is refractory to current therapeutic regimens. Despite advanced treatment strategies and improved molecular characterization, the prognosis for GBM patients continues to be dismal and the mean patient survival remains <2 years. A population of glioma-initiating cells (GICs) has been identified in GBM tumors, which can self-renew and give rise to the heterogeneous mixture of cells in the developing tumor. GICs possess potent tumor-initiating capacity and are considered to be responsible for the drug resistance associated with GBM [1]. The maintenance of GICs in a stem-like state requires the precise interplay of signaling pathways and transcriptional networks, which underscores the possible involvement of epigenetic factors in maintaining stem cell-like identity of GICs. Here we describe the role of Brahma-related gene 1 (BRG1), one of the catalytic subunits of the SWI/SNF chromatin remodeling complex, in the maintenance of stem cell identity and tumorigenic potential of GICs.

The SWI/SNF complex is an evolutionary conserved multi-subunit complex that is critical for gene regulation, differentiation, DNA repair, and development. The two catalytic subunits, BRM (also called BRAMA, SMARCA2) and BRG1 (also called BRAHMA RELATED GENE 1, SMARCA4), provide energy from ATP hydrolysis required to reposition and/or remodel nucleosomes at targeted loci to open or close chromatin and regulate transcription [2, 3]. The importance of BRG1 in embryonic development was demonstrated by the finding that BRG1 knockout in mice was embryonically lethal at the peri-implantation stage [4], and BRG1-negative embryonic stem (ES) cells have not yet been isolated. BRG1 promotes pluripotency of both mouse and human ES cells, and targeted knockdown of BRG1 induces cell differentiation [4,5]. In ES cells, the SWI/SNF or BAF complex has a unique composition (esBAF), and expression of the esBAF components in fibroblasts causes a reversal to a pluripotent state [5, 6]. Furthermore, the SWI/SNF complex not only enhances the efficiency of ESC pluripotency induced by the forced expression of Oct4, Sox2, KLF4, and c-Myc [7], but also can replace c-Myc in this pathway [7].

Interestingly, BRG1 is the sole catalytic subunit in the esBAF complex [8–10]. BRG1 binds to the promoters of various pluripotency specific genes such as Oct4, Nanog, and the polycomb group (PcG) of genes [5]. BRG1 has been found to have tumor suppressing [11,12] and tumor promoting activity [13,14] in a highly cancer-context-specific manner. BRG1 has been identified as a major tumor suppressor with silencing or loss-of-function mutations enriched in cancers such as lung, ovaries, skin, thoracic sarcoma, and lymphoma [12,15–18]. However, BRG1 mutations are relatively rare in GBM [19], and BRG1 appears to be overexpressed in glioma as compared to adjacent normal brain tissue [20]. Interestingly, high BRG1 expression was found to be associated with poor prognosis in many types of tumors and more aggressive tumors in several types of cancer using data from The Cancer Genome Atlas (TCGA) and other databases [21]. Although other BAF components have been found to regulate GIC stemness [22], the precise role of BRG1 in GICs remains poorly understood. In this article, we demonstrate that the BRG1 subunit of the human SWI/SNF complex is essential for maintaining the stem-like identity of GICs. Through targeted microarrays, we uncovered a novel mechanism by which BRG1 regulates

the expression of glycolysis and glucose transport genes in GICs by attenuating the expression of TXNIP, a negative regulator of glycolysis [23,24]. We also determined that BRG1 knockdown led to upregulation of the STAT3 signaling pathway and the subsequent downregulation of interferon-stimulated genes (ISGs). Moreover, we demonstrate that TXNIP is an STAT3-regulated gene. Inhibiting BRG1 by genetic and pharmacologic means promotes GIC differentiation and chemosensitizes GICs to temozolomide (TMZ) and carmustine. Our studies suggest that BRG1 may be a novel, druggable therapeutic target in GBM.

MATERIALS AND METHODS

Cell Culture

GBM6, GBMX10, and GBMX16 patient-derived xenolines (PDXs) were maintained as xenografts in immunocompromised mice [25]. GICs were isolated and maintained in flasks precoated with poly-D-lysine and laminin, and grown in NeuroBasal-A medium (Invitrogen, Carlsbad, CA) containing 2% B27 supplement, 2 mM L-glutamine, 100 units/ml penicillin, 100 g/ml streptomycin, EGF (20 ng/ml), and basic FGF (40 ng/ml).

Knockdown of BRG1 and TXNIP

pLKO.1 plasmids containing gene-specific or scrambled shRNA (control) were used to knockdown (KD) BRG1 or TXNIP expression (Open Biosystems, Thermo Scientific, Lafayette, CO). Lentivirus was produced by packaging in 293FT cells [26]. BRG1-KD or TXNIP-KD GICs were generated by transduction with shRNA-encoding lentivirus, selected with 5 μg/ml puromycin, and stable pools maintained without puromycin.

Inducible STAT3 Knockdown (iSTAT3-KD) and Restoration of STAT3 Expression

The doxycycline-inducible STAT3 knockdown system was used as described previously [27].

Cell Proliferation Assays

GICs were seeded in 96-well plates (4,000 cells/well) and the number of viable GICs was measured using the CellTiter-Glo luminescence viability assay (Promega, Madison, WI). TMZ was purchased from Sigma (St Louis, MO), carmustine from Toronto Research Chemicals (Toronto, Canada), and PFI-3 from Tocris (Minneapolis, MN).

Gene Expression Analysis

Total RNA was extracted using the QIAshredder and RNeasy mini kits (Qiagen Inc., Frederick, MD). Gene expression was determined by quantitative real-time PCR (qPCR) using gene-specific primers (Supporting Information, Table 1) and an iScript one-step RT-PCR kit with SYBR Green (Bio-Rad). Reaction parameters were as follows: cDNA synthesis at 50°C for 20 minutes, transcriptase inactivation at 95°C for 5 minutes, and PCR cycling at 95°C for 10 seconds and 60 °C for 30 seconds for 40 cycles.

Immunoblotting

Protein extracts (50 μg) were prepared from GICs in urea-based lysis buffer as previously described [28,29], separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore, Burlington, MA), and immunoblotted with the following antibodies: BRG1 and BRM (Proteintech, Rosemont, IL), TXNIP (Abcam, Cambridge, MA), STAT3 (BD Biosciences, San Jose, CA), pY705-STAT3 and GFAP (Abcam), CDK1, CDK2, Rb, Rb 107, Rb 110 (Cell Signaling, Danvers, MA), and Actin (Santa Cruz, Dallas, TX). Following addition of IRDye800CW goat anti-mouse IgG or IRDye680 goat anti-rabbit IgG, blots were visualized on an Odyssey infrared imaging system (LICOR Biosciences, Lincoln, NE).

Immunohistochemistry

Cells were grown in 8-well chamber slides (Millipore, Burlington, MA) precoated with poly-D-lysine and laminin to ~50% confluency and fixed with 4% paraformaldehyde and permeabilized with Triton X-100. After blocking with 5% goat serum, cells were incubated with antibodies against GFAP (Molecular Probes, Carlsbad, CA), S100B (Abcam, Cambridge, MA), and Olig2 (Abcam, Cambridge, MA) and subsequently stained with Alexa Fluor 488 or 594 (Molecular Probes, Carlsbad, CA). Images were captured on a laser-scanning confocal microscope (Zeiss model LSM700, San Diego, CA).

Tumor Xenografts in Mice

All animal experiments were performed with at least 5 mice in each experimental group in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the University of Tennessee Health Science Center. GICs were dissociated with HyQTase, resuspended in PBS, and enumerated on a cellometer (Nexelcom, Lawrence, MA). Heterotopic tumor xenografts were established in 5-week-old male NOD.Cg-$Prkdc^{scid}$ $Il2rgtm1Wjl/SzJ$ (NSG) mice (Jackson Laboratory, Bar Harbor, ME) by injecting control and BRG1-KD GICs expressing luciferase (10^6 cells/100 μl PBS) into the flanks. Tumor burden was assessed weekly by palpation with slide caliper measurement, and live animal imaging following luciferin injection and bioluminescence was analyzed with Living Image software (IVIS, Perkin Elmer, Waltham, MA) [30]. After confirming tumor initiation, TMZ was administered at 10 mg/kg of mice on alternate days by intraperitoneal (IP) administration. For orthotopic xenograft studies, luciferase-expressing GICs (5×10^5 cells in 100 μl PBS) were injected stereotactically into the superficial brain parenchyma through a burr hole in the skull, and tumor burden was assessed as previously described [31]. Tumor growth was analyzed using Graphpad Prism 7 software (La Jolla, CA). At 3 weeks following injection, animals were sacrificed.

Chromatin Immunoprecipitation (ChIP)

ChIP analysis was carried out using the ChIP-IT™ Express Enzymatic kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. In brief, chromatin was cross-linked with 1% formaldehyde (10 minutes at 22°C), sheared to an average size of ~200 bp by sonication (Bioruptor Pico, Diagenode, Denville, NJ), and immunoprecipitated with anti-STAT3 (Santa Cruz Biotechnology). The ChIP-PCR primers were designed to amplify a

proximal promoter region containing putative binding sites on the TXNIP promoter using ExPASy and the JASPAR database.

Nanostring Gene Expression Analysis

Total RNA from X16 and the X16 BRG1-KD cells was extracted using the QIAshredder and RNeasy mini kits (Qiagen Inc., Frederick, MD). Nanostring arrays were conducted with the PanCancer Immune Panel on the nCounter Analysis system (NanoString, Seattle, WA), and the data (GEO accession number GSE116545) were analyzed with nSolver software using a twofold cutoff value as previously described [32].

Data Analysis

At least three independent experiments were performed in triplicate, and data are presented as mean ± SEM. Analysis of variance or Student's t tests were performed and p 0.05 was considered to be statistically significant.

RESULTS

BRG1 Subunit of the SWI/SNF Complex is Expressed in GICs

We assessed the expression of the catalytic subunits of the SWI/SNF complex in a panel of GICs obtained from independent GBM tumors (GBM6, X10, and X16), which all have high tumor-initiating activity [27]. Immunoblotting with a BRG1-specific antibody showed that BRG1 was robustly expressed in all three GICs (Fig. 1A). In contrast, BRM was detected in both GBM6 and X16 GICs, but not in X10 GICs (Fig. 1A). The expression of BRG1 in all the three GICs indicates that BRG1, and not BRM, may be an essential component for GIC function. This finding is consistent with BRG1 but not BRM being essential for the maintenance of pluripotency in hESCs and mESCs [4,5], and that BRG1 is the only catalytic component found in the embryonic stem cells (esBAF) SWI/SNF complex [4,5,9].

BRG1 is Critical for Maintaining GIC Sternness

To elucidate the role of BRG1 in GBM6 and X16 GICs, we then generated BRG1-KD GICs by transducing GICs with lentivirus encoding scrambled or BRG1-shRNA RNAs. After puromycin selection, stable pools of GICs were maintained without puromycin. Immunoblotting showed decreased BRG1 expression in BRG1-KD cells, but BRM levels were unaffected (Fig. 1B). In contrast, BRG1 and BRM levels were not reduced in scrambled-shRNA treated cells (Fig. 1B). We then examined the effect of BRG1-KD on expression of the esBAF components in GICs, and found that BRG1-KD had subtle effects on some components of the esBAF complex in a GIC-specific manner (Fig. 1B), such as attenuating PBRM1 levels in GBM6 (40% decrease) but not in X16 GICs, and attenuating BAF155 levels in both GBM6 and X16 GICs (40%–50% decrease). Since GICs express a number of stem cell markers (CD44, OCT4, and Nanog), we also determined the effect of BRG1-KD on the expression of these marker genes by qPCR and found that BRG1-KD significantly attenuated their expression in both GBM6 and X16 GICs (Fig. 1C). Since we found that GBM6 and X16 GICs can be induced to differentiate into cells of the astrocytic lineage [33], we evaluated the expression of astrocytic marker, Glial fibrillary acidic protein (GFAP), following BRG1-KD by immunoblotting. BRG1-KD in both GBM6 and X16 GICs

significantly upregulated GFAP levels (Fig. 1D). In addition, BRG1-KD in GICs also increased expression of astrocytic marker S100B and oligodendroglial marker Olig2, as determined by immunohistochemistry (Fig. 1E). Taken together, these data strongly suggest BRG1 is critical for maintaining GICs in a stem-like, undifferentiated state.

BRG1-KD GICs Show Accelerated Cellular Proliferation in vitro and Produce Larger Intracranial Tumors

BRG1 plays an important role in cell cycle regulation and proliferation of hESCs [4,6]. We next examined the role of BRG1 on GIC proliferation by CellTiter-Glo assay, which quantifies the number of viable cells. BRG1-KD GBM6 and X16 GICs showed a marked increase in cell proliferation (more than twofold increase in cell number after 7 days) when compared to control GICs (Fig. 2A). The increased proliferation of BRG1-KD GICs was consistent with BRG1 maintaining GICs in a more quiescent dormant state; hence, BRG1-KD cells were more actively dividing. Previous studies show that BRG1 binds directly to the retinoblastoma (RB) protein to inhibit cell proliferation [34], and that BRG1 loss is associated with the inactivation of the function of the RB family members, p107 and p130, through their hyperphosphorylation. To elucidate the underlying mechanism by which BRG1 regulates GIC growth, we next analyzed the phosphorylation state of Rb1, p107, and p130. We observed increased p107 and p130 phosphorylation in the BRG1-KD GICs (Fig. 2B), but no change in Rb1 phosphorylation (data not shown). Additionally, Cyclin A2 expression increased in BRG1-KD GBM6 and X16 GICs, while CDK1 levels remained unchanged (Fig. 2C). Taken together these results suggest that BRG1 restrained GIC proliferation.

We next determined the biological role of BRG1 in vivo by examining the tumorigenic potential of BRG1-KD and control GICs by intracranial injection of luciferase-labeled cells into immunocompromised NSG mice. Tumor progression was determined at weekly intervals by live animal imaging after luciferin injection. BRG1-KD GICs produced tumors more rapidly (Fig. 2D) and the tumors were larger at the end-point of the study (Fig. 2E) as determined by bioluminescence. In addition, at necropsy BRG1 expression in BRG1-KD tumors was verified to be reduced in the tumor tissue by qPCR (Fig. 2F). Since BRG1-KD resulted in GFAP and S100B upregulation in vitro (Fig. 1D, 1E), we also evaluated GFAP and S100B expression in tumor tissue by immunohistochemistry. BRG1-KD X16 GIC-induced tumors had markedly higher GFAP and S100B protein expression as compared to the EV tumors, and Ki67 (a marker for proliferation) staining was also much higher in BRG1-KD tumors (Fig. 2G). These results suggest that BRG1 restrains GIC differentiation and proliferation in vivo and in vitro.

BRG1 Regulates an STAT3/TXNIP Pathway

To assess the role of BRG1 on cancer-related gene pathways in GICs, we conducted targeted microarrays on X16 BRG1-KD GICs and scrambled-shRNA transduced GICs serving as a control. Interestingly, as illustrated in the heat maps (Fig. 3A), multiple IFN-simulated genes (ISGs) were downregulated in BRG1-KD GICs, including *CXCL11, IRF7,* and *IL6,* whose differential expression were validated by qPCR (Supporting Information, Fig. S1). We recently demonstrated that these ISGs were negatively regulated by STAT3 in GICs [27],

suggesting that BRG1 might regulate a STAT3-dependent pathway. A functional link between STAT3 signaling and BRG1/SMARCA4 is further supported by protein–protein interaction (PPI) analysis of the BRG1-regulated genes (Supporting Information, Fig. S2), as well as by previous studies [35–37]. BRG1-KD resulted in increased expression of LIF (STAT3 pathway activator [42]) and decreased SOCS1 (STAT3 pathway repressor [38,39]) in GICs, which was validated by qPCR (Fig. 3A and Supporting Information, Fig. S1). Thus, these results provide further evidence that BRG1 regulates a STAT3-dependent pathway. In addition, TXNIP (Thioredoxin-interacting protein) expression was markedly upregulated in BRG1-KD GICs (Fig. 3A and Supporting Information, Fig. S1). TXNIP is involved in redox regulation and acts as a negative regulator of glycolysis [23,40–43]. To assess whether BRG1 regulated a STAT3 pathway leading to TXNIP upregulation, lysates from control (scrambled shRNA), and BRG1-KD GBM6 and X16 GICs were prepared and immunoblotted. As shown in Figure 3B, BRG1 levels were markedly reduced in BRG1-KD GBM6 and X16 GICs, while levels of pY705-STAT3 and TXNIP were increased. Since our data indicate that BRG1 alters TXNIP expression which regulates cellular glucose uptake [44–46], we then examined the effect of BRG1-KD on several genes encoding enzymes involved in glucose transport and glycolysis (GLUT1, FBP2, and PKM) and found BRG1-KD resulted in a significant downregulation of these genes (Fig. 3C). To determine whether TXNIP directly regulated the expression of these genes in GICs, we knocked down TXNIP expression in the BRG1-KD X16 GICs (Fig. 3D), and found that it restored their expression (Fig. 3E). Taken together, our data suggest that BRG1 may maintain glucose availability in GICs by downregulating TXNIP expression.

To directly determine the role of STAT3 on TXNIP expression in GICs, X16 and STAT3-KD GICs were examined for TXNIP expression. STAT3-KD reduced TXNIP gene (Fig. 4A) and protein expression (Fig. 4B) in the STAT3-KD GICs as compared to control GICs. Most importantly, rescue of STAT3 expression in STAT3-KD GICs restored TXNIP expression, indicating that STAT3 regulates TXNIP expression in GICs (Fig. 4A, 4B). Similar results were also observed in the X10 GICs (Supporting Information, Fig. S3). We then identified three putative STAT3 binding sites on the TXNIP promoter, and by chromatin immunoprecipitation (ChIP) assays with the GBM6 and X16 GICs found that STAT3 binds to the TXNIP promoter at these sites in the BRG1-KD GICs (Fig. 4C, 4D). BRG1 was previously reported to be essential in STAT3-mediated gene transcription [5,35,47], and our results suggest TXNIP expression in GICs is STAT3-dependent and lies downstream of BRG1, providing a mechanism of how BRG1 negatively regulates TXNIP expression in GICs.

We then related our findings on BRG1-regulated gene expression in GICs to gene expression patterns of GBM patient tumor specimens in the TCGA database. Among the molecular features defined by the PanCancer Atlas molecular classification [48,49], we found that BRG1 gene expression levels were positively correlated to the stemness index defined by mRNA expression patterns (mRNAsi) and to the IFN-score (Fig. 5), which assess the degree of oncogenic dedifferentiation [48] and activation of IFN response pathway, respectively [49]. These observations provide further evidence in supporting for a role of BRG1 in the regulating of genes involved in maintaining cancer sternness and the IFN response of GBM.

BRG1-KD Sensitizes X16 GICs to TMZ in vitro and In Vivo

Although the alkylating agent TMZ is front-line chemotherapy in the treatment of GBM [50], a significant clinical problem is intrinsic or adaptive resistance to TMZ [50]. As such, we next sought to determine if GICs are resistant to TMZ. To evaluate for TMZ sensitivity of GBM6 or X16 GICs, cells were treated with 300 and 500 pM TMZ, and cell proliferation was determined in Cell Titer-Glo assays. GBM6 GICs were highly sensitive to both TMZ concentrations and TMZ resulted in a marked reduction in cell viability (Fig. 6A). In contrast, X16 GICs were highly resistant to the effects of TMZ (Fig. 6A). TMZ resistance in GBM is linked to O^6-methylguanine methyltransferase (MGMT) expression, which is a DNA repair protein [50,51]. To assess the involvement of MGMT in the TMZ sensitivity of GICs, we next determined MGMT mRNA and protein expression and found significantly higher MGMT expression in X16 GICs as compared to GBM6 GICs (Fig. 6B, 6C). The SWI/SNF complex plays an important role in facilitating DNA repair [52–55], and BRG1 has been implicated in DNA double-strand break repair [53–55]. Thus, we hypothesized that BRG1-KD in GICs would prevent the repair of TMZ-induced DNA damage and sensitize GICs to TMZ. The X16 BRG1-KD GICs were treated with 500 pM TMZ and displayed significantly higher sensitivity to TMZ (Fig. 6D). Moreover, BRG1-KD in X16 GICs resulted in a reduction in MGMT expression consistent with their sensitization to TMZ (Fig. 6E).

To determine whether BRG1-KD sensitized GICs to TMZ in vivo, we conducted animal studies with control (scrambled shRNA) and BRG1-KD X16 GICs injected into the flanks of NSG mice. Following confirmation of tumor initiation by live animal imaging, mice were treated with TMZ. At 1 and 2 weeks posttreatment, TMZ markedly reduced tumor growth in BRG1-KD GICs (Fig. 6F), and at necropsy, the tumors were markedly smaller (Fig. 6G, 6H). Thus, despite X16 BRG1-KD GICs forming larger tumors, the tumors had a more differentiated phenotype and were sensitized to TMZ therapy.

Pharmacologic Inhibition of BRG1 Sensitizes X16 GICs to TMZ

PFI-3 is a competitive inhibitor of the SWI/SNF catalytic components with higher affinity for BRG1 than BRM [56]. We examined the effect of PFI-3 on TMZ sensitivity and found that the combination of TMZ and PFI-3 had a markedly greater effect on X16 GIC proliferation than either agent alone (Fig. 7A). Since BRG1-KD upregulated GFAP expression, we also examined the effect of PFI-3 on GFAP expression in X16 GICs and found that PFI-3 upregulated GFAP protein expression (Fig. 7B). The X10 GICs have been isolated from another PDX which was TMZ-resistant. Both X10 and X16 GICs have high MGMT expression compared to the GBM6 GICs (Fig. 6B, 6C). Most importantly, although X10 GICs were highly resistant to TMZ, the combination of PFI-3 and TMZ had a marked effect on cell proliferation (Fig. 7C). Similar to the findings with TMZ, although X10 and X16 GICs were resistant to carmustine (another DNA alkylating agent used to treat GBM patients [57]), a combination treatment of carmustine and PFI-3 rendered the X10 and X16 GICs sensitive to the drugs (Fig. 7D).

Discussion

In GBM, the subpopulation of neural stem-like GICs is considered responsible for tumor initiation and progression, inherent and acquired resistance to therapy, and intratumoral heterogeneity. Therefore, it is imperative to identify and elucidate the mechanisms deregulated in GICs. The SWI/SNF complex of chromatin remodelers are global regulators of gene expression, which are recruited to the promoters and enhancers of genes across the genome [58–61]. This process alters the positioning of histones and enables the recruitment of transcription factors that activate gene transcription [62,63]. The SWI/SNF complex plays critical roles during embryonic development, and regulates stem cell renewal and differentiation [9,64]. Of the two catalytic components, BRG1 is the sole catalytic component found to be expressed in the ESCs [5,8–10]. In the present study, we investigated the role of BRG1 in the maintenance and function of GICs. We found that the BRG1 protein was broadly expressed in GICs isolated from multiple GBM patients. In contrast, BRM was expressed in only some GICs. This observation suggests that BRG1 is a critical component in GICs and that BRM maybe dispensable, since all GICs studied display characteristics of stem cells and have high tumor-initiating capability.

Loss of the different components of the SWI/SNF complex has been implicated in several cancers but its role appears to be highly context dependent. In some cancers, loss of SWI/SNF complex components leads to tumor progression, while in others, it leads to tumor suppression [65–69]. To determine the role of BRG1 in GICs, BRG1 expression was knocked down by an shRNA-based approach in BRG1-expressing GICs. Similar to the findings described in ESCs [4,6], BRG1 loss in GICs significantly reduced stem cell markers (CD44, Oct4, and Nanog) and induced differentiation markers (GFAP, S100, and Olig2). CD44 expression has been previously shown to be BRG1-regulated [70,71]. These findings indicated that BRG1 is involved in maintaining GIC stemness; hence, BRG1 loss leads to a more differentiated state. We next examined whether BRG1 loss alters other components of the BAF complex. We observed subtle modulation of several members of the BAF complex, including BAF155 and PBRM1, in a GIC-specific manner. Thus, BRG1 loss has effects on the BAF complex, which may regulate stem cell identity in GICs due to destabilization of the BAF complex or aberrant activity of the residual complex [61,72,73].

Although BRG1-KD in ESCs and in GICs has been reported to reduce proliferation [4,6,22], BRG1 loss also has been shown to increase the proliferation of some cell lines [67,74–77]. Consistent with the latter reports, we found that BRG1-KD enhanced proliferation of both GBM6 and X16 GICs. Moreover, BRG1 loss resulted in hyperphosphorylation of the p107 and p130 members of RB-like proteins, which is consistent with reports that BRG1 mediates cell growth inhibition through the Rb pathway [34,76]. Since p107 controls G1/S phase transition, while p130 controls G1/G0 progression [78,79], BRG1 loss in association with p107 and p130 inactivation may promote proliferation of BRG1-KD GICs. Furthermore, BRG1 loss also led to increased expression of Cyclin A2, which interacts with CDK1/2 and promotes G1/S and G2/M cell cycle transition [80]. Taken together our findings strongly suggest that BRG1 plays an important role in restraining GIC proliferation, which may also contribute to GIC drug resistance since chemotherapeutic drugs target proliferating cancer cells [1,81]. Consistent with the finding that BRG1 loss increased GIC proliferation in vitro,

BRG1-KD GICs injected orthotopically into the brains of mice showed accelerated tumor growth. Although BRG1-KD tumors were larger and more proliferative (as indicated by Ki67 staining), the tumors were more differentiated since they had high GFAP, S100B, and Olig2 expression, which is consistent with the finding that BRG1 loss led to an increased ESC differentiation [5].

To elucidate the molecular pathways that BRG1 regulates in GICs, we conducted targeted microarrays with cancer-related gene panels on RNA prepared from X16 and the X16 BRG1-KD cells, and identified several BRG1-regulated genes, whose altered expression was validated by qPCR and immunoblotting. An interesting finding was that BRG1 loss upregulated *TXNIP* expression in GBM6 and X16 GICs. TXNIP is a major redox regulator that negatively regulates thioredoxin, an ROS scavenger [42,82]. TXNIP also blocks glucose uptake in cells by targeting the glycolytic pathway [42,82]. TXNIP has been implicated as a tumor suppressor, and TXNIP downregulation is associated with tumor aggressiveness [43,82,83]. Cancer stem cells (CSCs) have been found to have lower mitochondrial respiration and higher glucose requirements [81]. By modulating mitochondrial respiration, CSCs regulate ROS levels which are important in resistance to DNA damage [84]. Studies have further demonstrated that high glucose levels are critical for CSC survival and maintenance [85]. This led us to hypothesize that BRG1 regulates the expression of genes implicated in the glucose requirement of GICs, which was substantiated by the observation that BRG1-KD resulted in the downregulation of glucose transporter GLUT1, and FBP2 and PKM of the glycolytic pathway [86–88]. We found that silencing of TXNIP in BRG1-KD GICs restored GLUT1, FBP2, and PKM expression, indicating that BRG1 promotes the expression of these genes in GICs through the TXNIP-regulated pathway. Interestingly, BRG1 has been linked to cancer cell metabolism through reprogramming lipid synthesis in breast cancer cells [89]. In addition, BRG1 regulates the glycolytic pathway in cardiomyocytes, providing another link of BRG1 to cell metabolism [90].

BRG1 loss in GICs also downregulated ISG expression, and we recently showed that ISG expression was negatively regulated by a STAT3 pathway in GICs [27]. These results suggest an interaction of the STAT3 pathway with BRG1. PPI hub analysis of our microarray data provided additional evidence that BRG1 loss leads to activation of the STAT3 pathway. Furthermore, BRG1-KD in GICs increased LIF expression but decreased SOCS1 expression, which together would consequently result in STAT3 pathway activation. We also confirmed that BRG1-KD resulted in increased tyrosine-phosphorylated and total STAT3 protein levels in both the GBM6 and X16 GICs. The BRG1 subunit of the esBAF complex employs STAT3 in driving pluripotency genes such as Sox2, Oct4, and Nanog and is associated with the PRC2 complex to inhibit the expression of the Hox gene clusters that are essential for differentiation [5]. However, loss of BRG1 allows the PRC2 complex to silence the BRG1-mediated transcription of the stem cell markers [5]. Our findings suggest that increased STAT3 activity in BRG1-KD GICs contributed toward their differentiation, which is consistent with the findings in ESCs where BRG1 drives the transcription of the pluripotent genes through STAT3, and BRG1 loss allows STAT3 to induce transcription of genes involved in differentiation [5]. Furthermore, our findings also implicate STAT3 in regulating TXNIP expression. STAT3-KD in GICs reduced TXNIP expression, while rescue with STAT3 restored TXNIP expression. ChIP analysis showed increased STAT3 binding to

several sites on the TXNIP promoter in BRG1-KD GICs. These observations provide further insights into how BRG1 promotes GIC stem cell maintenance and inhibits differentiation. We found that BRG1-KD in GICs increased the STAT3 signaling pathway, which mediates the transcription of the genes promoting stem cell differentiation and proliferation. In addition, STAT3 induces the transcription of TXNIP, which then blocks increased glucose uptake essential for GIC maintenance. Interestingly, we have also observed an increase in TXNIP expression in xenograft tumors of BRG1-KD cells (Supporting Information, Fig. S4).

The SWI/SNF complex regulates various DNA repair pathways, including DNA double-strand breaks by enabling homologous recombination repair, and BRG1 loss sensitizes cancer cells to cisplatin and UV radiation [53,55]. The DNA alkylating agent TMZ is front-line chemotherapy in GBM patients following tumor resection. However, a significant clinical problem is inherent and acquired resistance of GBM patients to TMZ therapy. TMZ resistance is in part due to the expression of the DNA repair protein MGMT [91,92]. We found that while GBM6 GICs were sensitive to TMZ, X16 GICs were relatively TMZ-resistant and that MGMT expression in the GBM6 and X16 GICs correlated with TMZ resistance. Most importantly, BRG1 loss resulted in decreased MGMT expression and sensitized X16 GICs to TMZ treatment. Furthermore, although tumors produced by X16 BRG1-KD cells were larger, the tumors were more sensitive to TMZ treatment than the tumors produced by control GICs. Additionally, although BRG1-KD in vitro and in vivo promotes GIC proliferation and differentiation, BRG1-KD GICs are sensitized to chemotherapy, which is consistent with previous findings that BRG1 loss sensitizes cancer cell lines to chemotherapy [67,93]. GICs are considered responsible for chemoresistance and radioresistance in GBM, and increased DNA repair capacity [1]. We also used the small molecule inhibitor of the SWI/SNF catalytic subunits PFI-3 to pharmacologically inhibit BRG1 function. Interestingly, PFI-3 treatment previously was not found to phenocopy the effect that BRG1/BRM knockdown has on growth inhibition of various tumor cell lines [94]. We treated TMZ-resistant X16 GICs with PFI-3 and TMZ, and found significant cell growth inhibition compared to TMZ treatment alone. Furthermore, PFI-3 treatment attenuated MGMT expression. X10 GICs, which are BRM-deficient and MGMT-positive, were also TMZ resistant, but were sensitized by a combination of PFI-3 with TMZ. Similar to our findings with TMZ, PFI-3 also sensitized GICs to carmustine, another DNA alkylating agent. Our findings provide strong evidence that BRG1 plays critical roles in the drug-resistance of GICs. Through a process, yet unknown, BRG1 regulates MGMT expression in GICs, which in turn promotes drug resistance. Future studies will be directed toward understanding the BRG1/ MGMT-mediated drug resistance pathway in GBM which will be critical for developing improved therapeutic interventions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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SIGNIFICANCE STATEMENT

Glioma-initiating cells (GICs) are implicated in tumor heterogeneity and therapeutic resistance of GBM. Results of this study show that the BRG1 subunit of the SWI/SNF chromatin remodeling complex plays critical roles in maintaining GICs in a stem-like state. Furthermore, the authors identified a novel mechanism by which BRG1 regulates expression of TXNIP, which is a redox regulator and also negatively regulates glycolysis. It was found that BRG1 regulates TXNIP through a STAT3-dependent pathway and that BRG1 plays a critical role in the drug resistance of GICs and in GIC-induced tumorigenesis. Thus, the BRG1-STAT3-TXNIP axis is a potential therapeutic target in GBM.
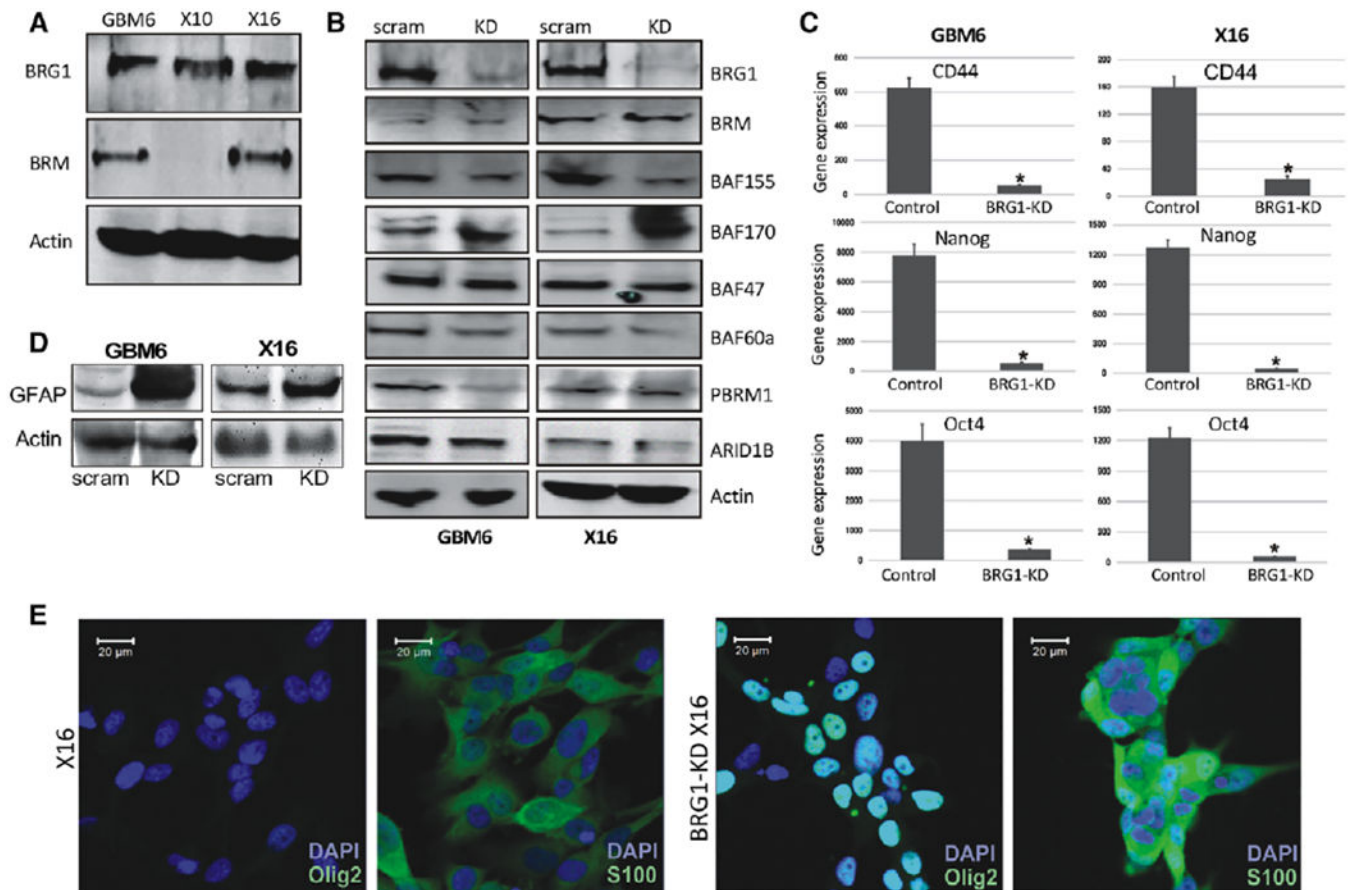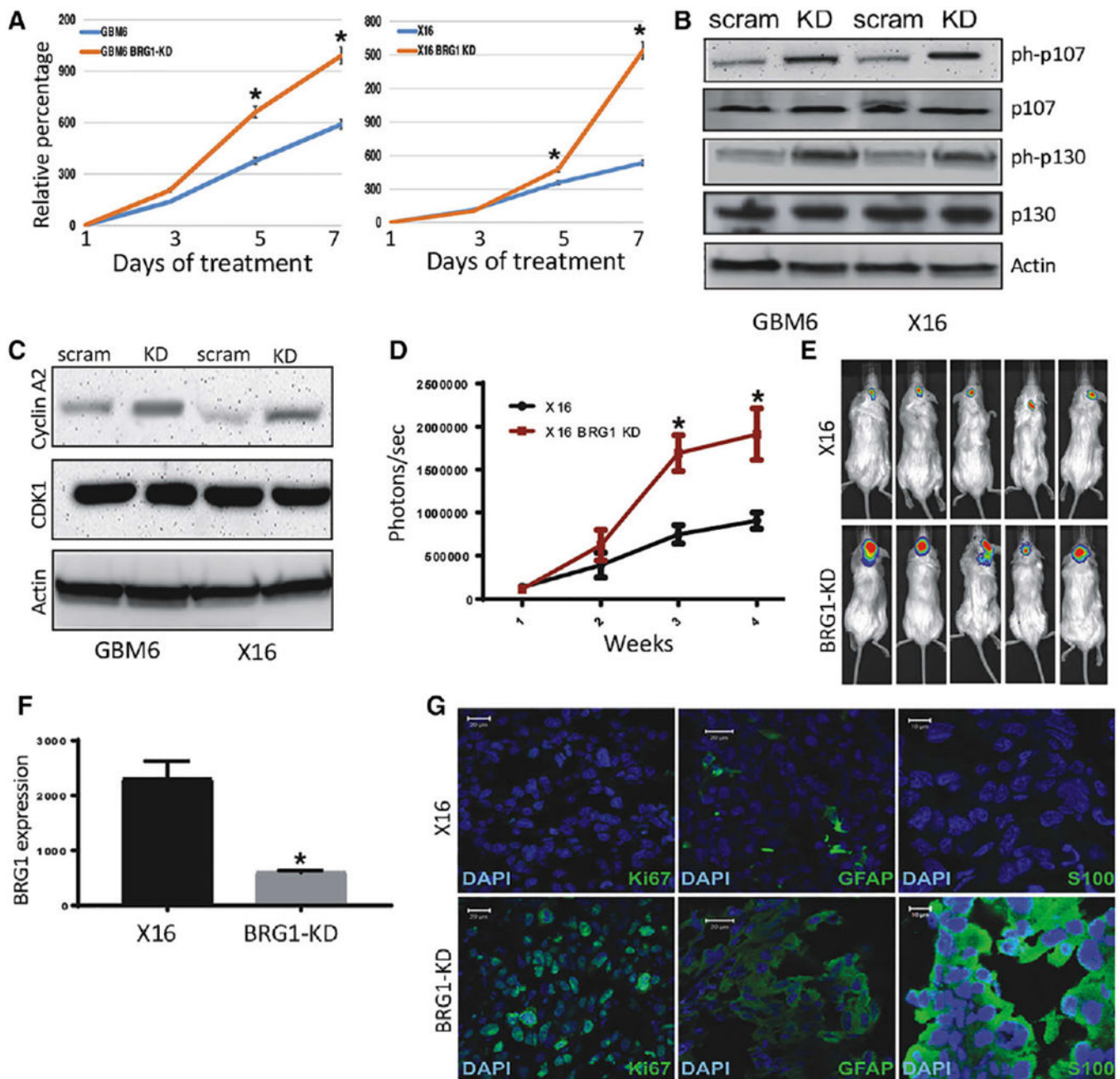
Figure 1.
Effect of BRG1-KD in GICs on BAF components and stem cell markers. **(A):** Protein lysates from GBM6, X10, and X16 GICs were immunoblotted for BRG1 and BRM. **(B):** Protein lysates from BRG1-KD GBM6 and X16 GICs were immunoblotted for core and accessory subunits of the BAF complex. The quantitation of the signals in GBM6 and X16 GICs versus BRG1-KD cells relative to actin, respectively, were BRG1 (0.1, 0.1); BAF155 (0.6, 0.5); BAF170 (1.7, 2.2); BAF47 (1.1, 1); BAF60A (0.9, 0.8); PBRM1 (0.6, 1); ARID1B (0.9, 1). **(C):** RNA was prepared from control and BRG1-KD GBM6 and X16 GICs, and CD44, Nanog and Oct4 expression was determined by qPCR. **(D):** Protein lysates from GBM6 and X16 GICs were immunoblotted for GFAP. **(E):** Immunohistochemistry for Olig2, S100B in X16 and X16 BRG1-KD GICs. A scale bar of 20 pm is provided for reference. $*p$ .05 was considered to be statistically significant.

Figure 2.
Effect of BRG1-KD on proliferation in vitro and tumorigenicity of GBM6 and X16 GICs.
(A): Proliferation of BRG1-KD GBM6 and X16 GICs was determined by CellTiter-Glo assays. **(B,C):** Protein lysates from control and BRG1-KD X16 GICs were immunoblotted for **(B)** phosphorylated and total Rb protein family members, and **(C)** Cyclin A2 and CDK1. **(D-G):** Tumorigenicity was assessed by injection of 10^6 tumor cells into the brains of NSG mice and live animal imaging was performed at weekly intervals. **(D):** Quantification of the bioluminescence signal detected at 1, 2, and 3 weeks postinjection. **(E):** Representative bioluminescent images of mice at 21 days postinjection. **(F):** RNA was prepared from

control and BRG1-KD X16 GIC-induced tumors and BRG1 gene expression was determined by qPCR. **(G):** Immunohistochemistry for GFAP, S100B, and Ki67 in X16 and X16-BRG1 KD tumor tissue. A scale bar of 20 μm is provided for reference. *p .05 was considered to be statistically significant.
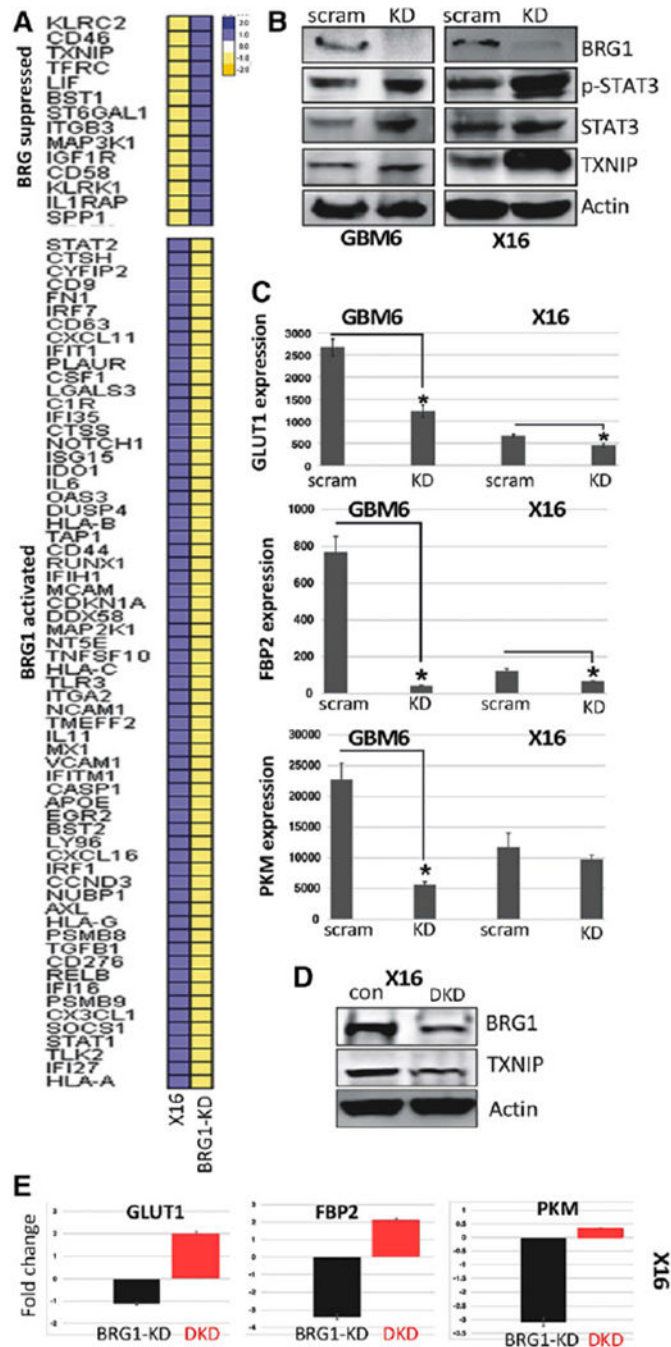
Figure 3.
Effect of BRG1-KD on gene and protein expression. **(A):** Total RNA was prepared from X16 and BRG1-KD GICs, and gene expression profiling was conducted on the nCounter Analysis System using the PanCancer Immune Panels, and heat maps of BRG1 suppressed and activated genes are shown. The scale reflects the levels of relative gene expression. **(B):** Protein lysates from control and BRG1-KD X16 GICs were immunoblotted for BRG1, STAT3, pTyr705-STAT3, and TXNIP. **(C):** RNA was prepared from control and BRG1-KD GICs, and expression of GLUT1, FBP2, and PKM was determined by qPCR. **(D):** Protein

lysates from control and BRG1-KD X16 GICs were immunoblotted for BRG1, TXNIP, and actin. **(E):** RNA was prepared from control X16 GICs, BRG1-KD X16 GICs, and X16 GICs with both BRG1 and TXNIP (DKD) knocked down, and GLUT1, FBP2, and PKM was determined by qPCR. Data were expressed relative to gene expression in control X16 GICs. *p .05 was considered to be statistically significant.
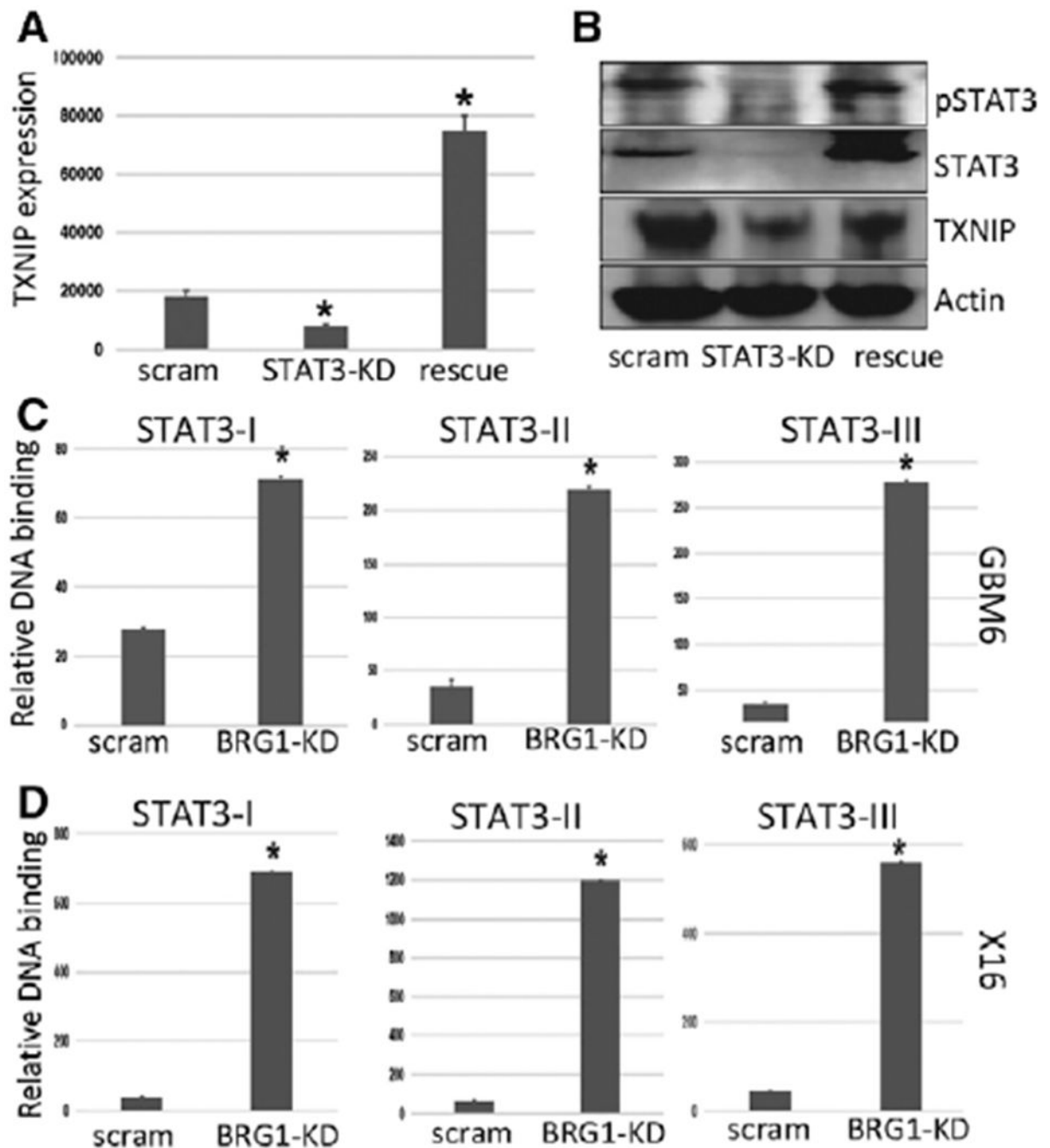
Figure 4.
TXNIP is an STAT3-regulated gene. GBMX16 GICs were transduced with a lentiviral vector containing a Dox-inducible shRNA against STAT3, and then transduced with wild-type (WT) STAT3 construct to restore STAT3 expression. **(A):** Total RNA was prepared from X16 GICs, and STAT3-KD GICs with or without STAT3 rescue, and TXNIP expression was determined by qPCR. **(B):** Protein lysates from control, STAT3-KD, and STAT3 rescued KD GICs were immunoblotted for pTyr-STAT3, total STAT3, TXNIP, and actin. **(C,D):** ChIP analyses of STAT3 binding to the STAT3-I, -II, and -III sites within the

TXNIP promoter in control and BRG1-KD (C) GBM6 GICs, and (D) X16 GICs. The ChIP-enriched DNA levels analyzed by qPCR were normalized to input DNA, followed by subtraction of nonspecific binding determined by control IgG. *p .05 was considered to be statistically significant.
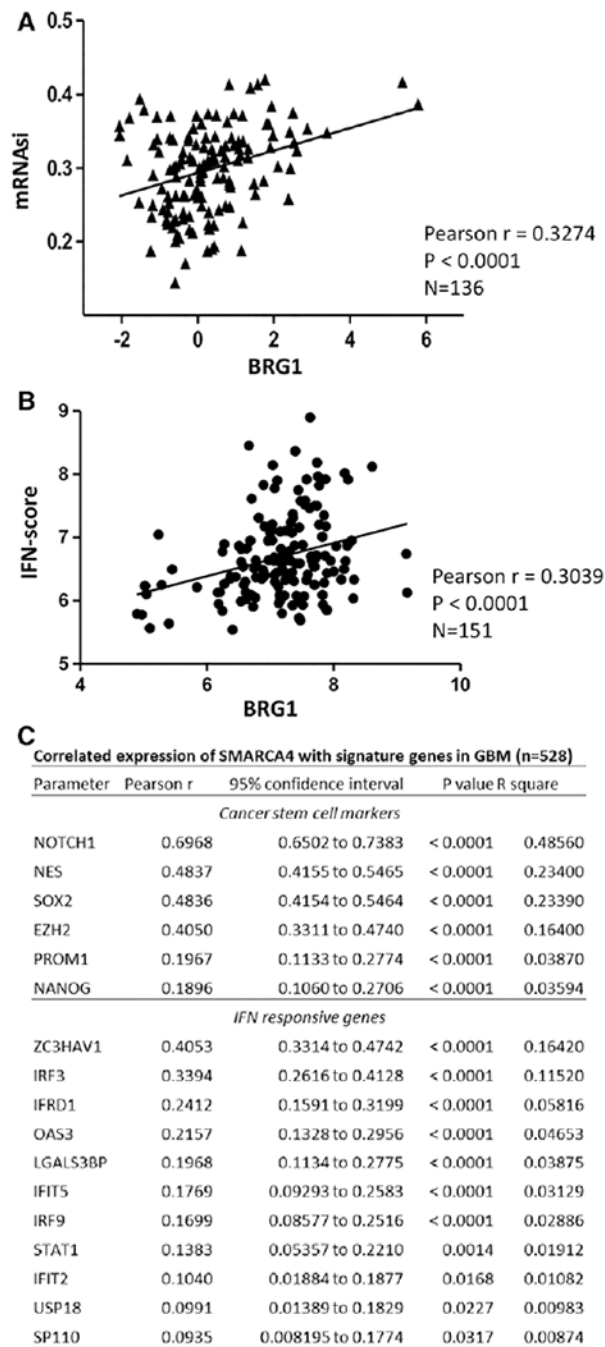
C

Correlated expression of SMARCA4 with signature genes in GBM (n=528)

Parameter	Pearson r	95% confidence interval	P value	R square
Cancer stem cell markers				
NOTCH1	0.6968	0.6502 to 0.7383	< 0.0001	0.48560
NES	0.4837	0.4155 to 0.5465	< 0.0001	0.23400
SOX2	0.4836	0.4154 to 0.5464	< 0.0001	0.23390
EZH2	0.4050	0.3311 to 0.4740	< 0.0001	0.16400
PROM1	0.1967	0.1133 to 0.2774	< 0.0001	0.03870
NANOG	0.1896	0.1060 to 0.2706	< 0.0001	0.03594
IFN responsive genes				
ZC3HAV1	0.4053	0.3314 to 0.4742	< 0.0001	0.16420
IRF3	0.3394	0.2616 to 0.4128	< 0.0001	0.11520
IFRD1	0.2412	0.1591 to 0.3199	< 0.0001	0.05816
OAS3	0.2157	0.1328 to 0.2956	< 0.0001	0.04653
LGALS3BP	0.1968	0.1134 to 0.2775	< 0.0001	0.03875
IFIT5	0.1769	0.09293 to 0.2583	< 0.0001	0.03129
IRF9	0.1699	0.08577 to 0.2516	< 0.0001	0.02886
STAT1	0.1383	0.05357 to 0.2210	0.0014	0.01912
IFIT2	0.1040	0.01884 to 0.1877	0.0168	0.01082
USP18	0.0991	0.01389 to 0.1829	0.0227	0.00983
SP110	0.0935	0.008195 to 0.1774	0.0317	0.00874

Figure 5.
BRG1 regulates sternness and IFN response genes in GBM patient samples. SMARCA4/ BRG1 expression in GBM patient samples in the TCGA database was correlated with the cancer sternness indices (mRNAsi) [48] **(A)** and the IFN-score [49] **(B)**. Signature genes of cancer stem cells and IFN-score that are significantly correlated with BRG1/SMARCA4 is listed in the table **(C)**.
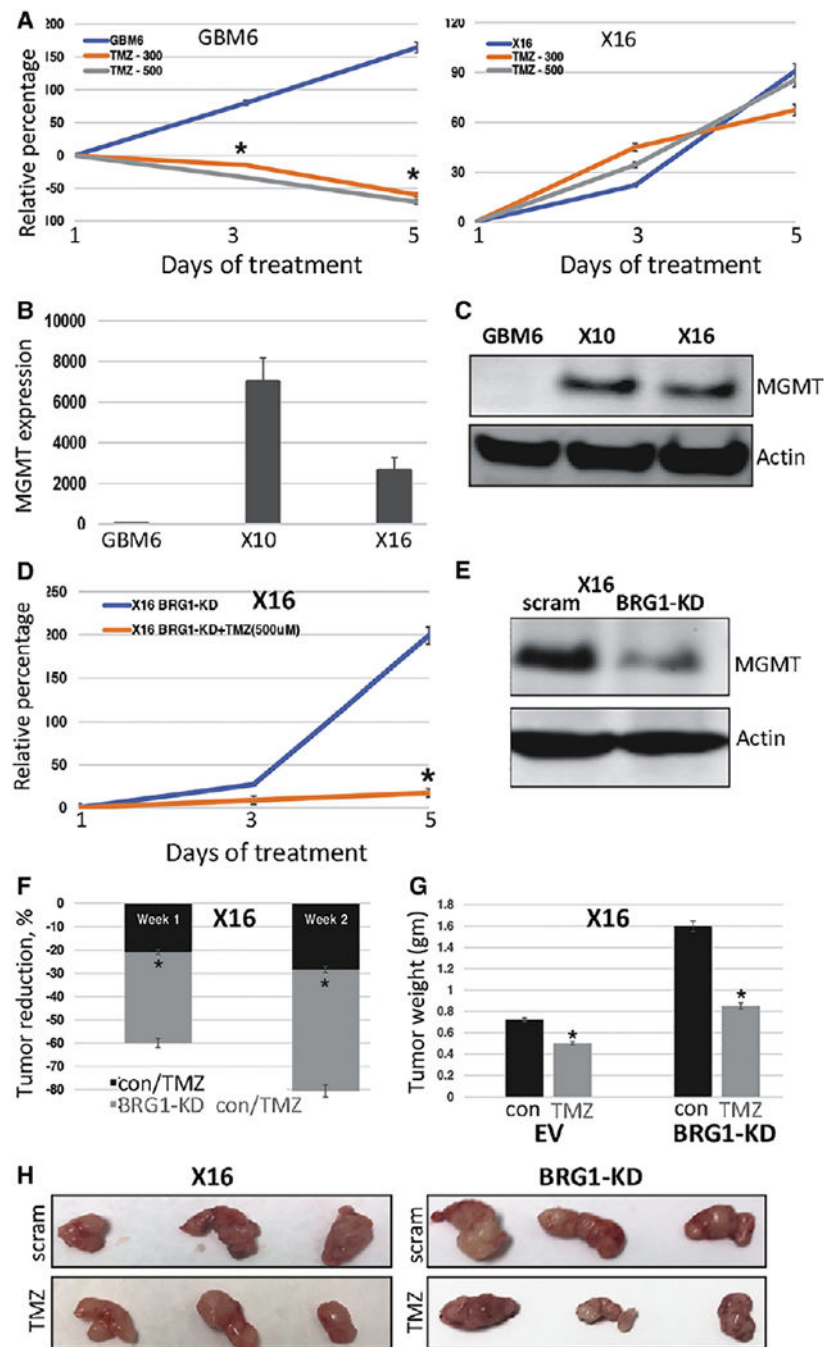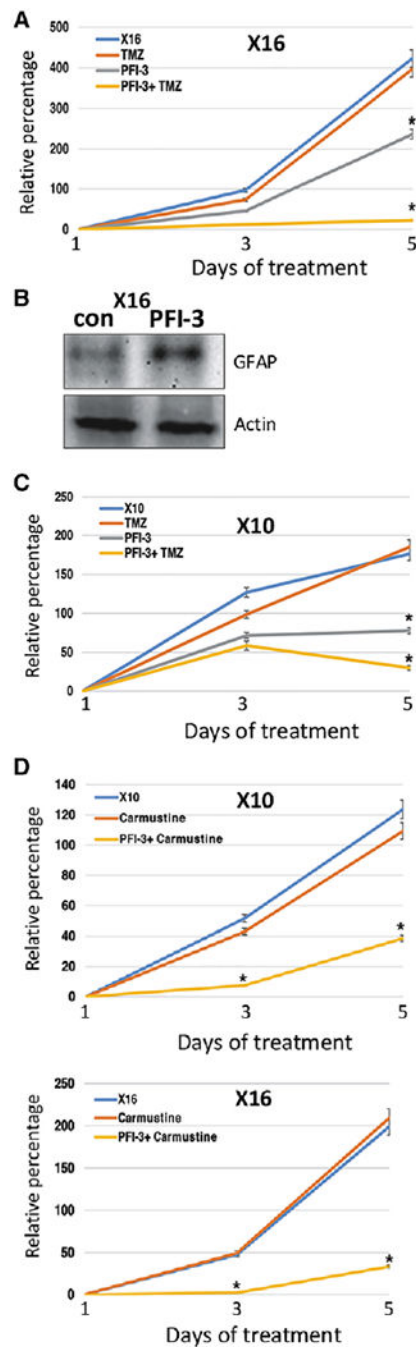
Figure 6.
BRG1 regulates GIC sensitivity to TMZ. **(A):** GBM6 and X16 GICs were treated with 300 or 500 μM of TMZ, and cell proliferation was determined by CellTiter-Glo assays. **(B):** Total RNA was prepared from GBM6, X10 and X16 GICs, and MGMT expression was determined by qPCR. **(C):** Protein lysates from GBM6, X10 and X16 GICs were immunoblotted for MGMT and actin. **(D):** Control and BRG1-KD X16 GICs were treated with or without 500 μM of TMZ, and cell proliferation was determined by CellTiter-Glo assays. **(E):** Protein lysates from control (scrambled shRNA) and BRG1-KD GICs were

immunoblotted for MGMT and actin. **(F):** The effect of TMZ on the tumorigenicity of control and BRG-KD GICs was assessed by injection of 10^6 tumor cells into the flanks of NSG mice and live animal imaging was performed at weekly intervals. After confirming tumor initiation by live animal imaging, mice were treated with TMZ by IP injection (10 mg/kg on alternate days), and the reduction in tumor volume was calculated and 1 and 2 weeks post-treatment. **(G)** Tumor weights and **(H)** photographs of tumors at necropsy. *p .05 was considered to be statistically significant.

Figure 7.
The BRG1 inhibitor PFI-3 sensitizes GICs to chemotherapy. **(A):** X16 GICs were treated with TMZ (500 μM) and PFI-3 (1 μM), and cell proliferation was determined by CellTiter-Glo assays. **(B):** Protein lysates from X16 GICs treated with PFI-3 were immunoblotted for GFAP and actin. **(C):** X10 GICs were treated with TMZ (500 μM) and PFI-3 (1 μM), and cell proliferation was determined by CellTiter-Glo assays. **(D):** X10 and X16 GICs were

treated with carmustine (10 μM) and PFI-3 (1 μM), and CellTiter-Glo assays performed. *p .05 was considered to be statistically significant.