SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Tumor samples

Adult human tumor samples were surgically obtained from 37 patients (mean, 49 yrs; range 20-88 yrs; 13 female, 24 male). Otherwise normal epileptic tissue resections were used as controls (n = 54, mean, 38 yrs; range 5-79 yrs; 28 female, 26 male). Tumors were graded by the attending neuropathologist in accordance with World Health Organization (WHO) established guidelines as ganglioglioma, oligodendroglioma, astrocytoma, mixed oligoastrocytoma and glioblastoma. Samples were obtained from patients who consented to tissue use, under protocols approved by both the University of Rochester-Strong Memorial Hospital and Johns Hopkins University Institutional Review Boards. Tumor specimens were divided into 3 different pieces; the first was dissociated for immunosorting and/or culture, a second was frozen in liquid nitrogen for RNA analysis, and the third one was fixed in 4% paraformaldehyde for immunohistochemical and histological verification of tumor phenotype.

Sample preparation

Samples were minced and digested by papain and DNase I in PIPES buffer, for 1 to 1.5 hours at 37°C. The samples were spun at 200*g* and their pellets recovered in 2ml of Dulbecco's minimum essential medium (DMEM)/F-12/N1. They were then dissociated by sequential trituration and passed through a 40 µm mesh into DMEM/F-12/N1 supplemented with 10% plasma-derived (PD) FBS (Cocalico Biologicals, Reamstown, PA) to stop the dissociation. The cell suspension was then diluted in 20 ml of DMEM/F12, mixed with 10 ml of Percoll in PBS, and fractionated by centrifugation at 15 000 g for 20 minutes. Cell fractions were harvested and washed in DMEM/F12. Cells were resuspended in DMEM/F-12/N1 media supplemented with bFGF (20ng/ml. Sigma), EGF (20ng/ml), PDGF-AA (20ng/ml; Sigma), and plated in cell suspension culture dish for overnight recovery. *Non-neoplastic GPCs* were isolated from matched grey and white matter dissociates of temporal lobes taken from 4 patients (temporal lobe epilepsy, ages 30-46 yrs). Separation of $A2B5^+$ and $A2B5^-$ cells was performed by MACS or FACS, 24-48h after dissociation. *Microglia* $CD11b^+$ microglia were extracted from non-neoplastic brain tissue by MACS using a similar procedure (n=4 patients, temporal lobe epilepsy, ages 9-53 yrs).

Cell Sorting

Glioma and non-neoplastic A2B5$^+$ GPCs were isolated using magnetic-beads (MACS) or fluorescent-activated (FACS) cell sorting followed by confirmation of the purity of the separated cell populations, as previously described (Nunes et al., 2003). Briefly, cells were suspended in DMEM/F12/N1 and incubated in A2B5 antibody supernatant (clone 105; American Type Culture Collection, Manassas, VA) for 30 to 45 minutes at 4°C on a shaker. The cells were washed three times with phosphate-buffered saline containing 0.5% bovine serum albumin and 2mM EDTA, then incubated with 1:4 diluted microbead-tagged rat anti–mouse IgM antibody (Miltenyi Biotech, Auburn, CA) (MACS) or with APC-tagged rat anti-mouse IgM antibody (FACS) for 30 minutes at 4°C. The A2B5 stained cells were washed, resuspended, and separated by MACS using either MS/RS or LS/VS positive selection columns (Miltenyi). APC-stained cells were incubated with DAPI (80ng/ml) and sorted (100 µm nozzle, low pressure) on a FACS Aria using FACS Diva software. Cell viability was determined both before and after sorting, using calcein (Molecular Probes, Eugene, Oregon). CD11b$^+$ microglia was isolated from adult human non-neoplastic tissue using a similar procedure. Briefly, following dissociation, cells were incubated with microbead labeled mouse anti-CD11b antibody for 30 minutes and magnetically sorted on LS columns as per manufacturer's instructions (Miltenyi Biotech, Auburn, CA). Acutely isolated cells were frozen for RNA extraction. The purity of sorted CD11b$^+$ microglia was verified using RCA-lectin staining (Vector Labs). *Glioma derived CD24/CD133-selected cells* were sorted using FACS-based procedures as described above. Briefly, cells were stained with CD24 (BD Pharmingen, clone ML5, 1/100), CD133/1-phycoerythrin (PE) (Miltenyi Biotec) followed by A647 conjugated anti-mouse IgG2a (Jackson Immunoresearch 1/500). The cells were then incubated with DAPI, sorted on a FACS Aria, and the purity was verified by flow cytometry.

Flow cytometry

For flow cytometry, 100,000 cells were resuspended in 100µl of flow cytometry (FC) buffer (PBS with 2mM EDTA and 0.5% BSA) and incubated for 20 minutes on ice with the following antibodies : CD133 (mouse IgG1; clone AC141-PE; Miltenyi Biotech; 1/10), CD24 (mouse IgG2a; BD Biosciences; 1/100), and A2B5 (mouse IgM; Chemicon; 1/300). Cells were washed in FC buffer, and incubated with secondary fluorescent-conjugated antibodies (goat anti-mouse IgM-APC conjugated; goat anti-mouse IgG2a-

APC) staining for 15 min on ice. Cells were washed once and resuspended in FC buffer supplemented with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen) to a final concentration of 80 ng/ml before analysis. Control conditions included unlabeled cells and cells labeled with appropriate isotypes control or secondary antibodies alone. Cells were analyzed on a FACS ARIA flow cytometer (BD Biosciences) using the FACS DIVA software and/or FlowJo.

Primary culture, propagation and clonogenicity

Establishment, growth and primary sphere formation of glioma cultures After dissociation, unsorted and A2B5 selected primary cells either derived from tumor samples or from epileptic tissue were seeded in 6 well cell suspension plate at clonal density (100 000-200 000 cells/ml) in DMEM/F12/N1 media containing FGF, EGF and PDGF (20ng/ml) as previously described. Growth factors and media were renewed 3 times per week. Using this strategy, we have established glioma cell lines with tumor stem/progenitor like cells features from various primary GBMs. Indeed, these cells were clonogenic *in vitro*, they expressed stem/progenitor cell markers, and they demonstrated neuronal and/or glial differentiation. In addition, these cells were tumorigenic *in vivo* after transplantation into the brain of immunodeficient mice and were able to generate xenograft tumors that recapitulate the histological and phenotypic features of the parental GBM.

Clonal assays For clonal experiments, gliomaspheres were dissociated to single cells and distributed to a 96-well plate directly after sorting at a density ranging from 5 to 100 cells/well with 0.2ml/well of DMEM/F12/N1 supplemented with bFGF, EGF and PDGF. Each well was fed with 50µl of fresh serum-free supplemented media every other day for up to 14 days. The number of tumor spheres (tight, spherical, non adherent masses >50µm in diameter) that formed subsequently per well was quantified after 14 days. Clonal growth was assessed visually with an inverted microscope.

Differentiation of glioma spheres Differentiation of neurospheres was achieved by plating individual spheres to poly-ornithine (Sigma) and laminin (Invitrogen) substrate in DMEM/F12/N1 supplemented with FBS 1% for up to 12 days. The cells were subsequently fixed in a solution of PFA 4% and stained using antibodies to GFAP (astrocytic marker), Olig2, CNPase (oligodendrocytic markers), Tuj1, MAP-2AB (neuronal markers) followed by Alexa-Fluor conjugated secondary antibodies (Invitrogen). A detailed list of the antibodies used is given in **Table S11.**

Telomeric Repeat Amplification Assay

The telomeric repeat amplification protocol (TRAP) assay was conducted as described (Roy et al., 2007; Roy et al., 2004), using the TRAPeze telomerase detection kit (Chemicon S7700), with the following modifications. The cells were collected by centrifugation, washed once with 1X `PBS and extracted with 1X cold CHAPS lysis buffer (supplemented with 100U/ml RNaseOUT). Sample extracts corresponding to 5000 or 20,000 cells were examined for telomerase activity using the following parameters: incubate at 30°C for 30 minutes then amplify by PCR in a thermocycler: 94°C for 30 sec denaturation, 59°C for 30 sec annealing, and 72°C for 1 min extension, for 33 cycles. 5 µl of loading dye was added to each sample and 25 µl of each sample was loaded onto a 12.5% non-denaturing PAGE gel. The gel was stained with SybrGreen at a dilution of 1:10,000 in 0.5X TBE for 40 minutes and then visualized with a 254 or 302 nm UV transilluminator.

IDH1 mutation analysis

The genomic region spanning wild-type R132 of *IDH1* was analyzed by direct sequencing, from genomic DNA, using the following primers, as previously described (Korshunov et al., 2009): FC-5′- ACCAAATGGCACCATACGA and RC-5′- TCCATACCTTGCTTAATGGGTGT. For analysis of IDH1 mutation on glioma-derived $A2B5^+$ sorted cells, IDH1 status was analyzed by direct sequencing, from cDNA, using the following primers: FC-5'-ACCAAATGGCACCATACGA and 773R-5'-ATGGCAACACCACCACCTTC.

Differential gene expression and pathway analysis

Extracted total RNA (Qiagen, Chatsworth, CA) was amplified using 3'-biased ribo-SPIA (NuGen Ovation) and hybridized onto Affymetrix U133 Plus2.0 microarrays as described (Sim et al., 2011; Wang et al., 2010). All analyses were performed in R/Bioconductor (Gentleman et al., 2004). Full analysis code is available on request (fjsim@buffalo.edu). Affymetrix U133+2 CEL files were preprocessed and normalized using RMA (Irizarry et al., 2003). Informative probe sets were determined using FARMS, which uses probe level information as repeated measures to quantify the signal-to-noise ratio of each given probe set (Talloen et al., 2007). Probe sets are called as informative when many of the probes within a probe set correlate with one another with respect to changes in expression across unclassified samples. Using FARMS, 25,175 probe sets

or 46% were classified as informative and were used in all further analysis. Sample-to-sample data exploration was performed using unsupervised hierarchical clustering and principle component analysis.

Linear models for assessing differential gene expression

Differential gene expression analysis was performed using a linear model approach and employing an empirical Bayes method for calculation of statistical significance (Bioconductor, limma package) (Smyth, 2004).The first model concentrated on identifying genes whose expression differed in the transformed glioma-derived A2B5$^+$ tumor progenitor cells (TPCs) from the non-transformed epilepsy-derived A2B5$^+$ glial progenitor cells. As such, samples were either designated tumor (A2B5$^+$ cells from tumor, n=20), normal (A2B5$^+$ cells from epilepsy, n=8), tissue dissociates (n=8) or CD11b$^+$ microglia (n=4). Following fitting of this linear model, we identified those probe sets whose expression were significantly enriched or depleted in tumor progenitors by at least 3 fold change and statistically significant following 1% FDR adjustment of p-values. Since the profiles of A2B5$^+$ TPCs suggested that the antigen is also expressed by human microglia, and given the high incidence of microglia in human GBM, we sought to exclude microglial transcripts from our database. To this end, we further filtered the tumor progenitor specific genes by including only those genes, which were similarly regulated in TPCs relative to CD11b-sorted microglia.

The second linear model focused on identifying those genes whose expression significantly varied within and between low and high-grade glioma A2B5$^+$ TPCs. WHO grade II, oligodendroglioma, astrocytoma, oligoastrocytoma, and ganglioglioma were grouped as low grade tumors (LG, n= 10) and WHO grade III and IV tumors (anaplastic astrocytoma, anaplastic oligodendroglioma, anaplastic oligoastrocytoma, GBM, small cell GBM, and gliosarcoma) were grouped as high grade (HG, n= 10). Significantly varying genes were identified using the same criteria (3 fold change, 1% FDR) and each phenotype was compared back to their native A2B5$^+$ progenitor and CD11b$^+$ microglia. In contrast, the third linear model focused on tumor phenotype specific differences. The linear model was designed with separate groups for each tumor subtype. The results for each of these linear model comparisons are summarized in the supplemental data.

Pathway-based functional analysis

To identify pathways of interest, we used three distinct types of functional analysis. Hypergeometric tests were performed using the differentially expressed genes

at >3 fold change and significance at 1% FDR (see above). Hypergeometric p-values were calculated for both gene ontology (GO) biological process annotations and KEGG pathways. The topGO package was used to identify over-represented GO terms using the 'elim' algorithm and Fisher statistics ($p<0.01$) (Alexa et al., 2006). Hypergeometric testing of KEGG pathways was performed using the GOstats package ($p<0.05$). Gene set enrichment analysis (GSEA) was performed using parametric GSEA (PGSEA package) (Furge and Dykema, 2006) on the Broad database of curated pathways (Molecular Signatures Database) databases (Subramanian et al., 2005). Linear models (described above) were then used to generate a moderated t-test statistic and FDR corrected p-values associated with specific gene set enrichment were calculated (5% FDR cut-off used for pathway significance). Functional analysis based on biological networks, functions and canonical pathways were also generated through the Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, www.ingenuity.com) using the differentially expressed genes at >3 fold change and significance at 1% FDR).

Real-time Polymerase Chain Reaction analysis

Extracted total RNA was amplified using ribo-SPIA based whole transcriptome based amplification (NuGen). The expression of 95 cell type marker and pathway-specific genes was assessed using a 96-gene Taqman low-density array (TLDA) (Applied Biosystems) (**Table S10**). The relative abundance of transcript expression was calculated by $\Delta\Delta C$ analysis, and the expression data normalized to GAPDH. Genes whose expression was not detected in more than half of the RNA samples were excluded. Statistical analysis of TLDA gene expression data was then performed using Bioconductor using a moderated t-test statistic with a 5% false discovery rate cut-off (Smyth, 2004).

Additional individual primers and probes were obtained as Assays-on-Demand from Applied Biosystems (www.allgenes.com). Statistical analysis was performed on log-transformed data and p-values calculated (1-way ANOVA followed by Tukey pairwise comparisons). P-values less than 0.05 were selected as significant.

Orthotopic transplants and in vivo tumorigenicity

Adult (5-10 week-old; 21-23 g) SCID/NOD and NSG mice (NOD/Shi-scid/IL-2Rγ^{null}) (Pearson et al., 2008) (Jackson Labs) were maintained in micro-isolator cages in a specific pathogen-free facility on standard 12-hour night and day cycles. Injections were performed according to institutional guidelines. Dissociated graft cell suspensions

from unsorted, positive and negative-sorted cells populations were diluted to a concentration of viable 100,000 cells/µl and placed on ice until transplantation. Animals were anesthetized with ketamine-xylazine and placed in a stereotaxic apparatus, and cells injected at 0.4 µl/min to the following coordinates: AP -0.98 mm from bregma; ML: + 2.2 mm; DV: - 2.2 mm. The syringe was left in place for 4 min following cell injection, and the wound then closed. After completion of the experiments, residual cells were grown in culture to validate their viability *in vitro*. Animals were subsequently examined for behavioral changes and weight loss, until the time of sacrifice, 6-15 weeks after injection. At that time, animals were terminally anesthetized, serially perfused via a transcardiac approach with saline solution and 4% paraformaldehyde, then their brains were removed, post-fixed for 2 h, serially cryoprotected in 6% and 30% sucrose, and frozen in cooled methyl-butane. The brains were serially sectioned at 14 µm by cryostat; sections were stored frozen for subsequent immunohistochemical and histological analysis.

Histological and immunohistochemical protocols

Human samples were immersed in paraformaldehyde 4% in phosphate buffer (PB) for 1-2 hrs, then gradually cryoprotected in sucrose 6% and 30% during 24h at 4°C, embedded with Tissue-Tek OCT and frozen. Serial 14 µm sections were cut on a Leica cryostat, dried at RT, then processed for immunohistochemical and histological analysis. Sections were rehydrated in PBS and permeabilized for 15 min with PBS containing 0.1% saponin and 1% normal goat/donkey serum (NGS/NDS). Sections were washed three times in PBS and then incubated for 1 h with PBS containing 0.05% saponin and 10% NGS/NDS. The primary antibodies were diluted in PBS containing 0.01% saponin and 5% NGS/NDS and incubated overnight. After three washes with PBS, sections were further incubated with a solution of secondary antibodies (in PBS containing 0.01% saponin and 5% NGS/NDS) using AlexaFluor488 and 594-labeled secondary antibodies (1/500 – 1/1000, Molecular Probes). Sections were counter stained with 4'-6-diamidino-2-phenylindole (DAPI; Invitrogen), and examined using either an Olympus BX51 epifluorescence or Fluoview 100 confocal microscope. Adjacent sections were typically stained with hematoxylin and eosin for histological assessment.

For immunohistochemical analysis of xenografts, transplanted cells were identified using antibodies to human nuclei (HNA), human GFAP, and human nestin. In order to examine the neoplastic status of the cells, the expression of Ki67, survivin

(Andersen et al., 2007), and P53 were assessed. To further compare the phenotype of xenografted cells to the original phenotype of the primary tumor, a panel of antibodies was used; these are listed in **Table S11**.

In vitro immunophenotyping

Glioma cells were plated in 24 well plate coated with poly-L-ornithine (Sigma) and laminin at a density of 25,000 to 50,000 cells per well. For proliferation analysis, glioma cells were cultured in SF media, allowed to attach for 12-24h, they were stained for A2B5, post-fixed with a solution of paraformaldehyde 4% and then subsequently stained for stem/progenitor cell markers like sox2 (1/500), Nestin (1/1000) and Olig2 (1/200), the mitotic marker Ki67 (1/250), the tumor marker survivin (1/300), the astrocytic marker GFAP (1/1000) **(Table S11)**. For differentiation assay, cells were cultured in DMEM/F12/N1 media containing either 1% FBS for up to 12 days. Cells were subsequently stained as described for the proliferation assay. For cell surface labeling (A2B5), cells were rinsed in PBS and then incubated for 1h with PBS containing 10% normal goat/donkey serum (NGS/NDS). The primary antibodies were diluted in PBS containing 5% NGS/NDS and incubated overnight. After three washes with PBS, cells were further incubated with a solution of secondary antibodies (in PBS containing 5% NGS/NDS) using AlexaFluor488 and 594-labeled secondary antibodies (1/500 – 1/1000, Invitrogen). Cells were incubated with a solution containing 4'-6-diamidino-2-phenylindole (DAPI) (1/1000), or post-fixed with PFA 4% for 10 minutes for subsequent immunolabeling for intracellular antigens.

Generation and validation of Six1 knock-down lentivirus

A set of lentiviral shRNA vectors containing 5 constructs with distinct target sequences was purchased from Open Biosystems. We first validated Six1-induced silencing constructs by transfection of multiple glioma cell lines and subsequent q-PCR analysis for Six1 mRNA expression. Only selected and validated constructs were packaged for viral production. A scrambled sequence (H. Ford) was cut from its original vector and cloned into pLKO.1 for further lentiviral generation. Subconfluent 293T cells were further co-transfected with equimolar amount of PLKO-scrambled or PLKO-Six1shRNA plasmid DNA, and a mixture of the packaging plasmids PAX2 (Addgene, Cambridge, MA) and vesicular stomatitis virus glycoprotein (VSVG) using Fugene HD (Roche Applied Science, Indianapolis, IN). Viral supernatants were collected at 48 and

72 hours by ultracentrifugation at 18000g for 3 hours, aliquoted and stored at -80°C. The viral titer was determined by transduction of HT1800 cells with serial dilutions of the viral supernatant and colony counting after puromycin selection (1 µg/ml).

Western blot

Western blotting was performed on both nuclear and cytoplasmic extracts, using the Pierce NE Kit. Cell lysates (10-30 µg protein) were electrophoresed using 8-12% SDS-PAGE gels, then transferred onto PVDF membranes (Bio-Rad). Blots were blocked with 5% non-fat dry milk in PBS containing 0.1% Tween-20 for 2 hrs at RT, then incubated overnight at 4°C with primary anti-Six1 (1:1000, Sigma). The blots were then washed and incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibodies; labeled proteins were detected using enhanced chemoluminescence (ECL) reagents (Amersham Biosciences). Anti-GAPDH (Sigma) was used as a positive loading control, and omission of the primary antibody and naïve antisera were used as negative controls.

Cell proliferation and cell cycle analysis

Six days after lentiviral shRNAi knock-down of Six1 (n=4), or transduction by scrambled control lentivirus, glioma cells were labeled with BrdU (30 µM) for 4 hours, fixed and then immunostained for BrdU. The number of $BrdU^+$ cells was counted and the proportion of $BrdU^+$ cells among all glioma cells was calculated. Values indicated means ± SEM; * $p < 0.05$ ** $p < 0.01$.

EdU labeling was performed using the Click-it Edu flow cytometry assay kit (Invitrogen; catalog#A10202), following manufacturer's instructions. Briefly, cells transduced with either scrambled or Six1-KD lentivirus were seeded in 12 well plates. Five days post-transduction, AraC was added 24 hours before the EdU pulse as a negative control to arrest cells in G_2M. The next day, cells were treated with 10µM of EdU for 4 hours, washed with 1% BSA in PBS, and fixed with Click-it fixative solution. Cells were then washed, saponin-permeabilized, and incubated in Click-it reaction cocktail solution for 30 min. The cells were then spun, washed and incubated in propidium iodide with RNase and incubated for 30 min at RT, so as to discern total DNA content. The experiments were repeated 3 times independently. Cells were then analyzed on a FACS ARIA flow cytometer (BD Biosciences) using the FACS DIVA software and/or FlowJo. After forward scatter and side scatter gating to remove debris and clusters, PI was used to isolate whole nuclei and exclude DNA fragments. Values

were reported as means ± S.E.M. Statistical analysis was performed using 1-way ANOVA followed by Tukey's multiple comparison test.

Cell death analysis

The effect of Six1 knock-down on glioma TPC viability was addressed using the Alexa-647 Annexin V Apoptosis Detection Kit (BD Pharmingen, San Diego, CA), according to the manufacturer's instructions. Glioma derived TPCs were transduced with either scrambled or Six1-KD lentivirus. Six days later, cells were harvested, resuspended in 200 μl of Annexin V binding buffer and incubated with 5 μl of Annexin for 15 min in the dark. The cells were then washed, resuspended in Annexin V binding buffer, incubated with DAPI (80 ng/ml) and analyzed using a FACS ARIA (BD Biosciences), with FlowJo cytometry analysis software. Values were reported as means ± S.E.M. Statistical analysis included 1-way ANOVA followed by Tukey's multiple comparison test.

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