Supporting Information

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SI Materials and Methods

Isolation of $CD133^+$ and $CD133^-$ Cells from Primary GBM Tissues. Following informed consent, tumor samples classified as glioblastoma (GBM), based on World Health Organization (WHO) criteria, were obtained from patients undergoing surgical treatment at Zhongshan Hospital of Fudan University with the approval of the Zhongshan Hospital Ethics Committee. Tumor grade and available cytogenetic information for each specimen are listed in Table S1. Within 1 h after surgical removal, tumor specimens were washed and enzymatically dissociated into single cells. After contaminating RBSs were lysed by brief incubation in hypotonic solution, the isolated tumor cells were briefly placed in DMEM and F12 media supplemented with B27 lacking vitamin A (Invitrogen) to permit recovery following enzymatic dissociation. $CD133^+$ and $CD133^-$ cells were separated through magnetic cell sorting with a CD133 Cell Isolation Kit (Miltenyi Biotec). The purity of sorted $CD133^+$ and $CD133^-$ cells was verified by flow cytometry: the purity of $CD133^+$ cells was greater than 85%, and the purity of $CD133^-$ cells was above 99%. $CD133^+$ cells were designated as glioma stem cells (GSCs), whereas $CD133^-$ cells were used as nonstem glioma cells.

Cell Cultures. The sorted $CD133^+$ cells were cultured in the DMEM/F12 media supplemented with B27 lacking vitamin A (Invitrogen), 2 μg/mL heparin (Sigma), 20 ng/mL EGF (Chemicon), and 20 ng/mL FGF-2 (Chemicon) for a short period before treatment and analysis. $CD133^-$ tumor cells were plated in DMEM with 10% (vol/vol) FBS for at least 12 h to permit cell survival because the viability of this subpopulation is decreased by suspension culture conditions. Before performing experiments with $CD133^-$ cells, DMEM with 10% FBS was replaced with supplemented DMEM/F12 media in order for experiments to be performed in identical media.

For comparing the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway in $CD133^+$ cells with $CD133^-$ cells, Western analysis, immunofluorescence and assay for monitoring PI3K activity were performed in $CD133^+$ and $CD133^-$ glioma cells cultured in supplemented DMEM/F12 media soon after cell isolation to avoid the adherence of $CD133^-$ cells to tissue culture plates and the neurosphere formation or aggregation of $CD133^+$ cells.

Immunohistochemistry. Human glioma specimens classified as grade I-IV based on WHO criteria were obtained from Zhongshan Hospital of Fudan University and Provincial Hospital Affiliated to Shandong University with approval of the hospitals' Ethics Committee. Paraffin-embedded sections were deparaffinized and rehydrated. Antigens were retrieved by boiling sections in Tris·EDTA buffer (pH 9.0) at 121 °C for 10 min. After the endogenous peroxidase was blocked by incubating with 3% H_2O_2 solution in PBS for 10 min, sections were blocked with 5% normal serum and were incubated with rabbit polyclonal anti-pY828-CD133 (1:200) at room temperature (RT) for 2 h. After extensive washing in TBST buffer [25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween-20], sections were incubated for 60 min with HRP-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology; 1:200). The immunoreactivity was visualized with 3, 3-diaminobenzidine (Dako). Sections were counterstained with hematoxylin (Sigma). The images were captured using the Motic Image Advanced 3.2 image analysis system. To quantify pY828-CD133 expression, we measured the immunostaining score of pY828-CD133 in 10 randomly selected fields per section. The scores for semiquantitative staining of the tissue sections were determined according to a total score (range, 0–8) that was obtained by combining the score of the percentage of positive cells and the score of the staining intensity, as previously described (1). Two separate individuals who were both blinded to the slides examined and scored each sample. An average value of the three scores is shown.

To test the binding specificity of the rabbit polyclonal anti-pY828-CD133 antibody, the antibody was preincubated with 100 μM phospho-Y828 peptide (RMDSEDV-pY^{828}-DDVETIP) or unphosphorylated Y828 peptide (RMDSEDVYDDVETIP) at RT for 1 h. Immunohistochemical staining was performed using vehicle-treated or peptide-treated anti-pY828-CD133 antibodies. A negative control was obtained by omitting primary antibody.

Western Analysis. Equal amounts of cell lysate were resolved by SDS/PAGE, transferred to PVDF membranes (Roche). Blocking was performed for 60 min with 5% nonfat dry milk in TBST and blotting was performed with primary antibodies for 12–16 h at 4 °C. Primary antibodies included: rabbit polyclonal anti-Akt (Cell Signaling; 1:1,000), rabbit polyclonal anti-pS473-Akt (Cell Signaling; 1:1,000), rabbit polyclonal anti-p threonine 308-Akt (Cell Signaling; 1:1,000), rabbit monoclonal anti-PI3K 85kDa regulatory subunit p85 (Cell Signaling; 1:1,000), rabbit polyclonal anti-PI3K catalytic subunit p110 (Cell Signaling; 1:1,000), rabbit polyclonal anti-Gli1 (Cell Signaling; 1:1,000), rabbit polyclonal anti-Src (Cell Signaling; 1:1,000), mouse monoclonal anti-CD133 (W6B3C1 clone) (Miltenyi Biotec; 1:1,000), goat polyclonal anti-CD133 C-terminal (Santa Cruz Biotechnology; 1:500), mouse monoclonal anti-Notch intracellular domain (BD Biosciences; 1:1,000), rabbit polyclonal anti-FLAG (Sigma; 1:3,000), and rabbit polyclonal anti-pY828-CD133 (1:2,000). Anti-pY828-CD133 antibody was raised against phosphopeptide RMDSEDV-pY^{828}-DDVETIP.

After extensive washing with TBST, the membranes were incubate for 1.5 h at RT with HRP-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology; 1:3,000), goat anti-mouse antibody (Santa Cruz Biotechnology; 1:2,000), or rabbit anti-goat secondary antibody (Sigma; 1:50,000), and signal was detected by enhanced chemiluminescence substrate (Pierce Biotechnology). For quantification, the Western blot films were scanned and were densitometrically analyzed using ImageJ Version 1.33u software.

Immunofluorescence. For immunostaining of undifferentiated tumor spheres, cells were fixed with 4% paraformaldehyde (PFA) for 20 min at RT, washed three times with PBS, and then blocked with a PBS-based solution containing 5% normal serum and 0.3% Triton X-100. Cells were incubated overnight at 4 °C with rabbit polyclonal anti-Nestin (Santa Cruz; 1:100). After being washed three times with PBS, cells were incubated with goat anti-rabbit Alexa 594 IgG (Invitrogen; 1:400). Nuclei were counterstained with Hoechst 33258 (Sigma; 10 μg/mL).

For examining the differentiation capacity of GSC, $CD133^+$ tumor cells were plated onto polylysine-coated coverslips in DMEM containing 10% FBS for 7 d. After cells had attached, spread out, and undergone distinct morphological changes, they were fixed, blocked, and incubated overnight at 4 °C with the appropriate antibody: mouse monoclonal anti-Map2 (Sigma; 1:200), rabbit polyclonal anti-GFAP (Millipore; 1:250) or mouse monoclonal anti-oligodendrocyte (IgM) (Sigma; 1:200). Cells were washed three times with PBS and incubated with the appropriate secondary antibody: goat anti-mouse Alexa 488 IgG (Invitrogen;

1:400), goat anti-rabbit Alexa 594 IgG (Invitrogen; 1:400), or goat anti-mouse Alexa 488 IgM (Invitrogen; 1:400). Nuclei were counterstained with Hoechst 33258 (Sigma; 10 μg/mL).

For immunostaining analysis of CD133 and p85 colocalization, $CD133^+$ and $CD133^-$ cells were fixed with 4% PFA for 20 min at RT, washed three times with PBS, and then blocked with a PBS-based solution containing 5% normal goat serum and 0.3% Triton X-100. Cells were coincubated overnight at 4 °C with rabbit polyclonal anti-p85 antibody (Millipore; 1:200) and mouse monoclonal anti-CD133 (W6B3C1 clone) (Miltenyi Biotec, cat# 130–092-395; 1:50). After being washed three times with PBS, cells were coincubated with goat anti-mouse Alexa 488 IgG (Invitrogen; 1:400) and goat anti-rabbit Alexa 594 IgG (Invitrogen; 1:400). Nuclei were counterstained with Hoechst 33258 (Sigma; 10 μg/mL).

Double immunofluorescence staining of pY828-CD133 and either pS473-Akt was performed on GBM frozen sections. Briefly, slides were fixed by 4% paraformaldehyde for 15 min at RT, blocked by 5% normal goat serum, and coincubated overnight at 4 °C with rabbit anti-pY828-CD133 (1:200) and mouse monoclonal anti-pS473-Akt (Cell Signaling; 1:200). Sections were washed three times with PBS and then coincubated with goat anti-rabbit Alexa 488 IgG (Invitrogen, 1:400) and goat anti-mouse Alexa 594 IgG (Invitrogen, 1:400). Nuclei were counterstained with Hoechst 33258 (Sigma; 10 μg/mL).

Immunofluorescent images were collected on a Leica TCS SP5 confocal microscope and analyzed using LAS AF software.

Plasmids. To knock down endogenous CD133 expression, the CD133 shRNA lentivirus vectors were generated by ligation of lentivirus vector pLL3.7 with oligonucleotides (5′-TGGCTTGGAATTATGAATTGTTCAAGAGACAATTCATAATTCCAAGCCTTTTTTC-3′) or (5′- TGCTCAGAACTTCATCACAATTCAAGAGATTGTGATGAAGTTCTGAGCTTTTTTC-3′) (underlines indicate the target sequence for CD133 shRNA1 or CD133 shRNA2). Beta-galactosidase (LacZ) shRNA lentivirus vector used for experimental control was generated by ligation of pLL3.7 vector with oligonucleotides (5′- TGTGACCAGCGAATACCTGTTTCAAGAGAACAGGTATTCGCTGGTCACTTTTTTC-3′) (underline indicates the target sequence for LacZ shRNA).

For ectopic expression of CD133, the LV-CD133-FLAG plasmid was constructed by inserting full-length human CD133 cDNA into the LV-FLAG lentivirus vector between BamHI and AgeI sites. CD133 phosphorylation mutants (Y818F, Y819F, Y828F, Y846F, and Y852F) were created using Takara MutanBEST mutagenesis kit. Mutated constructs were sequenced, and the correct ones were selected for further experiments. To generate shRNA-resistant CD133 (shR-CD133-FLAG) or shRNA-resistant Y828F (shR-Y828F-FLAG) lentivirus vectors, site-directed mutagenesis technique was used to introduce four mutations into the coding region of CD133 or Y828F (nucleotides 90–108) cognate to the CD133 shRNA1 target sequence (GGC*A*TGGAA*C*TA*C*GAGTT*A,* mutations italicized); the introduction of these mutations was confirmed by sequencing.

The prokaryotic expression plasmid GST-CD133(813-865) was constructed by inserting cDNA sequence encoding CD133 813–865 aa into pGEX-4T-1 prokaryotic expression vector between BamHI and XhoI sites. The prokaryotic expression plasmid GST-p85 was constructed by inserting full length human p85 cDNA sequence into pGEX-6p-1 prokaryotic expression vector between BamHI and SalI sites.

Lentivirus Production and Infection. Briefly, using calcium phosphate transfection, 293T cells were cotransfected with lentivirus vectors, and the packaging vectors RRE, REV and RSVG. Two days later, the supernatants were collected, filtered, concentrated, and used for experiments or frozen at −80 °C. Cells were transduced by using lentivirus with polybrene (Sigma; 8 μg/mL).

Immunoprecipitation. GBM tissues (Table S1) or cells were lysated in a modified radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris (pH 7.4), 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, protease inhibitor mixture, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM NaF]. Lysates were centrifuged and cleared by incubation with 25 μL of Protein G-Agarose (Roche) for 1.5 h at 4 °C. The precleared supernatant was subjected to immunoprecipitation (IP) using the indicated first antibodies at 4 °C overnight. Antibodies used in IP included: mouse monoclonal anti-CD133 (W6B3C1 clone) (Miltenyi Biotec) and rabbit polyclonal anti-p85 (Millipore). Then, the protein complexes were collected by incubation with 30 μL of Protein G-Agarose (Roche) for 2 h at 4 °C. In some cases, the precleared supernatants were immunoprecipitated directly with mouse monoclonal anti-FLAG M2 affinity Agarose gel (Sigma). The collected protein complexes were washed four times with IP buffer and analyzed by Western blotting using indicated antibodies. Antibodies included: rabbit monoclonal anti-PI3K regulatory subunit p85 (Cell Signaling; 1:1,000), rabbit polyclonal anti-PI3K catalytic subunit p110 (Cell Signaling; 1:1,000), mouse monoclonal anti-CD133 (W6B3C1 clone) (Miltenyi Biotec; 1:1,000), rabbit polyclonal anti-FLAG (Sigma; 1:3,000), and rabbit polyclonal anti-pY828-CD133 (1:2,000).

GST Pull-Down Assay. Ten micrograms of GST or GST-CD133(813-865) (CD133 C-terminal cytoplasmic domain; amino acids 813–865) protein purified from bacteria BL21 bound to GST beads were pretreated in a phosphorylation reaction [20 mM Hepes·NaOH (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT and 0.2 mM Na_3VO_4] with 0.25 μg recombinant active Src (Millipore) in the presence or absence of 0.1 mM ATP for 1 h at 30 °C. Then, the GST beads were washed, and the bound proteins were incubated with recombinant p85 (generated from *Escherichia coli* BL21) in binding buffer [20 mM Hepes-KOH (pH 7.6), 2.5 mM $MgCl_2$, 200 mM KCl, 0.5 mM EDTA, 1 mM DTT, 0.1% Nonidet P-40, 10% Glycerol, 1 mM PMSF, and 0.2 mM Na_3VO_4] at 4 °C for 4 h. After washing with binding buffer, the pull-down products were subjected to SDS/PAGE and were analyzed by Coomassie Blue staining or Western blotting using the indicated antibody. Antibodies included: rabbit monoclonal anti-PI3K regulatory subunit p85 (Cell Signaling; 1:1,000), rabbit polyclonal anti-pY828-CD133 (1:2,000), goat polyclonal anti-CD133 C-terminal (Santa Cruz Biotechnology; 1:500), and mouse monoclonal anti-p-tyrosine (BD Bioscience; 1:2,000).

Subcellular Fractionation. Membrane and cytoplasmic fractions, used for the assessment of p85 and p110 recruitment to membrane, were extracted with Subcellular Protein Fractionation Kit (Pierce Biotechnology) following manufacturer's instruction. Equal amounts of membrane and cytoplasmic fractions were resolved by SDS/PAGE and analyzed by Western blotting using the indicated antibody. Antibodies included: rabbit monoclonal anti-PI3K regulatory subunit p85 (Cell Signaling; 1:1,000), rabbit polyclonal anti-PI3K catalytic subunit p110 (Cell Signaling; 1:1,000), rabbit anti-EGFR (Cell Signaling, 1:1,000), and α-Tubulin (Sigma, 1:1,000). EGFR was used as the membrane marker and α-Tubulin was used as the cytoplasmic marker.

Neurosphere Formation Assay. For single-cell neurosphere formation assay, 24 h after treatment with the indicated lentivirus, cells were trypsinized and single-cell suspensions were cultured in 96-well plates (one cell per well) containing supplemented DMEM/F12 medium. After 10 d, the percent of wells with neurospheres was quantified. For secondary neurosphere formation assay, the established neurospheres were dissociated into single cells and were cultured in 96-well plates (one cell per well). The percent of wells with secondary neurospheres was counted after 10 d.

For bulk-culture neurosphere formation assay, 24 h after the treatment with the indicated lentivirus, cells were trypsinized and single-cell suspensions were cultured in 96-well plates (1, 10, or 100 cells per well) containing supplemented DMEM/F12 medium. After 10 d, the number of neurospheres per well was quantified.

Assay for Monitoring PI3K Activity. PI3K activity was evaluated with a PI3 kinase ELISA kit (Echelon Biosciences, K-1000s) according to the manufacturer's protocol. This kit measures PI3K activity as a conversion of PI(4,5)P2 into PI(3,4,5)P3. Briefly, PI3K bound to protein A Sepharose beads was incubated for 1–2 h with PI(4,5)P2 substrate at RT in 50 μL buffer A [20 mM Tris·HCl (pH 7.4), 4 mM $MgCl_2$, 10 mM NaCl, and 25 μM ATP]. The beads were removed by centrifugation, and the supernatant or known concentrations of PI(3,4,5)P3 were incubated for 1 h with 50 μL PI (3,4,5)P3-binding reagent and then transferred to a detection plate coated with PI(3,4,5)P3. A peroxidase-linked secondary detection reagent was used to detect PI (3,4,5) P3 detector protein binding to the plate. The peroxidase substrate with reaction product was measured by absorbance at 450 nm. The amount of PI(3,4,5)P3 produced was calculated from a standard curve prepared from known concentrations of lipid product.

Tumor Formation Assays. For studies comparing the tumor-initiating capacity of $CD133^+$ cells versus $CD133^-$ cells, cells (100,000, 50,000, 10,000, 5,000, 1,000, or 500 cells) were intracranially injected into 6- 8-wk-old nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice. For CD133 knockdown, ectopic expression, or rescue studies, 72 h after lentivirus infection, cells were counted and certain number cells were intracranially injected into NOD/SCID mice. Mice were maintained up to 180 d or until the development of neurologic signs that significantly inhibited their quality of life (e.g., ataxia, lethargy, seizures, inability to feed). After the mice were killed, their brains were collected, fixed in 4% PFA, paraffin embedded, and sectioned. Tumor formation was determined by systematic histological analysis of H&E-stained sections.

Analysis of GBM Subtype. Briefly, RNA from patient samples was extracted and profiled on Affymetrix Human Exon 1.0 ST Gene Chips according to the manufacturer's protocol. Proneural, neural, classical, and mesenchymal GBM subtypes were determined by clustering of expression data from the Affymetrix HuEx array platform using the previously published gene marker (2).

1. Ji H, et al. (2009) EGF-induced ERK activation promotes CK2-mediated disassociation of alpha-Catenin from beta-Catenin and transactivation of beta-Catenin. *Mol Cell* 36(4): 547–559.

2. Verhaak RG, et al.; Cancer Genome Atlas Research Network (2010) Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* 17(1):98–110.

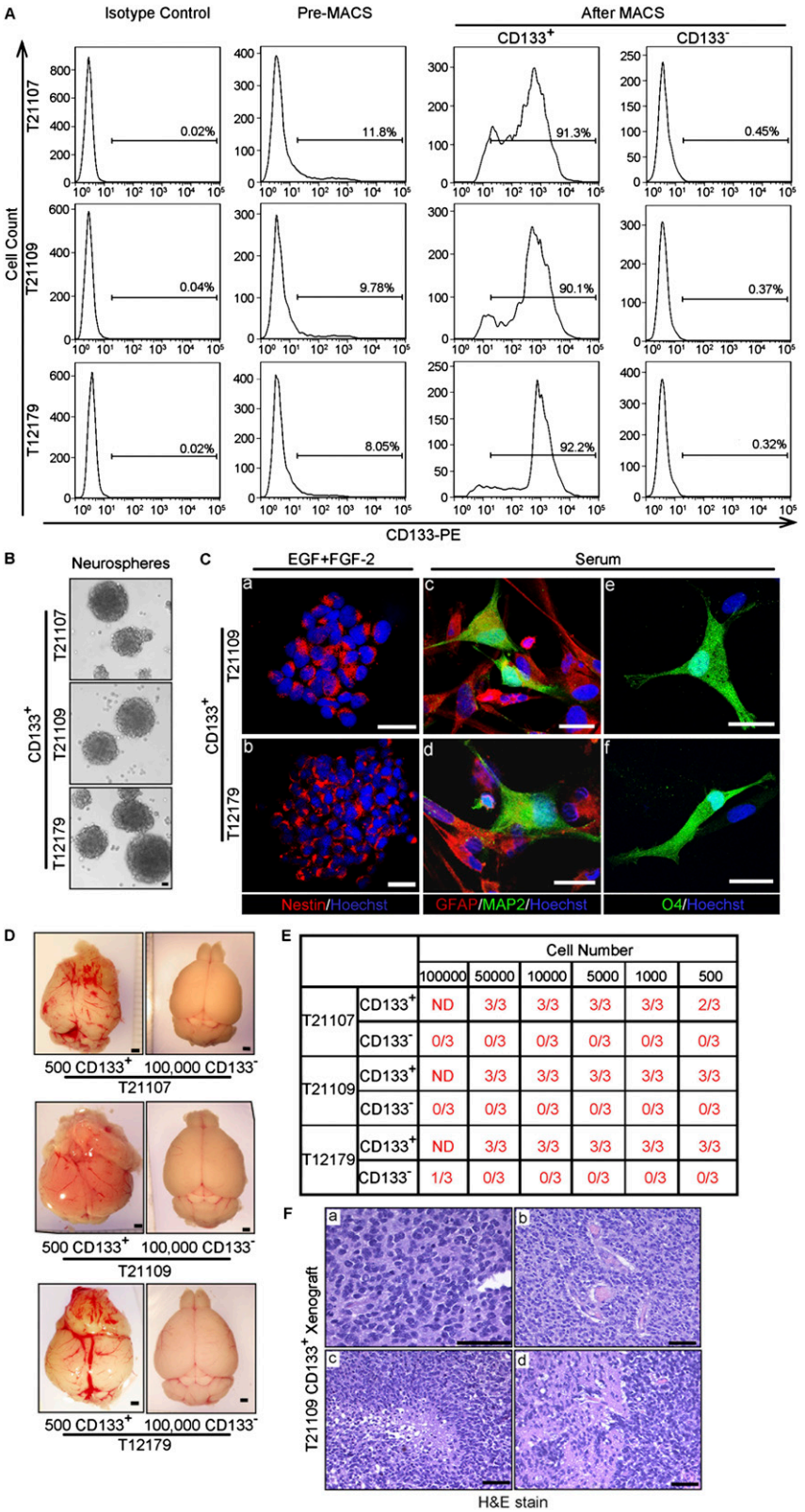

Fig. S1. Characterization of CD133$^+$ and CD133$^-$ cells from human GBM specimens. (*A*) FACS analysis of CD133 expression before and after sorting of CD133$^+$ and CD133$^-$ fractions. CD133$^+$ cells were isolated from freshly dissociated GBM samples T21107, T21109, and T12179 by magnetic beads. The percentage CD133$^+$ cells were determined by FACS analysis relative to cells labeled with a fluorophore-conjugated IgG isotype control antibody. (*B*) Representative images of neurospheres from CD133$^+$ cells isolated from GBM specimens (T21107, T21109, and T12179). (Scale bar, 10 μm.) (*C*, *a* and *b*) CD133$^+$ cells expressed neural stem cell marker Nestin (red), as assessed by immunofluorescence. (*c*–*f*) Multilineage differentiation capacity of CD133$^+$ cells was evaluated by staining for the GFAP astrocyte marker (*c* and *d*, red), the MAP2 neuron marker (*c* and *d*, green), and the oligodendrocyte (O4) oligodendrocytic marker (*e* and *f*, green), shown

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as representative immunofluorescence images. Nuclei were counterstained with Hoechst 33258. EGF+FGF-2, supplemented DMEM/F12 condition; serum, the addition of serum. (Scale bars, 30 μm.) (*D–F*) An in vivo–limiting dilution tumor formation assay (using 100,000, 50,000, 10,000, 5,000, 1,000, or 500 cells per mouse) was performed to compare the tumor-initiating capacity of $CD133^+$ glioma cells with $CD133^-$ glioma cells. Mice were killed when they were moribund or 180 d after implantation. Tumor formation was determined by histology. (*D*) Gross histology demonstrates highly vascular tumors in tumors derived from 500 $CD133^+$ cells but not from 100,000 $CD133^-$ cells. (Scale bars, 1 mm.) (*E*) The table displays number of mice developing tumors. ND, not detected. (*F*) H&E staining of xenograft derived from 500 T21109 $CD133^+$ cells shows histological features of GBM, such as cellular pleomorphism (*a*), vascular proliferation (*b*), necrosis (*c*), and invasion into surrounding brain tissue (*d*). (Scale bars, 50 μm.) MACS, magnetic activated cell sorting.

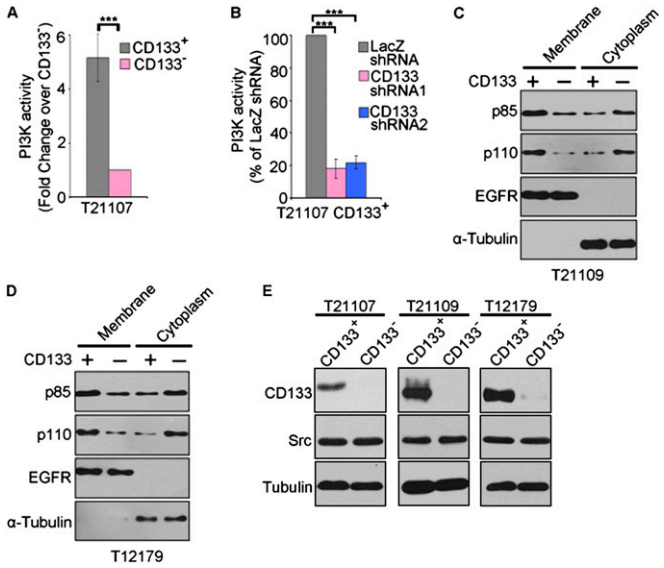


Fig. S2. CD133 regulates PI3K signaling. (*A*) The PI3K activity of $CD133^+$ cells versus $CD133^-$ cells derived from GBM specimen T21107 were assessed using a PI3 kinase ELISA kit. Values are normalized to that of matched $CD133^-$ cells. Results are expressed as mean ± SD from three separate experiments; $^{***}P < 0.001$. (*B*) Targeting CD133 decreases PI3K activity in $CD133^+$ cells. Seventy-two hours after lentivirus infection, the PI3K activity of T21107 $CD133^+$ infected with the indicated lentivirus were measured using a PI3 kinase ELISA kit. Values are normalized to that of $CD133^+$ cells treated with LacZ shRNA, which is set as 100%. Results are expressed as mean ± SD from three separate experiments; $^{***}P < 0.001$. (*C* and *D*) Membrane and cytoplasmic distribution of p85 and p110 in $CD133^+$ cells and $CD133^-$ cells derived from GBM specimens T21109 (*C*) or T12179 (*D*) was determined by immunoblotting. EGFR was used as the membrane marker, and α-Tubulin was used as the cytosolic marker. (*E*) The level of tyrosine kinase Src protein expression in matched $CD133^+$ and $CD133^-$ cells isolated from GBM samples. Whole-cell lysates were analyzed by Western blotting. Tubulin was blotted as a loading control.

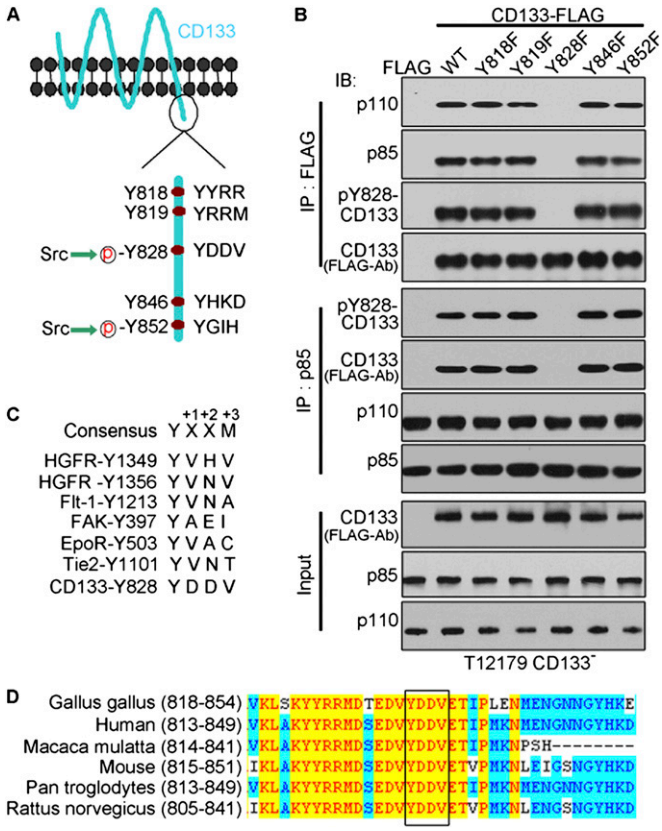
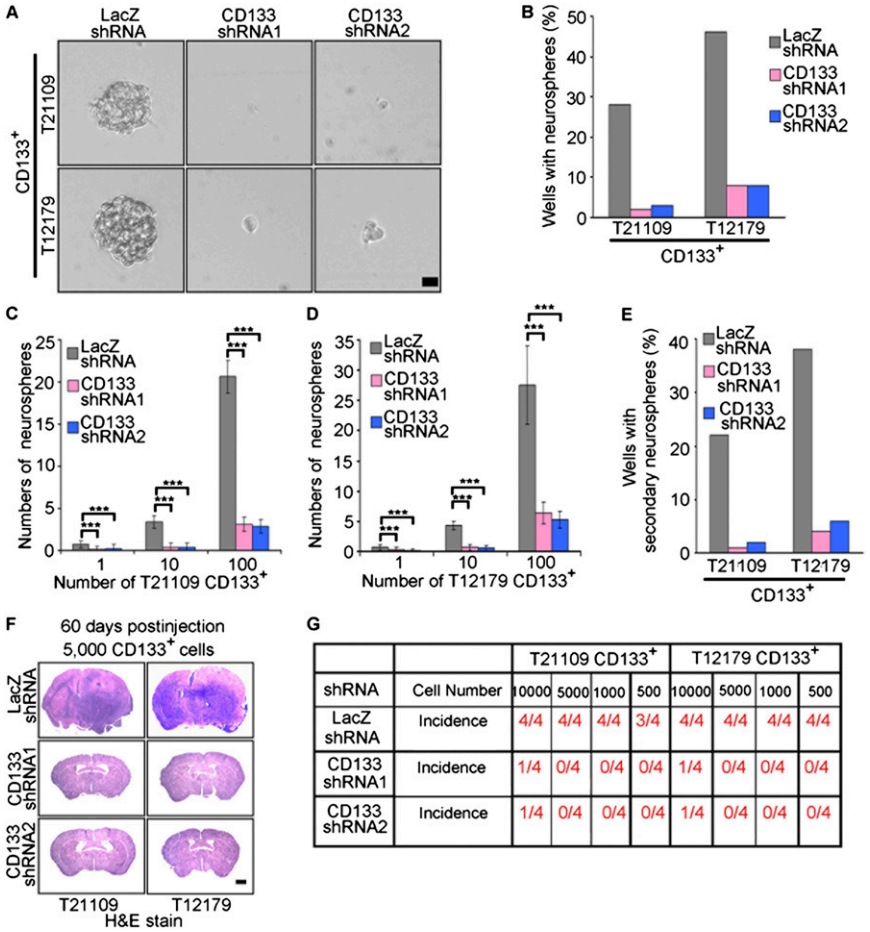


Fig. S3. Y828 phosphorylation of CD133 mediates the interaction between CD133 and p85. (*A*) Schematic illustration of relative positions of tyrosine residues in CD133 C-terminal cytoplasmic domain. Y819RRM conforms to the known p85 consensus-binding motif YXXM. Y828 and Y852 are phosphorylated by Src family kinases. (*B*) Coimmunoprecipitation analysis to determine the effect of Y828 mutation on the interaction between CD133 and p85. The lysates of CD133^{-} cells expressing wild-type CD133 or its mutants were subjected to IP using anti-CD133 or anti-p85 antibodies, followed by immunoblotting with anti-CD133, anti-p110, anti-p85, anti-FLAG, or anti-pY828-CD133 antibodies. (*C*) Comparison of amino acid residues flanking tyrosine 828 of CD133 with consensus- and nonconsensus p85–binding motifs. Two nonconsensus p85–binding motifs on the hepatocyte growth factor receptor (HGFR) as well as those on the endothelium-specific vascular endothelial growth factor receptor Flt-1, focal adhesion kinase (FAK), the erythropoietin receptor (EpoR), and angiopoietin receptor Tie2 are shown in comparison with residues flanking Y828 of CD133. There is a strong preference for hydrophobic or neutral residues in the Y +1 and Y +3 positions. Amino acid residues are designated according to the single-letter code: A, alanine; C, cysteine; E, glutamate; H, histidine; I, isoleucine; M, methionine; N, glutamine; V, valine; Y, tyrosine; D, aspartic acid; X, any amino acid residue. (*D*) Sequence alignment of CD133 C-terminal sequence shows amino acid conservation. Invariant residues are represented by yellow highlighting. Variant residues are represented by azure letters. The YDDV sequence shown in box is conserved in orthologs from Gallus gallus to Human.



		T21109 CD133+				T12179 CD133+			
shRNA	Cell Number	10000	5000	1000	500	10000	5000	1000	500
LacZ shRNA	Incidence	4/4	4/4	4/4	3/4	4/4	4/4	4/4	4/4
CD133 shRNA1	Incidence	1/4	0/4	0/4	0/4	1/4	0/4	0/4	0/4
CD133 shRNA2	Incidence	1/4	0/4	0/4	0/4	1/4	0/4	0/4	0/4

Fig. S4. CD133 knockdown suppresses neurosphere formation and tumor formation of CD133$^+$ glioma cells. (*A* and *B*) Single-cell neurosphere formation assay of CD133$^+$ cells with CD133 knockdown. CD133$^+$ cells infected with the indicated shRNA lentivirus were plated at 1 cell/well in 96-well plates. Ten days later, neurospheres were counted and representative pictures were taken. (*A*) Representative images of spheres are shown. (*B*) Data are mean of the percentage of neurosphere-containing wells in each group. (Scale bar, 10 μm.) (*C* and *D*) Bulk-culture neurosphere formation assay of CD133$^+$ cells with CD133 knockdown. A total of 1, 10, or 100 CD133$^+$ cells infected with LacZ shRNA, CD133 shRNA1, or CD133 shRNA2 lentivirus were cultured in 96-well plates. Ten days later, the number of neurospheres per well was counted. The total number of neurospheres per well is significantly decreased with CD133 knock down. Results are expressed as mean ± SD from three separate experiments; ***$P < 0.001$. (*E*) Secondary neurosphere formation assay of CD133$^+$ cells infected with LacZ shRNA, CD133 shRNA1, or CD133 shRNA2 lentivirus. Data are mean of the percentage of neurosphere-containing wells in each group. Targeting CD133 in CD133$^+$ cells decreases the secondary neurosphere formation efficiency. (*F*) Targeting CD133 in CD133$^+$ cells decreases tumorigenesis. A total of 5,000 CD133$^+$ cells infected with the indicated lentivirus was injected into mice brains. Sixty days after injection, histological section of mouse brain shows tumors formation by cells infected with LacZ shRNA but not CD133 shRNAs. (*G*) GSCs are less tumorigenic when CD133 are targeted. In vivo–limiting dilution tumor formation assay (using 10,000, 5,000, 1,000, and 500 cells per mouse) was performed using CD133$^+$ cells expressing LacZ shRNA, CD133 shRNA1, or CD133 shRNA2. Mice were killed when they were maintained up to 180 d or moribund after implantation. Tumor formation was determined by histology. The table displays number of mice developing tumors.

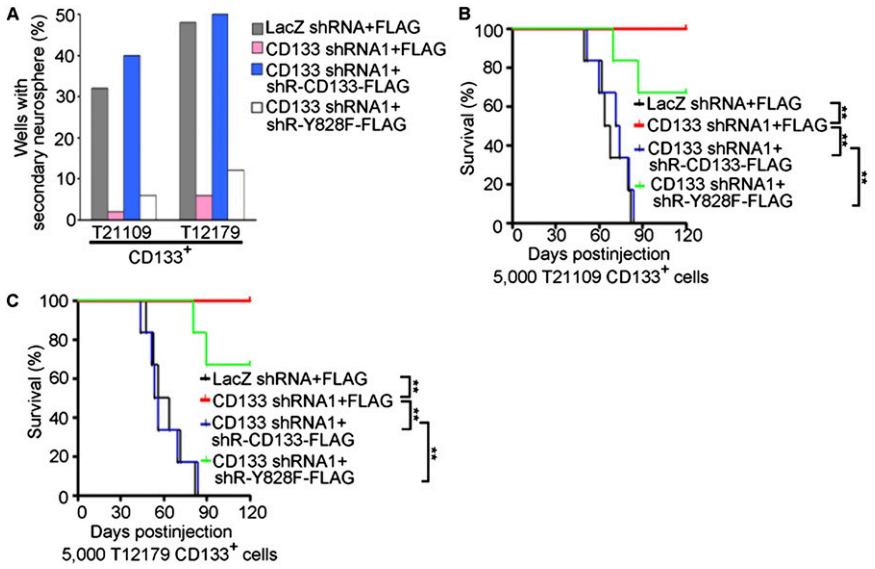


Fig. S5. Y828 phosphorylation is required for CD133 maintaining glioma stem cell tumorigenesis. (*A*) The secondary neurosphere formation efficiency of $CD133^+$ cells expressing LacZ shRNA, CD133 shRNA1, CD133 shRNA1+shRNA-resistant WT CD133, or CD133 shRNA1+shRNA-resistant Y828F. Data are mean of the percentage of neurosphere-containing wells in each group. (*B* and *C*) $CD133^+$ cells expressing LacZ shRNA, CD133 shRNA1, CD133 shRNA1+shRNA-resistant WT CD133, or CD133 shRNA1+shRNA-resistant Y828F were intracranially implanted into immunocompromised mice brain (5,000 cells per mouse). Mice were killed when they were moribund or 180 d after implantation. Survival of mice ($n = 6$) was evaluated by Kaplan-Meier analysis (**$P < 0.01$, log rank test).

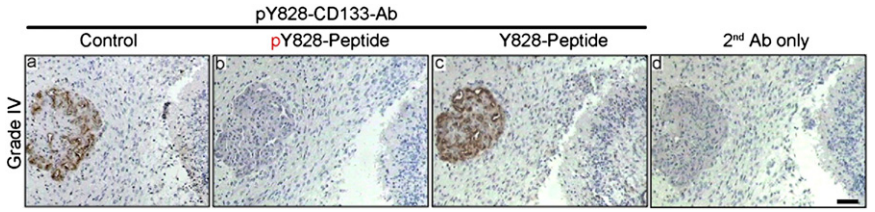


Fig. S6. Rabbit polyclonal anti-pY828-CD133 antibody (Ab) specifically recognizes pY828-CD133 in GBM specimens. Paraffin sections from a human GBM were stained with the anti-pY828-CD133 antibody preincubated with vehicle control (*A*), phospho-Y828 peptide (pY828-peptide) (*B*), or unphosphorylated Y828 peptide (Y828-peptide) (*C*). Section processed with omission of primary antibody served as the negative control (*D*). (Scale bar, 50 μm.)

Table S1. Pathologic and cytogenetic characteristics of brain tumors related to the experimental procedures

Specimen	Age (y)	Sex	Pathologic histology	Subtype	Primary/ recurrence	Tumor features				
						MIB1, %	PTEN	P85	P110	MGMT
T21107	74	F	GBM	NL	Primary	35	WT	WT	WT	Positive
T21109	45	F	GBM	PN	Primary	40	WT	WT	WT	Positive
T12179	68	F	GBM	Mes	Primary	45	LOSS	WT	WT	Positive
T18756	66	M	GBM	PN	Primary					
T22588	32	M	GBM	Mes	Primary					
T22122	46	M	GBM	Mes	Primary					
T228490	44	F	GBM	NL	Primary					
T12013	59	M	GBM							
T10140	34	M	GBM							
T12129	73	M	GBM							
T11278	59	M	GBM							
T11293	58	M	GBM							
T12328	45	F	GBM							
T12260	67	M	GBM							
T12173	78	F	GBM							
T12152	53	M	GBM							
T09217	41	F	GBM							
T12022	56	M	GBM							
T11149	68	F	GBM							

Patient and pathological information associated with brain tumor samples is provided. LOSS, deletion; Mes, mesenchymal; MGMT, O-6-methylguanine-DNA methyltransferase; MIB, Ki-67; NL, neural; PN, proneural; PTEN, phosphatase and tensin homolog.