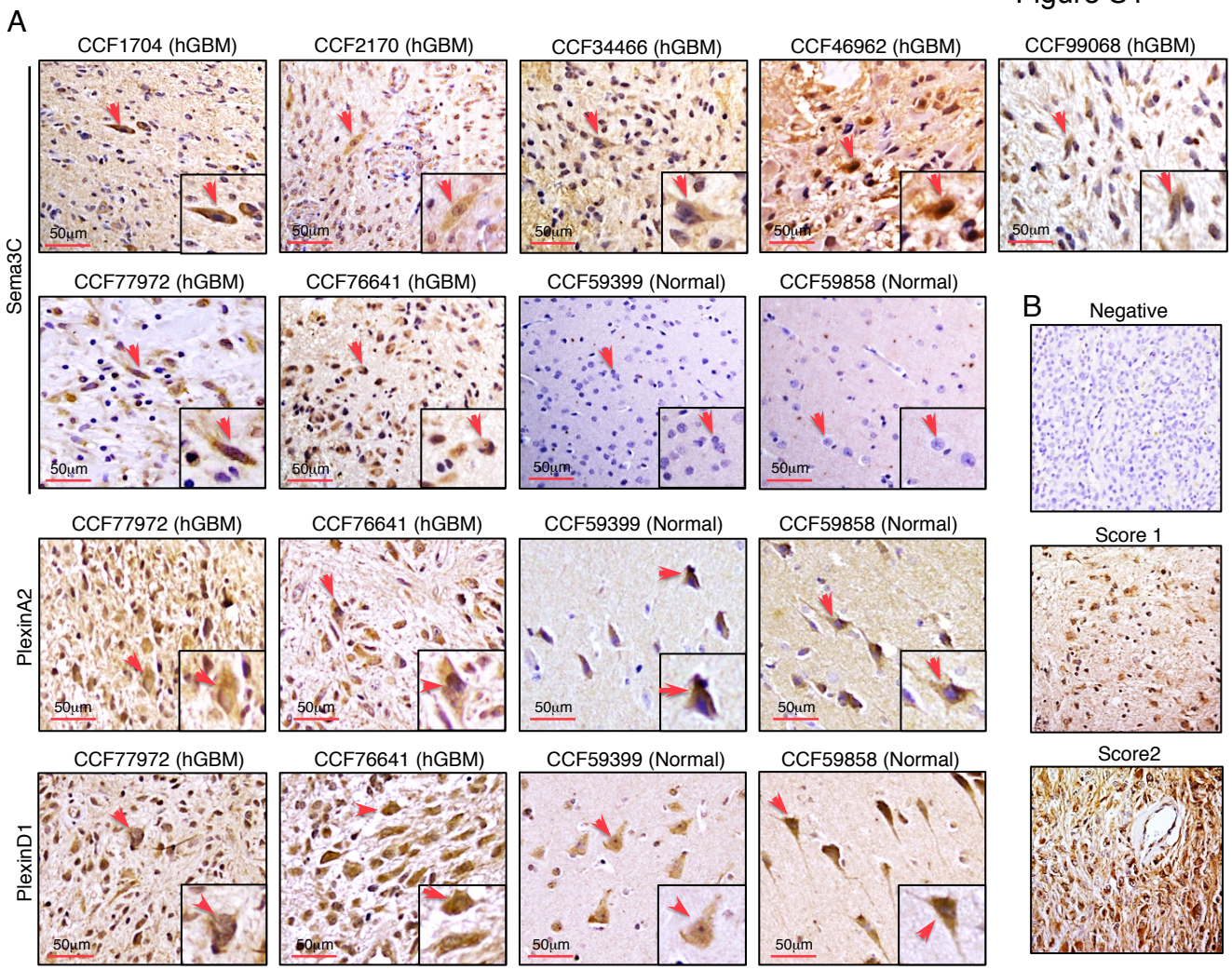
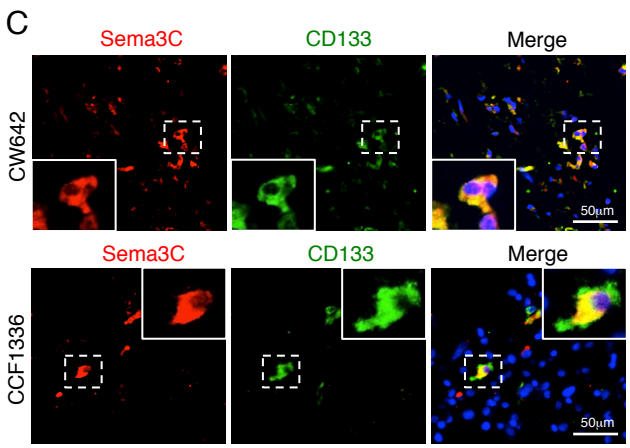
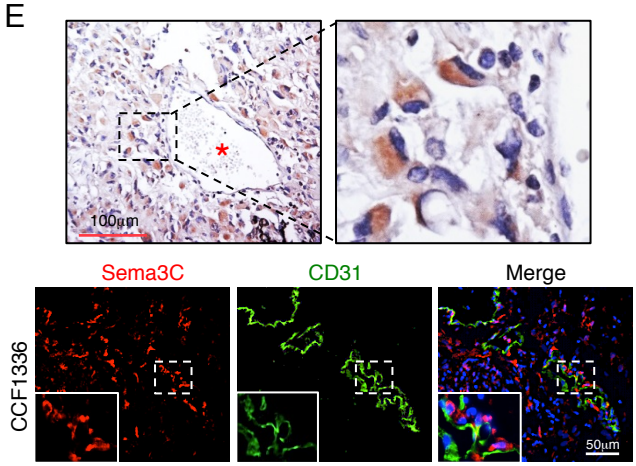
Figure S1


A
CCF1704 (hGBM)
CCF2170 (hGBM)
CCF34466 (hGBM)
CCF46962 (hGBM)
CCF99068 (hGBM)
Sema3C
CCF77972 (hGBM)
CCF76641 (hGBM)
CCF59399 (Normal)
CCF59858 (Normal)
PlexinA2
PlexinD1
50μm
B
Negative
Score 1
Score2


C
Sema3C
CD133
Merge
CW642
CCF1336
50μm


E
100μm
Sema3C
CD31
Merge
CCF1336
50μm

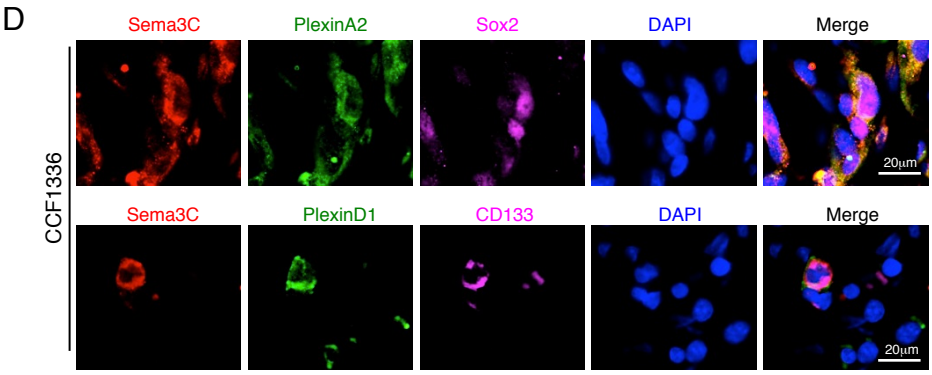

D
Sema3C
PlexinA2
Sox2
DAPI
Merge
CCF1336
20μm
Sema3C
PlexinD1
CD133
DAPI
Merge
20μm

Figure S2

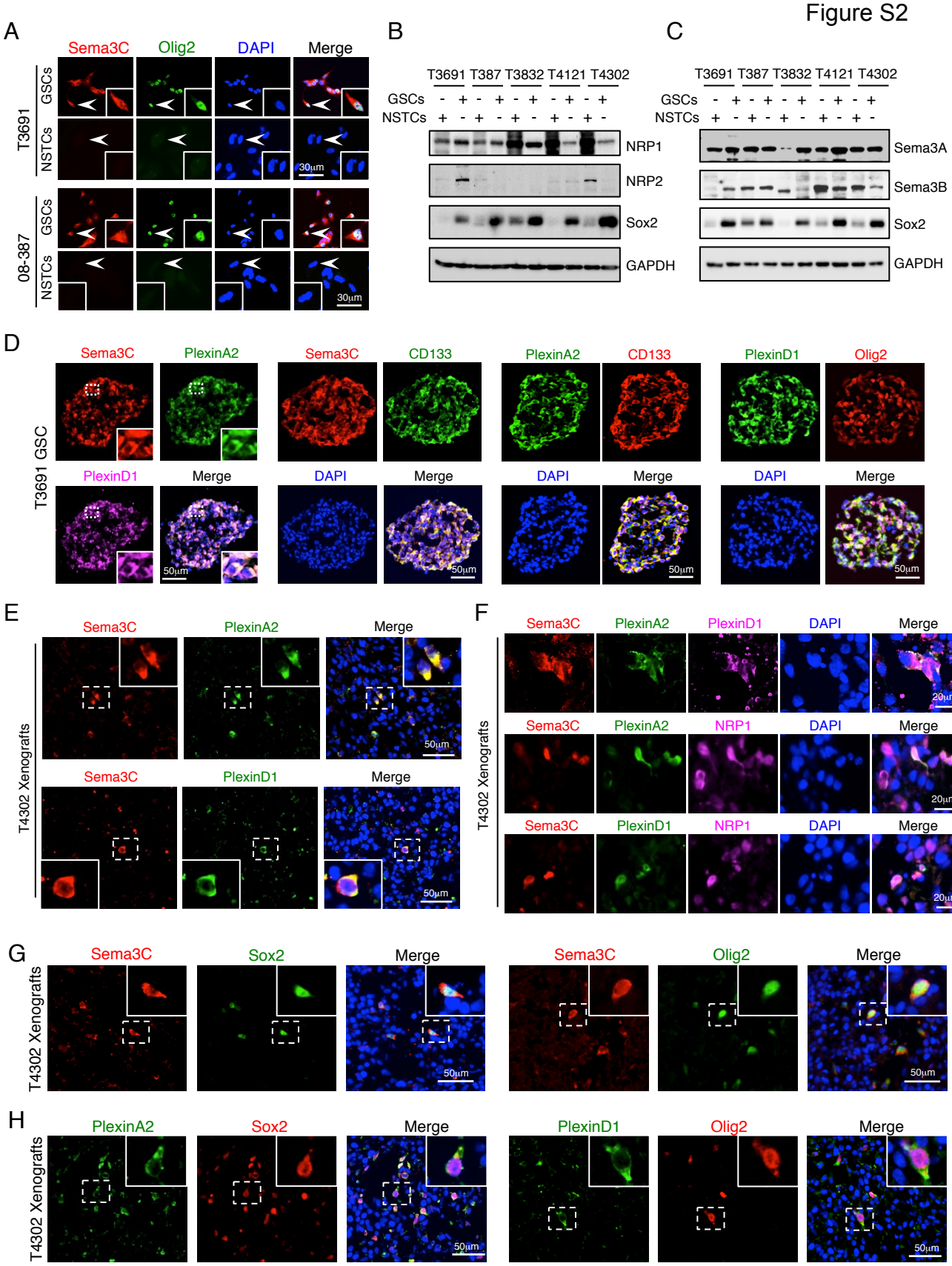

A
Sema3C
Olig2
DAPI
Merge
T3691
GSCs
NSTCs
08-387
30μm
B
T3691 T387 T3832 T4121 T4302
GSCs
NSTCs
NRP1
NRP2
Sox2
GAPDH
C
T3691 T387 T3832 T4121 T4302
GSCs
NSTCs
Sema3A
Sema3B
Sox2
GAPDH
D
T3691 GSC
Sema3C
PlexinA2
CD133
PlexinD1
Olig2
Merge
DAPI
50μm
E
T4302 Xenografts
Sema3C
PlexinA2
PlexinD1
Merge
50μm
F
T4302 Xenografts
Sema3C
PlexinA2
PlexinD1
NRP1
DAPI
Merge
20μm
G
T4302 Xenografts
Sema3C
Sox2
Olig2
Merge
50μm
H
T4302 Xenografts
PlexinA2
Sox2
PlexinD1
Olig2
Merge
50μm

Figure S2

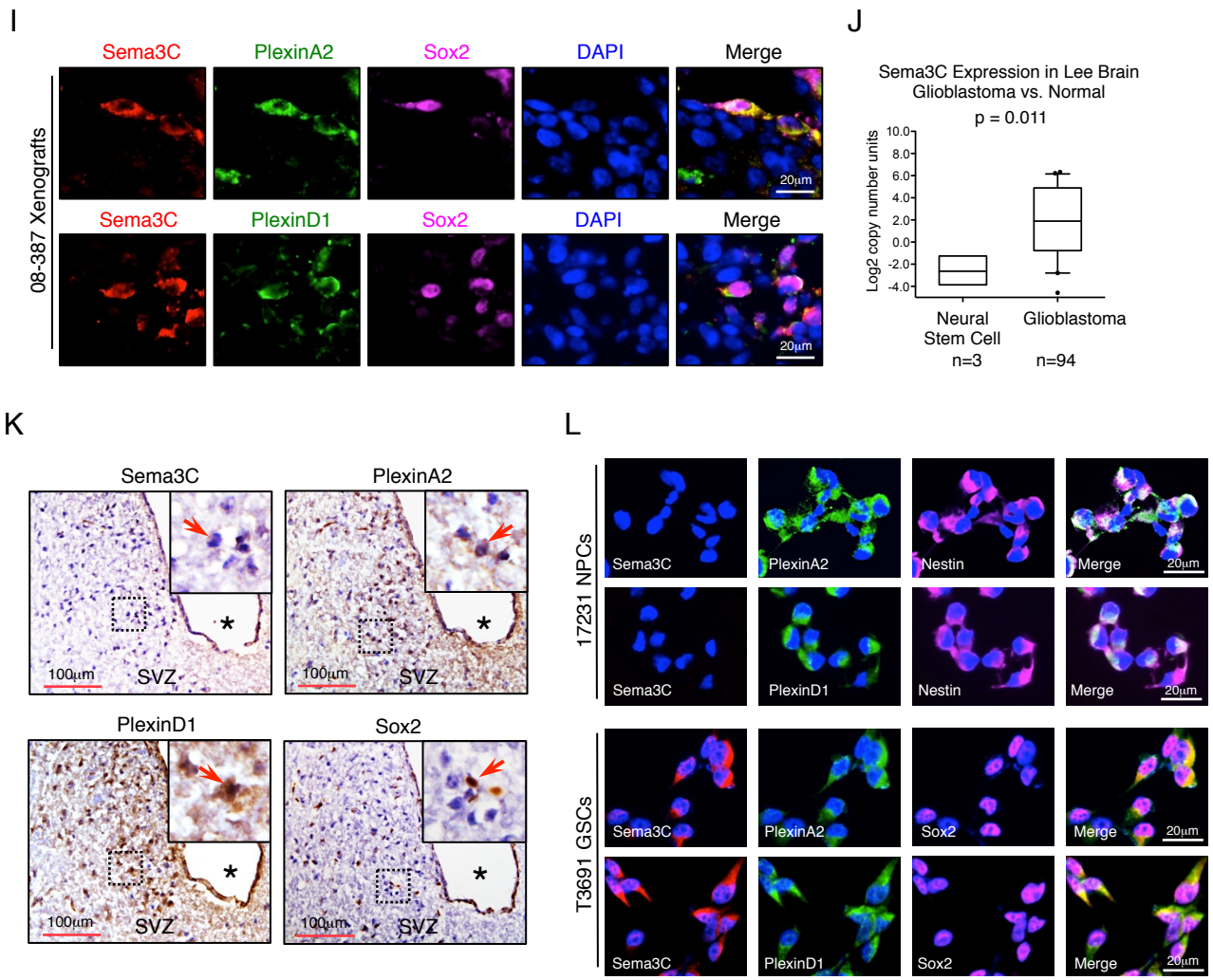

I
Sema3C
PlexinA2
Sox2
DAPI
Merge
08-387 Xenografts
20μm
Sema3C
PlexinD1
Sox2
DAPI
Merge
20μm
J
Sema3C Expression in Lee Brain Glioblastoma vs. Normal
p = 0.011
Log2 copy number units
10.0
8.0
6.0
4.0
2.0
0.0
-2.0
-4.0
Neural Stem Cell
n=3
Glioblastoma
n=94
K
Sema3C
PlexinA2
100μm
SVZ
PlexinD1
Sox2
L
17231 NPCs
T3691 GSCs
Sema3C
PlexinA2
Nestin
Merge
20μm
PlexinD1
Sox2

Figure S3

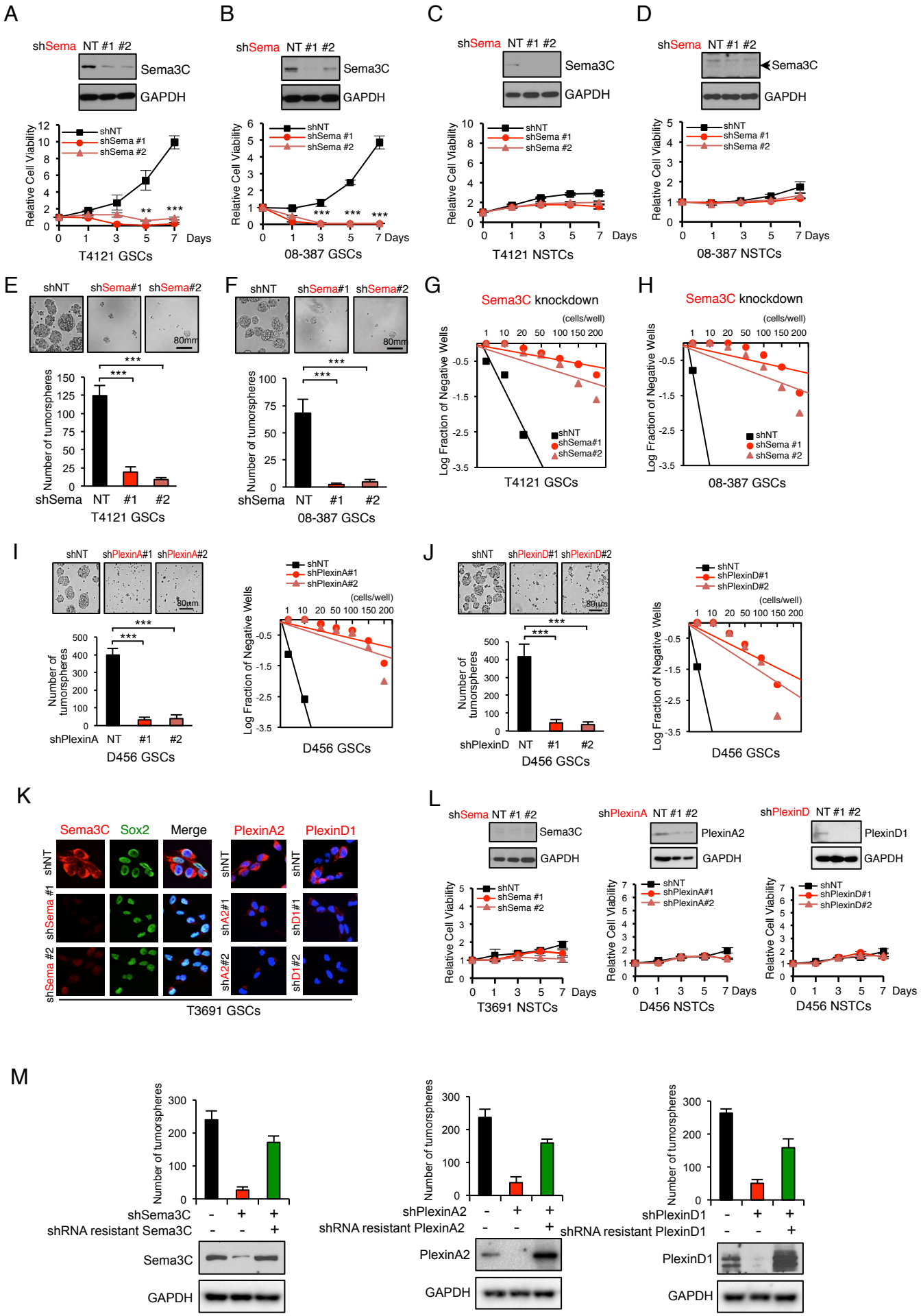

A
shSema NT #1 #2
Sema3C
GAPDH
Relative Cell Viability
shNT
shSema #1
shSema #2
Days
T4121 GSCs
B
08-387 GSCs
C
T4121 NSTCs
D
08-387 NSTCs
E
shNT
shSema#1
shSema#2
80mm
Number of tumorspheres
shSema NT #1 #2
F
G
Sema3C knockdown
(cells/well)
Log Fraction of Negative Wells
H
I
shPlexinA NT #1 #2
shPlexinA#1
shPlexinA#2
D456 GSCs
J
shPlexinD NT #1 #2
shPlexinD#1
shPlexinD#2
K
Sema3C
Sox2
Merge
PlexinA2
PlexinD1
T3691 GSCs
L
shPlexinA
shPlexinD
T3691 NSTCs
D456 NSTCs
M
shSema3C
shRNA resistant Sema3C
shPlexinA2
shRNA resistant PlexinA2
shPlexinD1
shRNA resistant PlexinD1
PlexinA2
PlexinD1

Figure S3

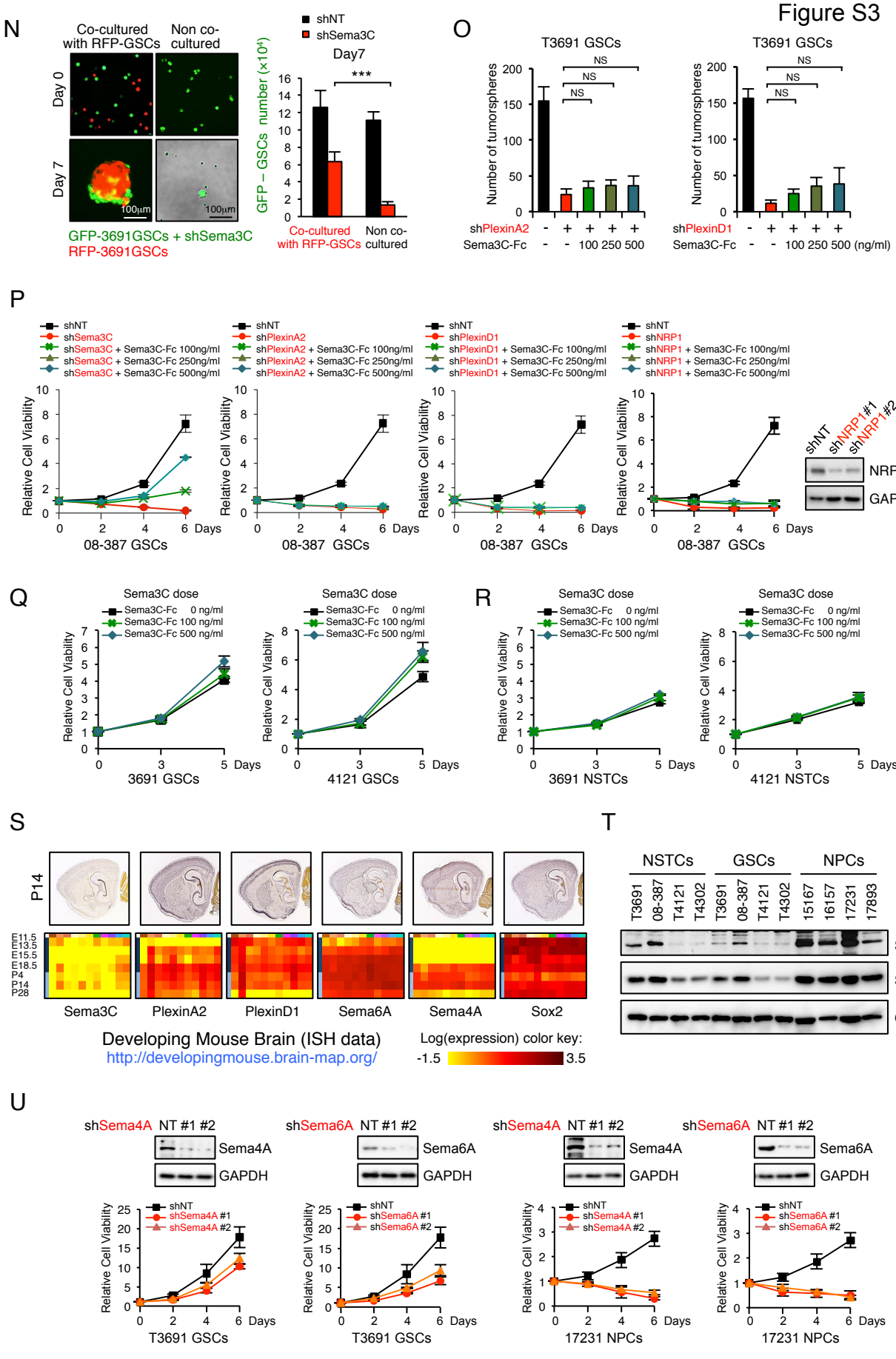

N
Co-cultured with RFP-GSCs
Non co-cultured
Day 0
Day 7
100μm
GFP-3691GSCs + shSema3C
RFP-3691GSCs
shNT
shSema3C
Day7
***
GFP – GSCs number (×10⁴)
Co-cultured with RFP-GSCs
Non co-cultured
O
T3691 GSCs
Number of tumorspheres
NS
shPlexinA2 - + + + +
Sema3C-Fc - - 100 250 500
shPlexinD1 - + + + +
Sema3C-Fc - - 100 250 500 (ng/ml)
P
shNT
shSema3C
shSema3C + Sema3C-Fc 100ng/ml
shSema3C + Sema3C-Fc 250ng/ml
shSema3C + Sema3C-Fc 500ng/ml
shPlexinA2
shPlexinA2 + Sema3C-Fc 100ng/ml
shPlexinA2 + Sema3C-Fc 250ng/ml
shPlexinA2 + Sema3C-Fc 500ng/ml
shPlexinD1
shPlexinD1 + Sema3C-Fc 100ng/ml
shPlexinD1 + Sema3C-Fc 250ng/ml
shPlexinD1 + Sema3C-Fc 500ng/ml
shNRP1
shNRP1 + Sema3C-Fc 100ng/ml
shNRP1 + Sema3C-Fc 250ng/ml
shNRP1 + Sema3C-Fc 500ng/ml
Relative Cell Viability
Days
08-387 GSCs
shNT
shNRP1#1
shNRP1#2
NRP1
GAPDH
Q
Sema3C dose
Sema3C-Fc 0 ng/ml
Sema3C-Fc 100 ng/ml
Sema3C-Fc 500 ng/ml
3691 GSCs
4121 GSCs
R
3691 NSTCs
4121 NSTCs
S
P14
E11.5
E13.5
E15.5
E18.5
P4
P14
P28
Sema3C
PlexinA2
PlexinD1
Sema6A
Sema4A
Sox2
Developing Mouse Brain (ISH data)
http://developingmouse.brain-map.org/
Log(expression) color key:
-1.5
3.5
T
NSTCs
GSCs
NPCs
T3691
08-387
T4121
T4302
T3691
08-387
T4121
T4302
15167
16157
17231
17893
Sema4A
Sema6A
GAPDH
U
shSema4A NT #1 #2
Sema4A
GAPDH
shSema6A NT #1 #2
Sema6A
GAPDH
shNT
shSema4A #1
shSema4A #2
shSema6A #1
shSema6A #2
T3691 GSCs
17231 NPCs

Figure S4

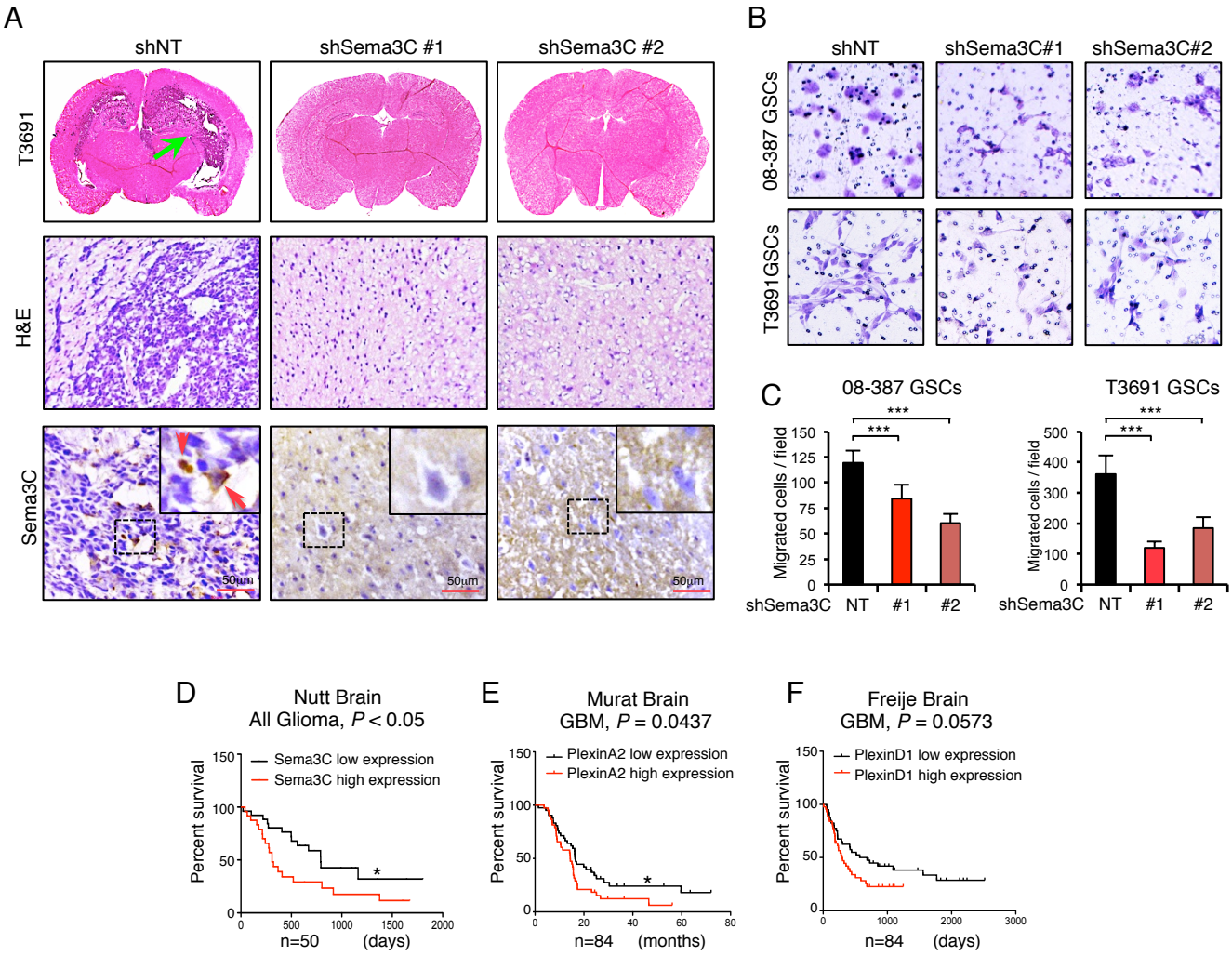

A
shNT
shSema3C #1
shSema3C #2
T3691
H&E
Sema3C
50μm
B
shNT
shSema3C#1
shSema3C#2
08-387 GSCs
T3691GSCs
C
08-387 GSCs
T3691 GSCs
Migrated cells / field
***
shSema3C NT #1 #2
D
Nutt Brain
All Glioma, P < 0.05
Sema3C low expression
Sema3C high expression
Percent survival
n=50 (days)
E
Murat Brain
GBM, P = 0.0437
PlexinA2 low expression
PlexinA2 high expression
n=84 (months)
F
Freije Brain
GBM, P = 0.0573
PlexinD1 low expression
PlexinD1 high expression
n=84 (days)

Figure S5

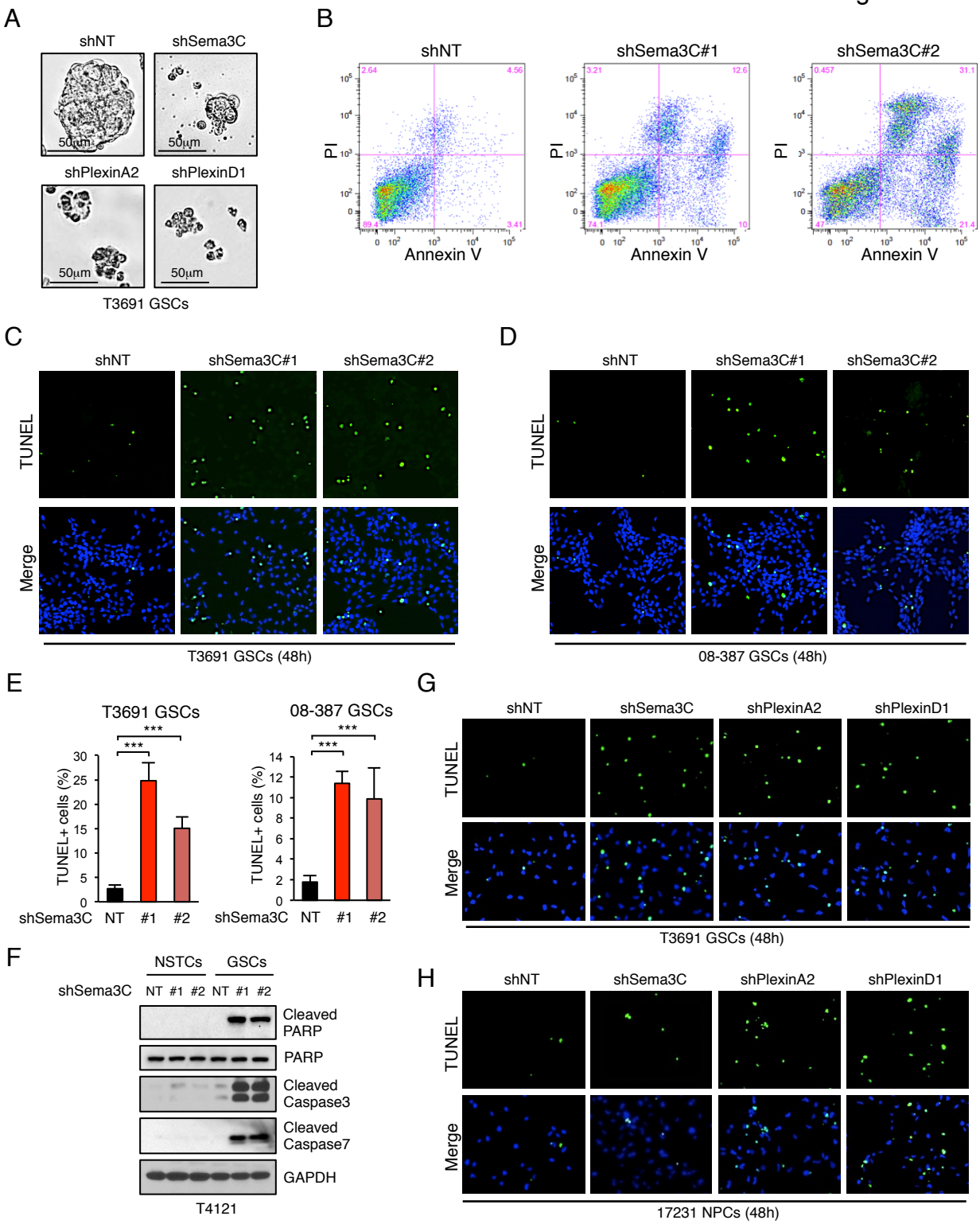

A
shNT
shSema3C
shPlexinA2
shPlexinD1
50μm
T3691 GSCs
B
shNT
shSema3C#1
shSema3C#2
PI
Annexin V
C
shNT
shSema3C#1
shSema3C#2
TUNEL
Merge
T3691 GSCs (48h)
D
shNT
shSema3C#1
shSema3C#2
TUNEL
Merge
08-387 GSCs (48h)
E
T3691 GSCs
08-387 GSCs
TUNEL+ cells (%)
***
shSema3C NT #1 #2
F
NSTCs
GSCs
shSema3C NT #1 #2 NT #1 #2
Cleaved PARP
PARP
Cleaved Caspase3
Cleaved Caspase7
GAPDH
T4121
G
shNT
shSema3C
shPlexinA2
shPlexinD1
TUNEL
Merge
T3691 GSCs (48h)
H
shNT
shSema3C
shPlexinA2
shPlexinD1
TUNEL
Merge
17231 NPCs (48h)

Figure S6

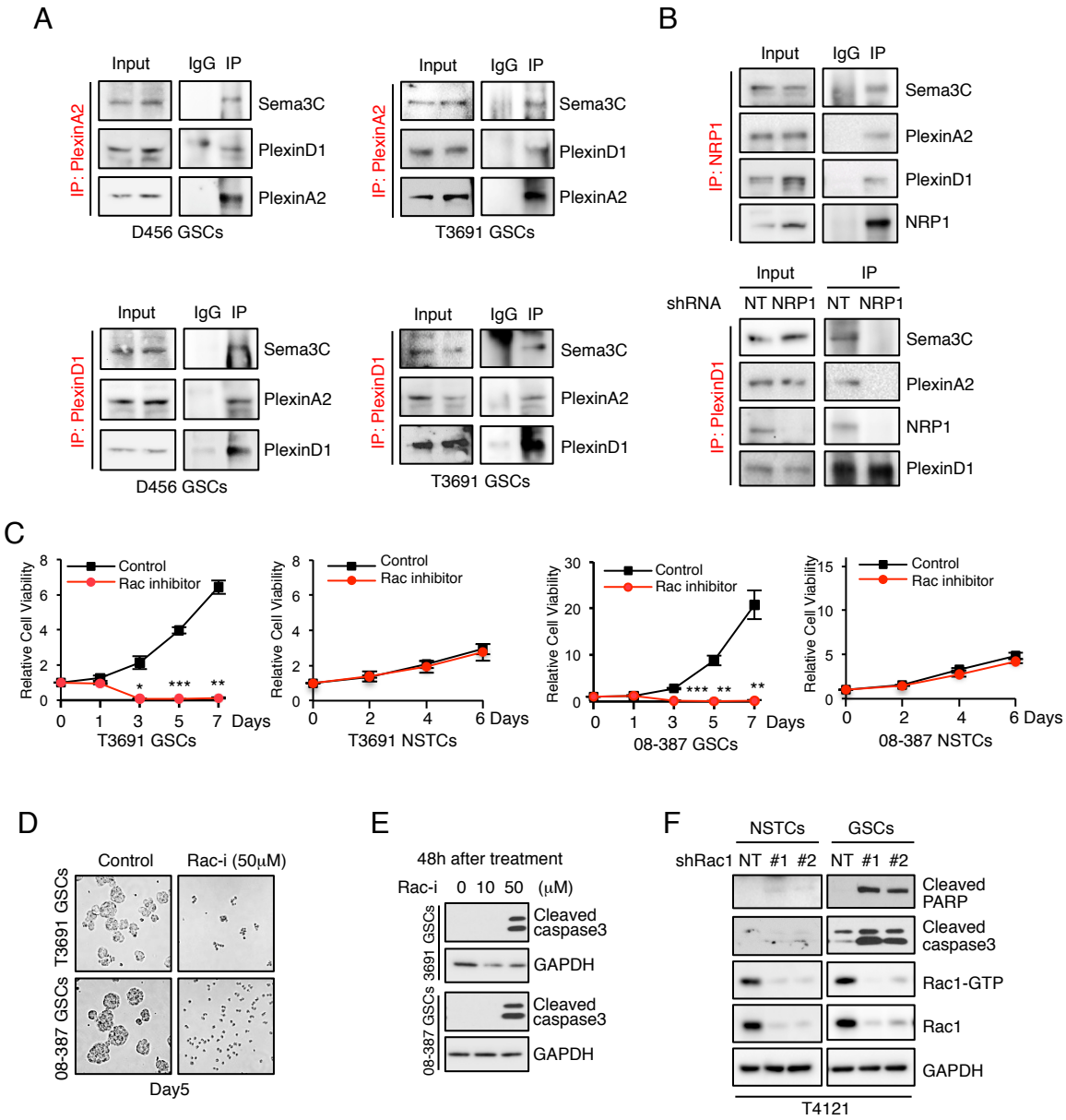
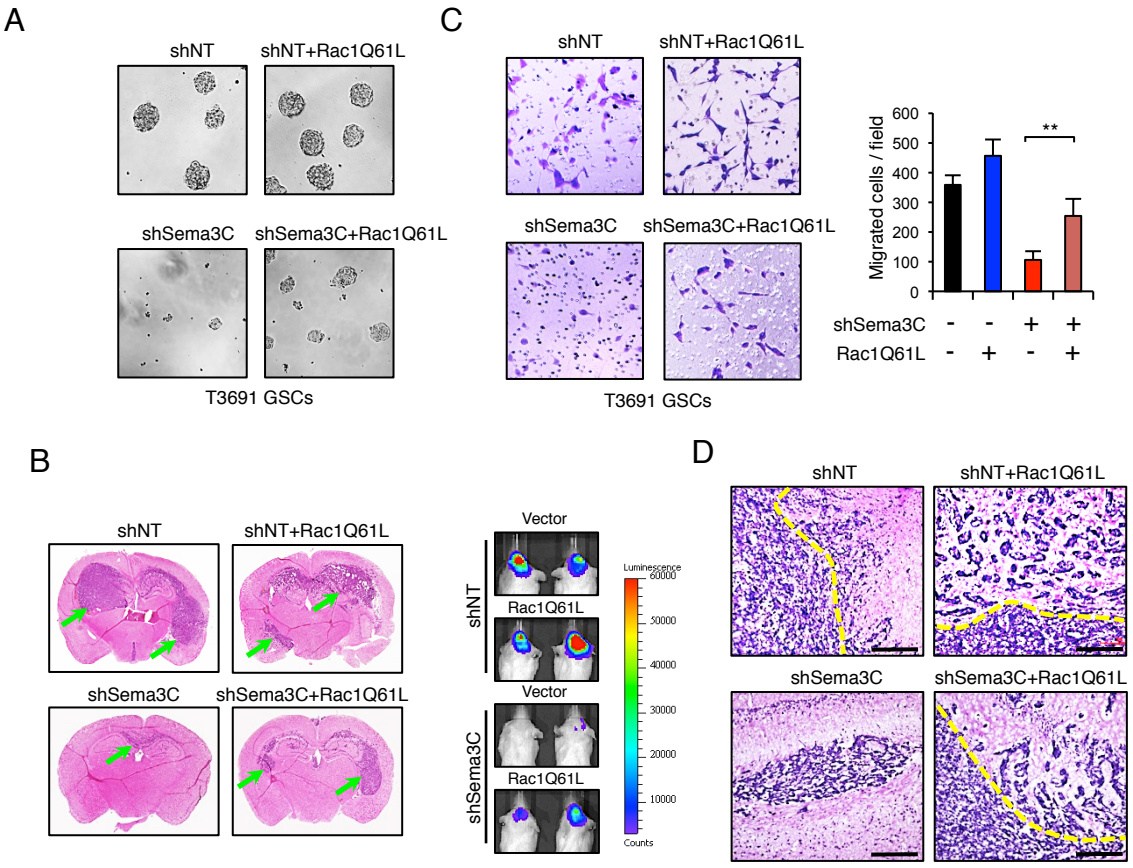

A
Input
IgG IP
IP: PlexinA2
Sema3C
PlexinD1
PlexinA2
D456 GSCs
T3691 GSCs
IP: PlexinD1
B
IP: NRP1
NRP1
shRNA
NT NRP1
C
Control
Rac inhibitor
Relative Cell Viability
Days
T3691 GSCs
T3691 NSTCs
08-387 GSCs
08-387 NSTCs
D
Control
Rac-i (50μM)
Day5
E
48h after treatment
Rac-i 0 10 50 (μM)
Cleaved caspase3
GAPDH
F
NSTCs
GSCs
shRac1 NT #1 #2
Cleaved PARP
Rac1-GTP
Rac1
T4121

Figure S7


A
shNT
shNT+Rac1Q61L
shSema3C
shSema3C+Rac1Q61L
T3691 GSCs
C
shNT
shNT+Rac1Q61L
shSema3C
shSema3C+Rac1Q61L
T3691 GSCs
Migrated cells / field
600
500
400
300
200
100
0
**
shSema3C - - + +
Rac1Q61L - + - +
B
shNT
shNT+Rac1Q61L
shSema3C
shSema3C+Rac1Q61L
Vector
Rac1Q61L
Luminescence
60000
50000
40000
30000
20000
10000
Counts
D
shNT
shNT+Rac1Q61L
shSema3C
shSema3C+Rac1Q61L

SUPPLEMENTAL DATA

Figure S1, related to Figure 1. Sema3C and Its Receptors Are Co-expressed In Stem Cell Marker+ GBM Cells

(A) Immunohistochemical (IHC) staining of Sema3C, PlexinA2 and PlexinD1 in human primary glioblastoma (GBM) and normal brain. Paraffin-embedded sections were stained with specific anti-Sema3C, anti-PlexinA2 or anti-PlexinD1 antibody, detected using an ABC kit, and then counterstained with hematoxylin.

(B) Representative images of Sema3C staining intensities used for scoring a human GBM tissue array.

(C and D) Immunofluorescence (IF) staining of Sema3C, PlexinA2/D1 and glioma stem cell (GSC) markers CD133 and Sox2 in frozen sections of human primary GBM. Nuclei were counterstained with DAPI (blue).

(E) IHC staining of Sema3C (up) or IF staining of Sema3C in relation to blood vessels (CD31) (down) in human primary GBM tissues. Asterisk denotes vessel lumen.

Figure S2, related to Figure 2. GSCs Preferentially Co-express Sema3C and Its Receptors

(A) IF staining of Sema3C with GSC marker Olig2 in GSCs and matched non-stem tumor cells (NSTCs). GSCs derived from T3691 and 08-387 GBM were cultured as an attached monolayer. Sema3C was labeled in red, Olig2 in green. Nuclei were counterstained with DAPI (blue).

(B and C) Immunoblot (IB) analysis of NRP1 and NRP2 (B), Sema3A and Sema3B (C) proteins in GSCs and matched NSTCs derived from five different GBM.

(D) IF staining of Sema3C, PlexinA2, PlexinD1, and GSC markers including CD133 and Olig2 in GSC tumorspheres. Nuclei were counterstained with DAPI (blue).

(E and F) Co-localization of Sema3C, PlexinA2/D1 and NRP1 in a subpopulation of tumor cells in GSC-derived GBM xenografts.

(G-I) IF staining of Sema3C, PlexinA2/D1 and GSC markers including Sox2 and Olig2 in GSC-derived GBM xenografts. Nuclei were counterstained with DAPI (blue).

(J) Sema3C mRNA expression in human neural stem cells and GBM were examined using the Oncomine database.

(K) IHC staining of Sema3C, PlexinA2, PlexinD1 and Sox2 in the subventricular zone (SVZ) in adult mouse brain. Sections were counterstained with hematoxylin. * indicates the ventricle.

(L) IF staining of Sema3C (red), PlexinA2/D1 (green) and Sox2 or the neural progenitor cell (NPC) marker Nestin (purple) in GSCs (T3691) and NPCs (17231). Nuclei were counterstained with DAPI (blue).

Figure S3, related to Figure 3. GSC Viability and Self-Renewal Depend on Sema3C Secretion

(A-H) Effects of Sema3C knockdown by two different target shRNAs on cell viability in GSCs (A and B) and matched NSTCs (C and D), tumorsphere formation capacity of GSCs (E-H). Cells transduced with shNT or shSema3C were plated in triplicate wells (1×10^3 per well), and then assessed by cell titer assay for cell viability (A-D). Representative images and number of tumorspheres derived from GSCs expressing shNT or shSema3C are shown (E and F). For the limiting dilution assay, GSCs expressing shNT or shSema3C were plated into 96-well plates with various seeding densities (1–200 cells per well, 12 wells per each condition) (G and H). Seven days later, each well was evaluated for the presence or absence of tumorspheres.

(I-J) Effects of PlexinA2 (I) or PlexinD1 (J) knockdown with two different shRNA sequences on tumorsphere formation capacity of GSCs. The limiting dilutation assay was performed as above in G-H.

(K) IF staining of Sema3C, PlexinA2 or PlexinD1 in GSCs transduced with shNT and two different shRNAs targeting Sema3C, PlexinA2 or PlexinD1. Nuclei were counterstained with DAPI (blue).

(L) Effects of Sema3C, PlexinA2 or PlexinD1 knockdown with two different shRNA sequences on cell viability of NSTCs.

(M) shRNA resistant mutants of Sema3C, PlexinA2 or PlexinD1 rescued self-renewal in T3691 GSCs in which Sema3C, PlexinA2 or PlexinD1 were knocked down, respectively.

(N) GFP-GSCs expressing shSema3C were co-cultured or not co-cultured with RFP-GSCs at a 1:1 ratio. GFP-GSCs were counted after 7 days co-culture (right). Representative images of mixed cells on day 0 and day 7 are shown (left).

(O) T3691 GSCs transduced with shNT (-), shPlexinA2 or shPlexinD1 were cultured with different doses of recombinant human Sema3C protein (Sema3C-Fc). GSC tumorsphere quantification is shown.

(P) 08-387 GSCs transduced with shNT, shSema3C, shPlexinA2, shPlexinD1 or shNRP1 were cultured with different doses of recombinant human Sema3C protein (Sema3C-Fc). GSC viability was assessed by cell titer assay.

(Q and R) Effects of recombinant human Sema3C protein (Sema3C-Fc) on cell viability of two different GSC populations (Q) and two different NSTC populations (R).

(S) In situ hybridization database (Developing Mouse Brain) [http://developingmouse.brain-map.org/] showed expression patterns of Sema3C, PlexinA2, PlexinD1, Sema4A, Sema6A and

Sox2 in developing mouse brain. Sema3C was expressed at lower levels than PlexinA2/D1, Sema4A/6A and Sox2 during brain development. ISH: In Situ Hybridization.

(T) Immunoblot (IB) analysis of Sema4A and Sema6A proteins in four GSCs, matched NSTCs and four human NPC lines.

(U) Effects of Sema4A or Sema6A knockdown with two different shRNA sequences on cell viability of GSCs and NPCs.

All data are means ± standard deviation (SD) (n = 3). **p < 0.01, ***p < 0.001.

Figure S4, related to Figure 4. Targeting Sema3C Suppresses GSC-Mediated Tumor Growth and Improves Animal Survival

(A) Representative images of cross-sections (hematoxylin and eosin [H&E] stained) of mouse brains bearing GSCs (T3619) transduced with shNT or shSema3C were harvested on day 33 after transplantation (A, top). Green arrow indicates a large tumor in the brain of a mouse transplanted with GSCs expressing shNT. Histological analysis of brain tumors derived from GSCs expressing shNT or shSema3C (A, middle). IHC staining of Sema3C in GBM xenografts derived from GSCs expressing shNT or shSema3C (A, bottom). Sections were counterstained with hematoxylin. Red arrows indicate Sema3C positive cells.

(B and C) Transwell migration assay of GSCs (08-387 or T3691) transduced with shNT or shSema3C in upper chambers coated with stem cell Matrigel to lower chambers containing conditioned media. Cells that migrated through the chambers were stained and the representative images are shown in (B). Quantification indicates that knockdown of Sema3C resulted in decreased GSC migration (C).

(D-F) Correlation of Sema3C, PlexinA2 or PlexinD1 mRNA expression with survival of glioma or GBM patients in Oncomine databases. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure S5, related to Figure 5. Sema3C Depletion Induces Apoptosis of GSCs But Not NPCs (A) Representative images of GSC tumorspheres transduced with shNT, shSema3C, shPlexinA2 or shPlexinD1 are shown. Knockdown of Sema3C, PlexinA2 or PlexinD1 resulted in GSC death.

(B) Evaluation of apoptosis by Annexin V-FITC/IP staining followed by flow cytometry analysis. Representative plots of Annexin V-FITC/IP staining of GSCs transduced with shNT or shSema3C for 48 hr are shown. Knockdown of Sema3C by two separate shRNAs induced GSC apoptosis.

(C-E) Representative images of TUNEL staining of apoptotic cells in T3691 (C) and 08-387 (D) GSCs transduced with shNT or shSema3C for 48 hr are shown. Quantitation of TUNEL positive cells/total cells is shown (E). Data are means ± standard deviation (SD) (n = 3). ***$p < 0.001$.

(F) IB analysis of cleaved-caspase3, -caspase7 and -PARP proteins in GSCs and matched NSTCs with Sema3C knockdown by two separate shRNAs.

(G and H) Representative images of TUNEL staining of apoptotic cells in GSCs (G) or NPCs (H) expressing shNT, shSema3C, shPlexinA2 or shPlexinD1 for 48 hr are shown.

Figure S6, related to Figure 6. Sema3C Activates Rac1 To Promote Survival of GSCs

(A) Co-immunoprecipitation (Co-IP) of Sema3C and PlexinA2/D1 in D456 (left) or T3691 (right) GSCs. IgG represents a control antibody used for IPs. For IP-immunoblotting data, antibodies used for IP were labeled red. Three hundred micrograms of lysates were used for each IP reaction and total lysates (20 μg) were used as input controls.

(B) Co-IP of Sema3C, PlexinA2, PlexinD1 and NRP1 in T3691 GSCs (up). Knockdown of NRP1 abrogated the interaction between Sema3C and PlexinA2/D1 in T3691 GSCs (down).

(C-E) GSCs and matched NSTCs were treated with vehicle control (DMSO) or Rac1 specific inhibitor NSC23766 (Rac-i, 50μM). Cell viability was assessed by cell titer assay (C). Data are means ± standard deviation (SD) (n = 3). Representative images of GSC tumorsphere formation after treatment with Rac inhibitor (Rac-i) are shown (D). IB analysis of cleaved-caspase3 in GSCs treated with Rac inhibitor for 48 hr (E). *p < 0.05, **p < 0.01, ***p < 0.001.

(F) IB analysis of cleaved-PARP and -caspase3 in GSCs and matched NSTCs transduced with shNT or two separate shRac1. Active GTP-Rac1 by pull-down assay is shown.

Figure S7, related to Figure 7. Ectopic Expression of a Constitutively Active Rac1 in GSCs Rescued the Phenotype Caused by Sema3C Disruption

(A) GSCs were transduced with vector control or Flag-Rac1Q61L and then targeted with shNT or shSema3C. Representative images of tumorspheres are shown.

(B) GSCs (08-387, labeled with luciferase) were transduced with vector control or Flag-Rac1Q61L, and then infected by shNT or shSema3C lentiviruses. 48 hr after infection, GSCs were transplanted into the brains of immunocompromised mice. Representative images of cross sections (H&E stained) of mouse brains from the indicated experimental groups harvested 23 days post-transplantation are shown (left). Real-time images from different animals on day 23 are shown (right).

(C) GSCs treated as (A) were assessed for cell migration. Cells that migrated through the chambers were stained and representative images are shown (left). Quantification of migrated cells is shown (right). Data are means ± standard deviation (SD) (n = 3). **p < 0.01

(D) Representative images are cross sections (H&E stained) of mouse brains from the indicated experimental groups. Yellow dashed lines indicated boundaries of in situ tumor cells and invaded tumor cells. Scale bar represents 200μM.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Isolation and Culture of GSCs and NPCs

GBM surgical specimens were collected for this study in accordance with a Cleveland Clinic Institutional Review Board-approved protocol. GSCs and NSTCs were isolated and characterized from GBM surgical specimens or xenografts as previously described (Cheng et al., 2013; Eyler et al., 2011; Guryanova et al., 2011; Lathia et al., 2010; Li et al., 2009). Briefly, tumors were disaggregated using the Papain Dissociation System (Worthington Biochemical) according to the manufacturer's instructions. Isolated cells were recovered in stem cell medium (Neurobasal-A medium with B27 supplement, 10 ng/ml EGF and 10 ng/ml bFGF) for at least 6 hr and then sorted by magnetic cell sorting for GSCs using the surface marker CD133 (Miltenyi Biotec.). CD133+ GSCs were cultured in stem cell medium as described above. CD133- NTSCs were cultured in DMEM with 10% fetal bovine serum as the viability of NSTCs is decreased by suspension culture conditions. The cancer stem cell phenotype of GSCs was confirmed by functional assays of self-renewal (serial neurosphere passage), stem cell marker expression, differentiation induction, and tumor propagation as previously described (Eyler et al., 2011; Guryanova et al., 2011; Li et al., 2009). Four human neural progenitor cell lines (15167, 16157, 17231 and 17893) were cultured and maintained in suspension culture or propagated on the Becton Dickinson stem cell Matrigel-coated dishes or coverslips in supplemented neurobasal stem cell media.

Immunofluorescence Staining, Immunohistochemistry and Immunoblot Analysis

Immunofluorescent (IF) staining of cells and tissues sections was performed as described (Guryanova et al., 2011). Briefly, cultured cells or frozen sections of tumorspheres, GBM xenografts and surgical specimens were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich) for 15 min then blocked with 10% normal goat or donkey serum (Vector) with 0.3% Triton X-100

(Bio-Rad) in PBS for 60 min at room temperature. Samples were incubated with primary antibodies overnight at 4°C followed by the appropriate secondary fluorescently labeled antibodies (Invitrogen Molecular Probes) for one hour at room temperature. Nuclei were counterstained with DAPI. Images were taken with a wide-field fluorescence microscope (Leica). Immunohistochemistry (IHC) staining of tissue section was performed with an ABC kit using DAB (3,30-Diaminobenzine) detection (Vector Lab) as previously described (Guryanova et al., 2011). Human GBM specimens were provided by Cleveland Clinic Department of Pathology under an approved IRB protocol. Tissue microarrays were purchased from US Biomax Inc. Phosphokinase array was purchased from R&D Systems. Immunoblot analysis was performed as previously described (Huang et al., 2011). Cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (Roche). Protein samples were resolved by SDS-PAGE and blotted onto PVDF membranes. Blots were incubated with primary antibodies overnight at 4°C followed by HRP-linked species-specific antibodies (Santa-Cruz). Specific antibodies against Sema3C (Pierce, R&D or Abcam), Sema3A (Abcam), Sema3B (Cell Signaling), Sema4A (Pierce), Sema6A (Pierce), PlexinA2 (Cell Signaling or R&D), PlexinD1 (Pierce), NRP1/NRP2 (Cell Signaling), CD133/1 (Miltenyi), CD31 (Dako), GFAP (Dako), Sox2 (Millipore or Santa Cruz), Nestin (Abcam), Olig2 (R&D), Flag (Sigma-Aldrich), Cleaved PARP/Caspase3/Caspase7, pAkt1, pERK1/2, p65, p-p65, CyclinD1, XIAP, Survivin, GAPDH (Cell Signaling), Rac1 (Millipore) and Rac1-GTP (New East Biosciences) were used for the IF staining, IHC or immunoblot analysis.

Differentiation Assay

GSCs were cultured on Matrigel-coated coverslips or dishes and induced to differentiate through withdrawal of EGF and or addition of serum (5% FBS in DMEM). At indicated time points, cells were harvested for immunoblot analysis or fixed for IF staining as described above.

DNA Constructs and Lentiviral Transfection

Lentiviral clones expressing NT shRNA, Sema3C, PlexinA2, PlexinD1, NRP1, Sema4A, Sema6A or Rac1 shRNAs were acquired from Sigma-Aldrich. Two of five shRNAs for each gene that displayed high knockdown efficiency (>80% reduction) were used for all related experiments. A lentiviral construct expressing constitutively active Rac1 Q61L (Flag-Rac1Q61L) was generated by cloning the DNA fragment of Rac1Q61L (Addgene) into the *EcoR1* and *BamH1* sites of pCDH-puro vector. Viral particles were produced in 293FT cells with the pPACK set of helper plasmids (System Biosciences) in stem cell media. Viral stocks were concentrated by precipitation with PEG-8000 and titered according to the manufacturer's instructions. For rescue experiments, GSCs were transduced with Flag-Rac1Q61L lentiviral construct or pCDH-EGFP control vector, and allowed to recover for 48 hr. Cells were selected by exposure to puromycin for 7 days, and then these stable cells expressing Flag-Rac1Q61L or EGFP were transduced with shSema3C or NT shRNA via lentiviral infection. 48 hours post infection, cells were plated to assess cell proliferation, self-renewal or used for in vivo experiments.

Orthotopic Mouse Xenografts

Intracranial transplantation of GSCs to establish GBM xenografts was performed as described (Eyler et al., 2011; Guryanova et al., 2011; Li et al., 2009). Briefly, 48 hr after lentiviral infection, GSC cells were counted and 2×10^4 cells were implanted into the right frontal lobes of athymic nude mice or NSG mice. For the survival experiments, animals were maintained until manifestation of neurological signs or for 180 days post-transplantation. To monitor tumor growth, GSCs stably expressed firefly luciferase and mice were monitored by bioluminescence imaging longitudinally. To compare tumor growth, different experimental groups of mice implanted with GSCs were harvested on same day as indicated after GSC transplantation. All animal procedures conformed to the Cleveland Clinic IACUC-approved protocol.

Necropsy

Mice were euthanized and necropsied when exhibiting signs of declining neurologic status or performance status. Animals were anesthetized and underwent cardiac perfusion with PBS. Brains were harvested and fixed with 4% PFA overnight at 4°C, post-fixed in 70% ethanol, cryopreserved in 30% sucrose and cryosectioned for staining and fluorescence analysis, or embedded with paraffin and sectioned for histological analysis.

Cell Viability Assays

For cell proliferation assay, 1×10^3 lentiviral infected or NSC23766 treated cells were plated into each well of 96-well plates. Cell titers were determined after the indicated number of days using the Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega). All data were performed in triplicate and normalized to day 0 and presented as mean ± standard deviation.

TUNEL Assay

A TUNEL assay-based in situ cell death detection kit (ApopTag®Red In Situ Apoptosis Detection Kit, Millipore) was used to detect apoptotic cell death, both in vitro and in vivo, following the protocols recommended by the manufacturer. Nuclei were counterstained with DAPI (blue). The results were observed under a fluorescence microscope.

Rac1 Activation Assay

Rac1 activity was assessed using the Rac1 Activation Assay Kit (Millipore) according to the manufacturer's instructions. Briefly, cell lysates were clarified by centrifugation at 14000 g at 4°C for 10 min. Equal volumes of lysates were incubated with beads to pull down activated Rac1 proteins. After incubation at 4°C for 1 hour, the beads were washed three times with cold MLB buffer. The Rac1 proteins were eluted with a sample buffer and subjected to SDS-PAGE gel electrophoresis. Western blot analysis was performed using anti-Rac1 antibodies (Millipore).

Immunoprecipitation

Cells were collected and lysed in lysis buffer supplemented with protease inhibitors, incubated on ice for 15 min, and cleared by centrifugation at 14,000g at 4°C for 10 min. Protein lysate was subjected to immunoprecipitation with the agarose-immobilized antibody (1 mg of antibody as indicated, or isotype control antibodies) overnight at 4°C.

SUPPLEMENTAL REFERENCES

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