Supplementary Information for:

Targeting JNK for therapeutic depletion of stem-like glioblastoma cells

Ken-ichiro Matsuda[1, 2#], Atsushi Sato[1, 2*#], Masashi Okada[1], Keita Shibuya[1], Shizuka Seino[1], Kaori Suzuki[1, 3], Eriko Watanabe[1], Yoshitaka Narita[3], Soichiro Shibui[3], Takamasa Kayama[2], Chifumi Kitanaka[1, 4, 5*]

Departments of [1]Molecular Cancer Science and [2]Neurosurgery, Yamagata University School of Medicine, Yamagata, 990-9585, Japan

[3]Neurosurgery Division, National Cancer Center Hospital, Tokyo, 104-0045, Japan

[4]Oncology Research Center, Research Institute for Advanced Molecular Epidemiology, Yamagata University, Yamagata, 990-9585, Japan

[5]Global COE program for Medical Sciences, Japan Society for Promotion of Science, Tokyo, 102-8472, Japan

*Corresponding Authors: Chifumi Kitanaka and Atsushi Sato

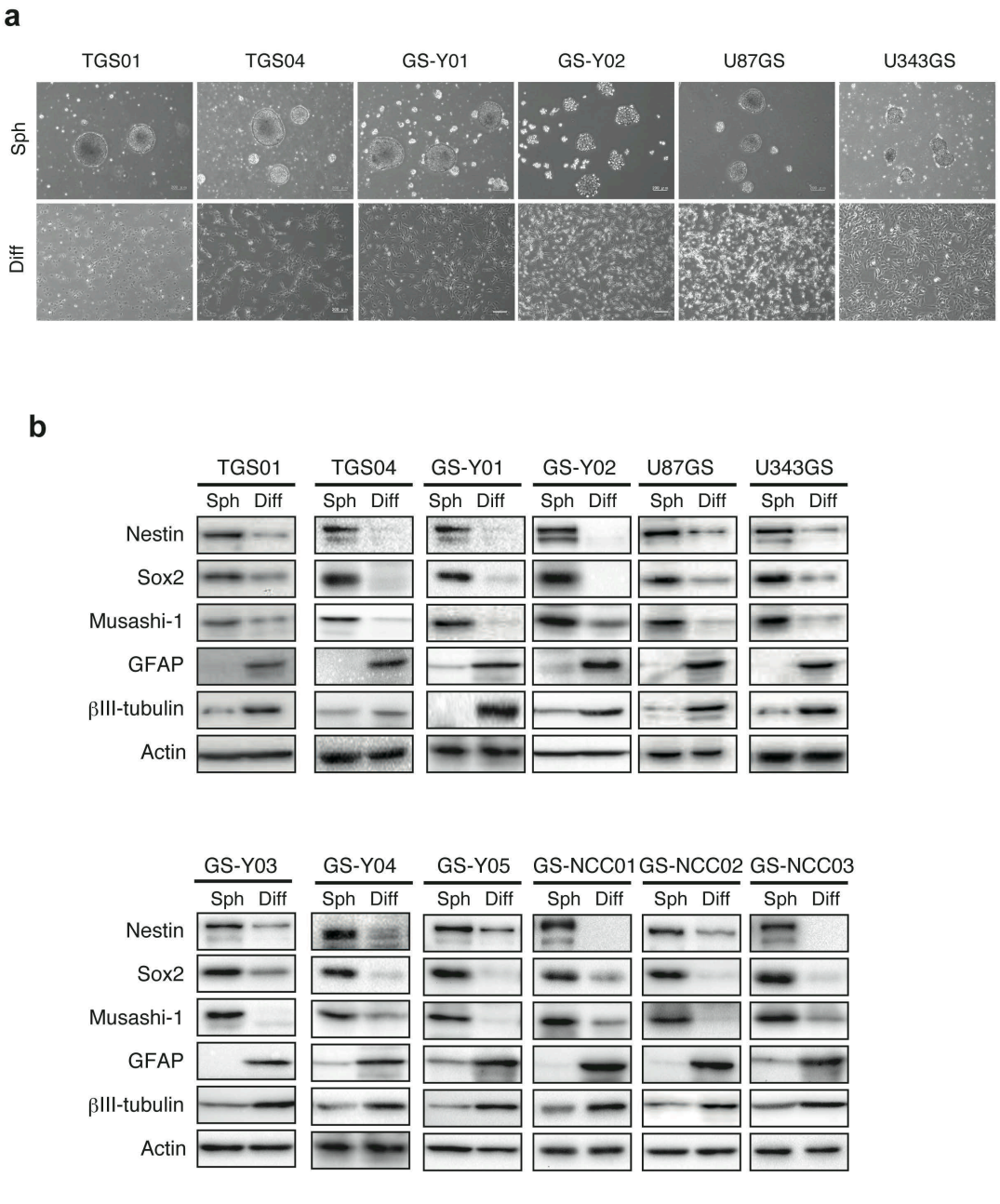
Department of Molecular Cancer Science, Yamagata University School of Medicine, Yamagata, 990-9585, Japan. Tel: +81-23-628-5212; Fax: +81-23-628-5215

E-mail: ckitanak@med.id.yamagata-u.ac.jp, asato@med.id.yamagata-u.ac.jp

#These authors contributed equally to this work

SUPPLEMENTARY FIGURES

Supplementary Figure 1



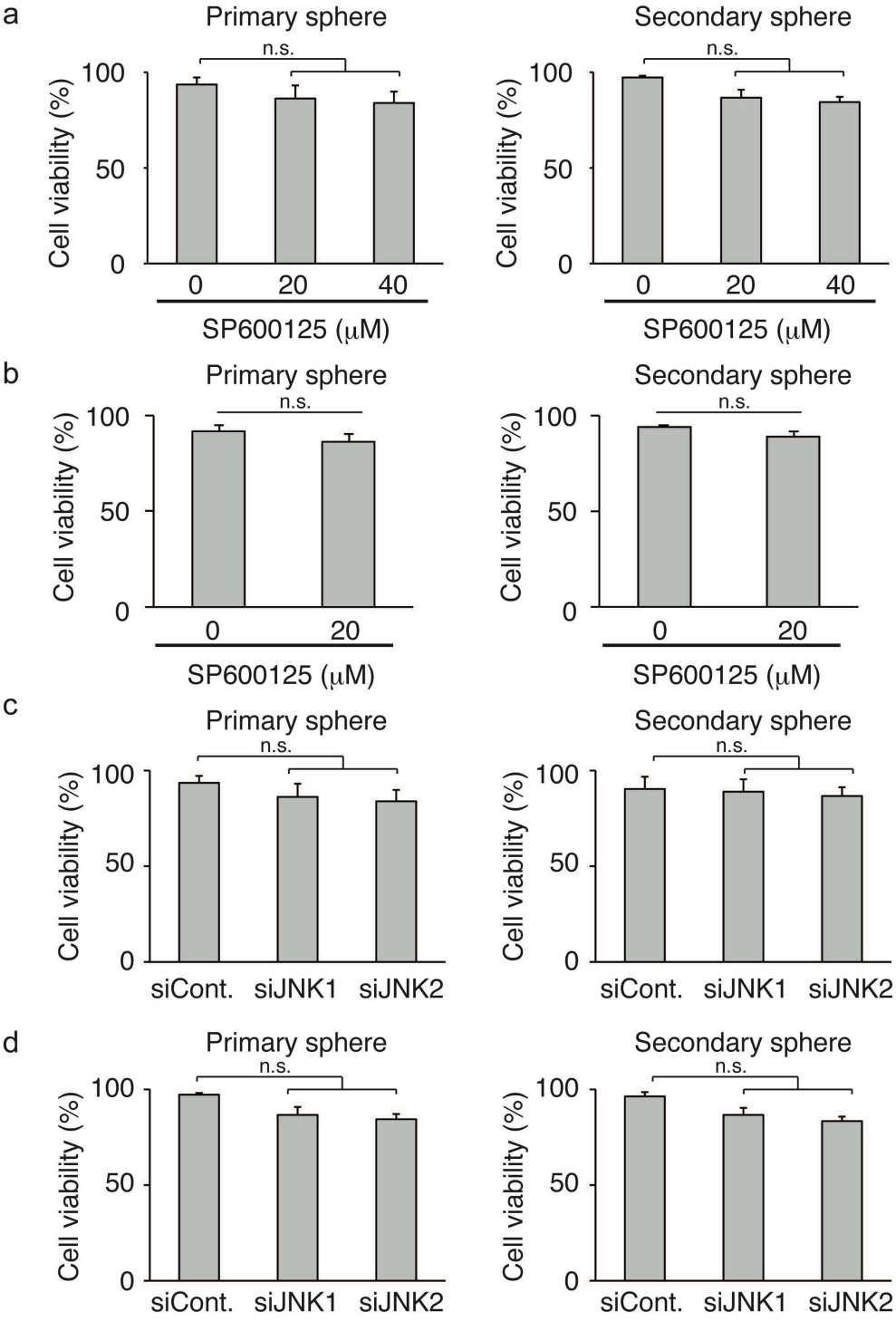
Supplementary Figure 1

Self-renewal capacity and multi-lineage differentiation potential of stem-like glioblastoma cells used in this study.

(**a**) Phase-contrast micrographs of stem-like glioblastoma cells cultured in the sphere culture condition (*top,* Sph) and in the differentiation-inducing culture condition (*bottom,* Diff). Scale bars, 200 μm.

(**b**) Immunoblot analysis of stem cell and differentiation-marker expression in stem-like glioblastoma cells cultured in the sphere culture condition (*left lanes*, Sph) and in the differentiation-inducing culture condition for 7 days (*right lanes,* Diff).

Supplementary Figure 2

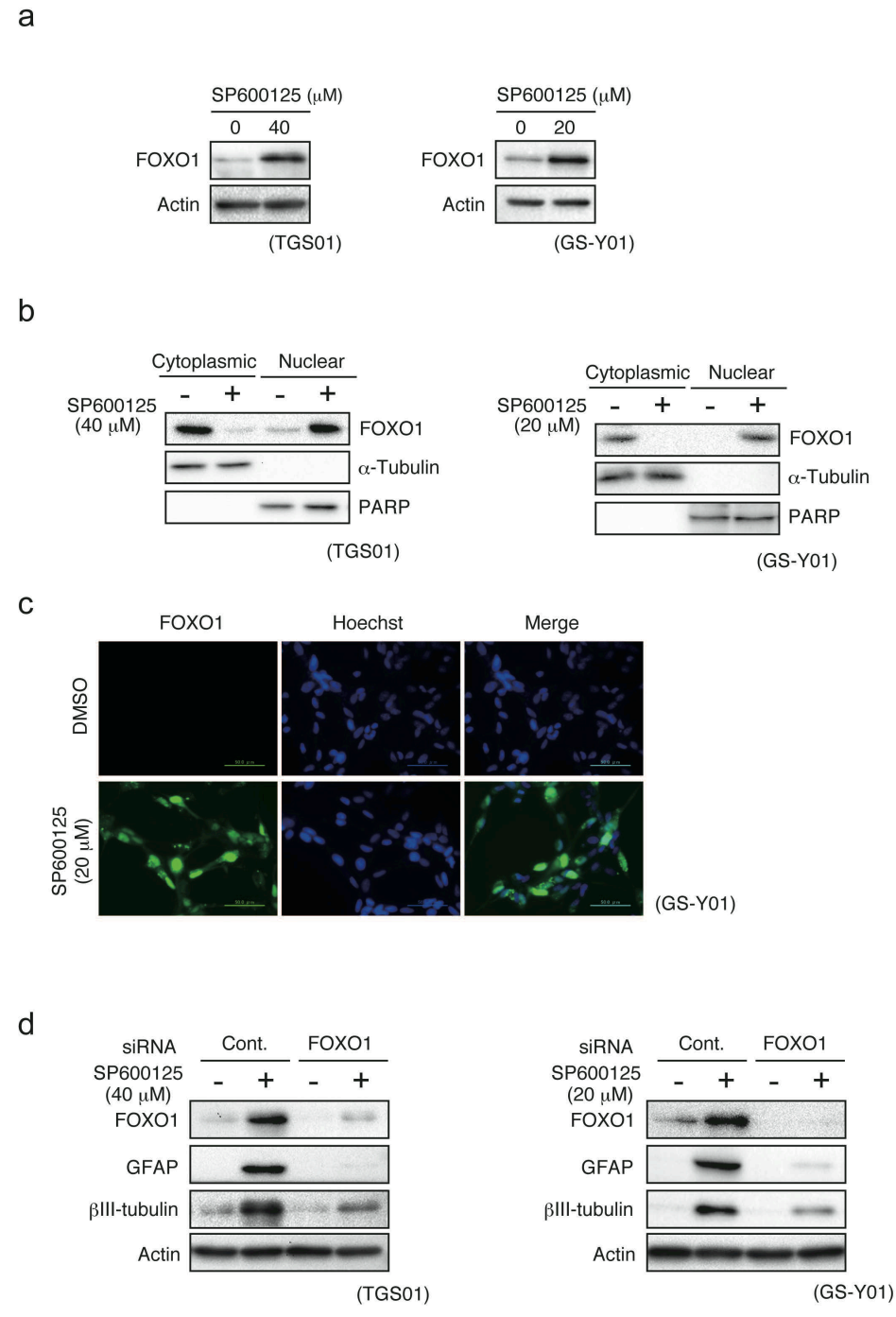


Supplementary Figure 2

Effect of JNK inhibition on viability of TGS01 and GS-Y01 stem-like glioblastoma cells.

Cellular viability of TGS01 (**a, c**) and GS-Y01 (**b, d**) cells analysed in the serial sphere formation assays of Fig. 2**a** and Fig. 2**e** was examined using the dye exclusion method. Values represent mean ± s.d. from 3 experiments. n.s., not statistically significant.

Supplementary Figure 3



Supplementary Figure 3

JNK prevents stem-like glioblastoma cell differentiation via inhibition of FOXO1 activation.

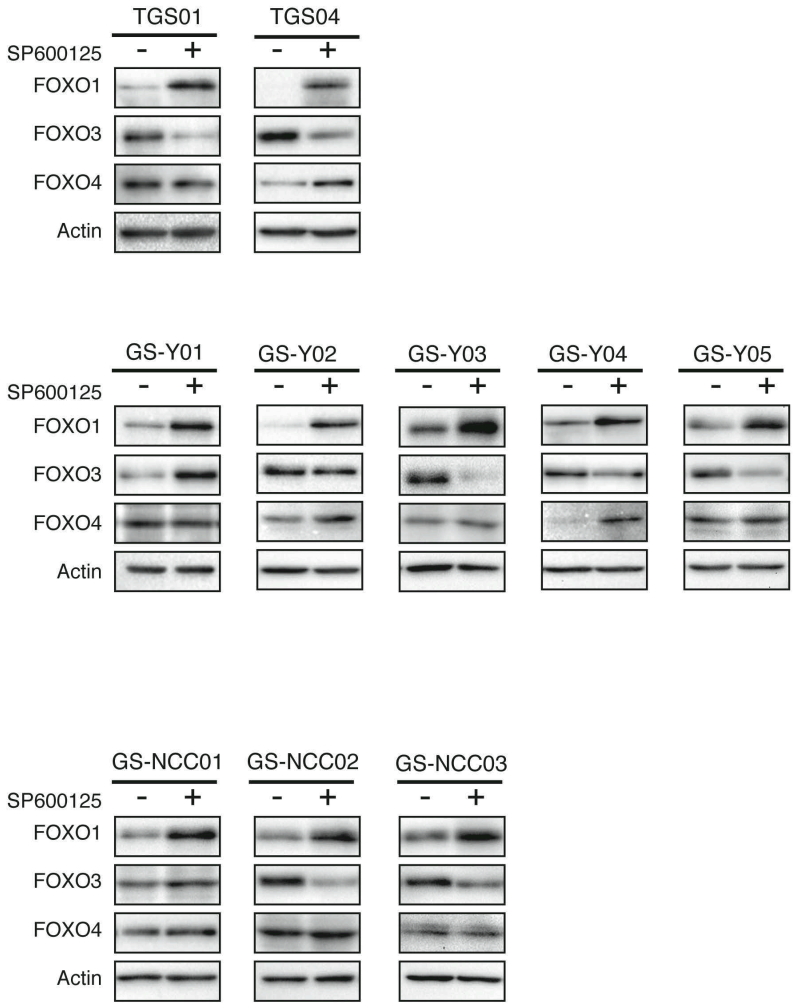
(**a**) TGS01 and GS-Y01 cells cultured in the absence or presence of the indicated concentrations of SP600125 for 3 days were analysed for FOXO1 expression by immunoblot analysis.

(**b**) TGS01 and GS-Y01 cells treated as in (**a**) were subjected to cellular fractionation followed by immunoblot analysis for FOXO1 as well as for cytoplasmic (α-tubulin) and nuclear (PARP) marker proteins.

(**c**) GS-Y01 cells cultured in the presence of SP600125 or the control vehicle (DMSO) for 3 days were subjected to immunofluorescence staining of FOXO1 (green). Nuclei were counterstained with Hoechst 33342 (blue). Scale bars, 50.0 μm.

(**d**) TGS01 and GS-Y01 cells transfected with the control (Cont.) or FOXO1 siRNA were treated with or without SP600125 for 3 days beginning 6 h after transfection. Cells were then subjected to immunoblot analysis with the indicated antibodies.

Supplementary Figure 4

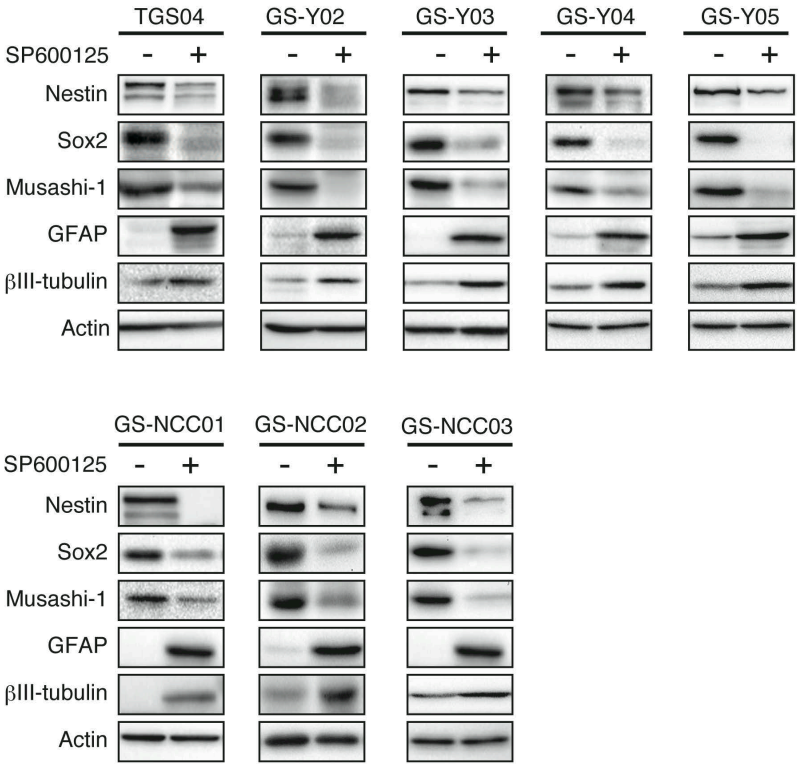


Supplementary Figure 4

Effect of JNK inhibition on expression of FOXO transcription factors in stem-like glioblastoma cells.

The indicated stem-like glioblastoma cells were treated with or without SP600125 (40 μM for TGS01 and 20 μM for the others) for 3 days and then subjected to immunoblot analysis for the expression of FOXO family members.

Supplementary Figure 5

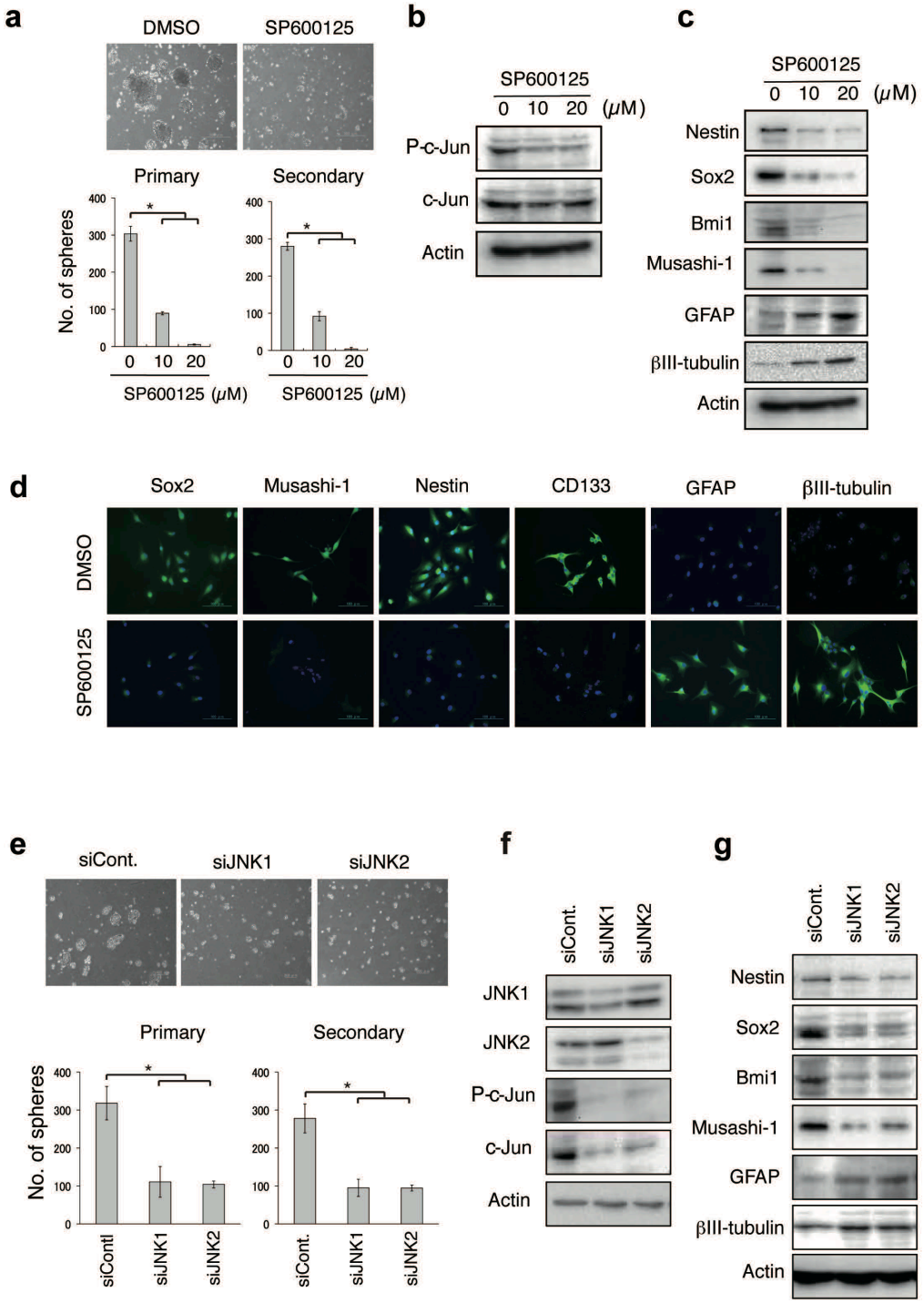


Supplementary Figure 5

JNK activity is required for maintenance of undifferentiated state in stem-like glioblastoma cells.

The indicated stem-like glioblastoma cells treated with or without SP600125 (20 μM) for 3 days were subjected to immunoblot analysis for stem cell and differentiation-marker expression.

Supplementary Figure 6



Supplementary Figure 6

JNK is required for maintenance of stem-like cells (U87GS) derived from the U87 human glioblastoma cell line.

(**a**) U87GS cells cultured in the presence of the indicated concentrations of SP600125 for 3 days were subjected to primary and secondary sphere formation assays. The data are presented as the number of tumourspheres formed (mean ± s.d. from 3 experiments, *bottom*). Representative phase-contrast micrographs of the primary spheres formed by cells treated with SP600125 (20 μM) and the control vehicle (DMSO) are shown (*top*). Scale bars, 500 μm. **P* < 0.05

(**b** - **c**) Immunoblot analysis of phospho-c-Jun (P-c-Jun) (**b**) and stem cell/differentiation-marker (**c**) expression after treatment of U87GS cells with the indicated concentrations of SP600125 for 3 days.

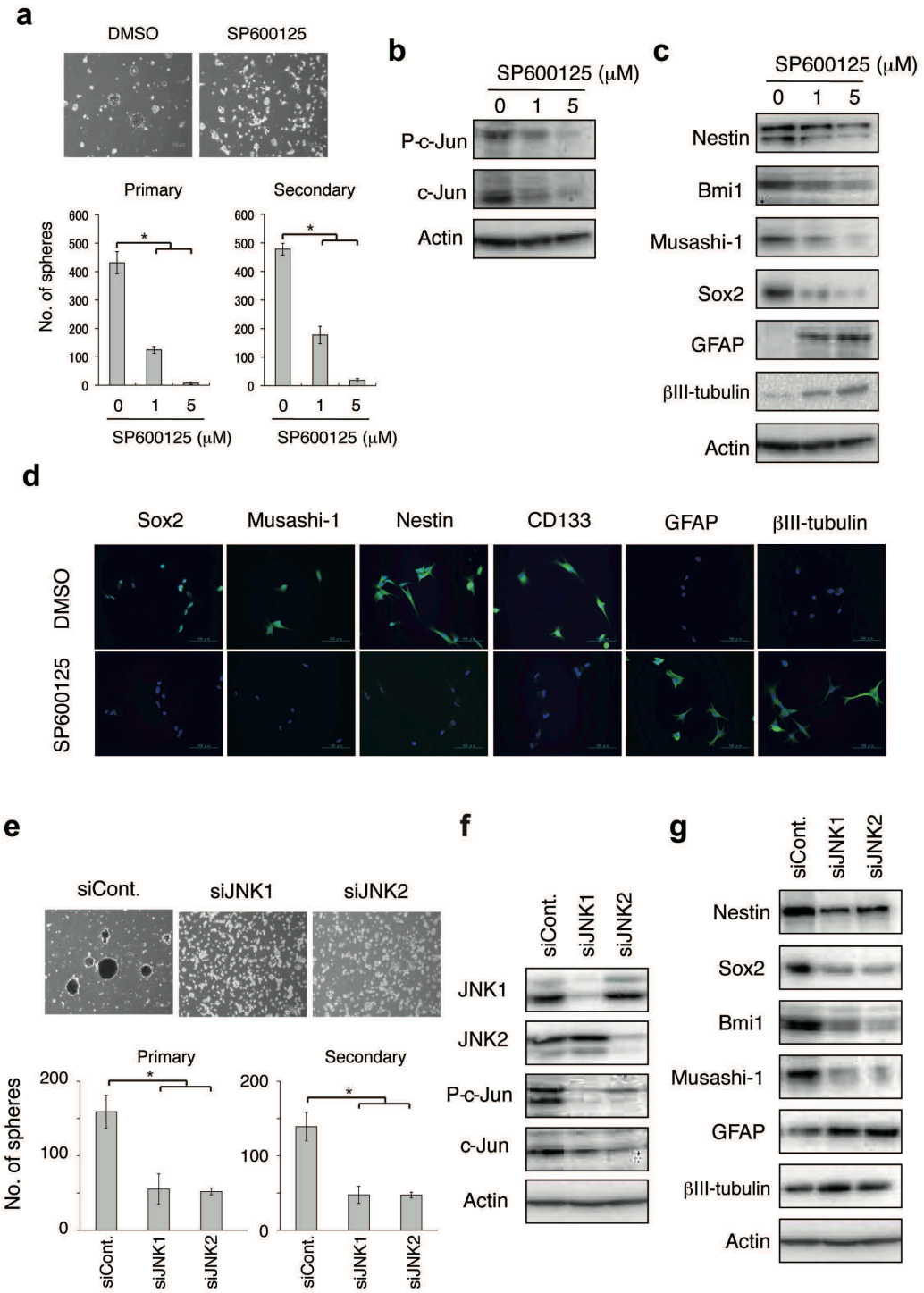
(**d**) U87GS cells treated with SP600125 (20 μM) or the control vehicle (DMSO) were subjected to immunofluorescence analysis for the expression of the indicated stem cell and differentiation markers. Scale bars, 100 μm.

(**e**) U87GS cells transfected with the indicated siRNAs were subjected to primary and secondary sphere formation assays 3 days after transfection. The data are presented as the number of tumourspheres formed (mean ± s.d. from 3 experiments, *bottom*). Representative phase-contrast micrographs of the primary sphere assay are shown (*top*). Scale bars, 200 μm. **P* < 0.05

(**f**) Effect of JNK knockdown on the JNK signalling pathway in U87GS cells.

(**g**) Effect of JNK knockdown on stem cell and differentiation-marker expression in U87GS cells.

Supplementary Figure 7



Supplementary Figure 7

JNK is required for maintenance of stem-like cells (U343GS) derived from the U343 human glioblastoma cell line.

(**a**) U343GS cells cultured in the presence of the indicated concentrations of SP600125 for 3 days were subjected to primary and secondary sphere formation assays. The data are presented as the number of tumourspheres formed (mean ± s.d. from 3 experiments, *bottom*). Representative phase-contrast micrographs of the primary spheres formed by cells treated with SP600125 (5 μM) and the control vehicle (DMSO) are shown (*top*). Scale bars, 200 μm. **P* < 0.05

(**b** - **c**) Immunoblot analysis of phospho-c-Jun (P-c-Jun) (**b**) and stem cell/differentiation-marker (**c**) expression after treatment of U343GS cells with the indicated concentrations of SP600125 for 3 days.

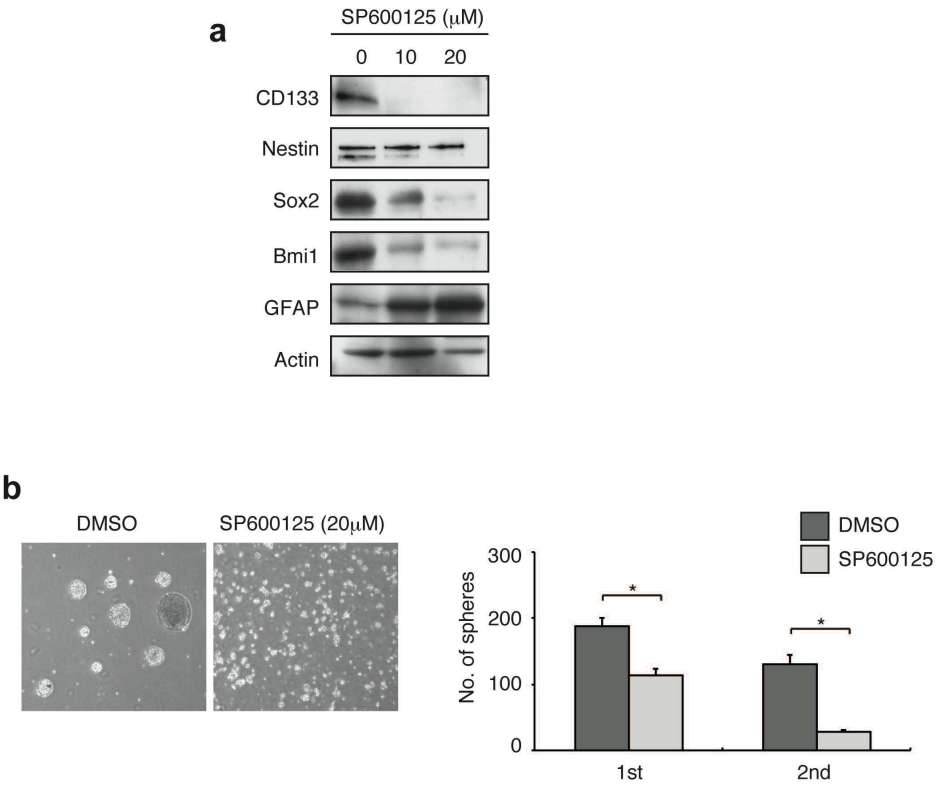
(**d**) U343GS cells treated with SP600125 (5 μM) or the control vehicle (DMSO) were subjected to immunofluorescence analysis for the expression of the indicated stem cell and differentiation markers. Scale bars, 100 μm.

(**e**) U343GS cells transfected with the indicated siRNAs were subjected to primary and secondary sphere formation assays 3 days after transfection. The data are presented as the number of tumourspheres formed (mean ± s.d. from 3 experiments, *bottom*). Representative phase-contrast micrographs of the primary sphere assay are shown (*top*). Scale bars, 200 μm. **P* < 0.05

(**f**) Effect of JNK knockdown on the JNK signalling pathway in U343GS cells.

(**g**) Effect of JNK knockdown on stem cell and differentiation-marker expression in U343GS cells.

Supplementary Figure 8



Supplementary Figure 8

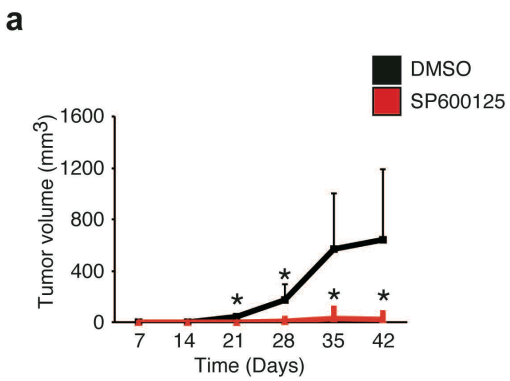
Effect of JNK inhibition on glioblastoma cell cultures propagated under the stem cell culture condition as self-renewing spheres for brief periods but from which cell lines were not established.

(**a**) Tumourspheres derived directly from a surgical specimen of a glioblastoma patient and formed under the sphere culture condition were dissociated and cultured in the presence of the indicated concentrations of SP600125 for 3 days under the sphere culture condition and then subjected to immunoblot analysis for stem cell (CD133, nestin, Sox2, and Bmi1) and differentiation (GFAP)-marker expression.

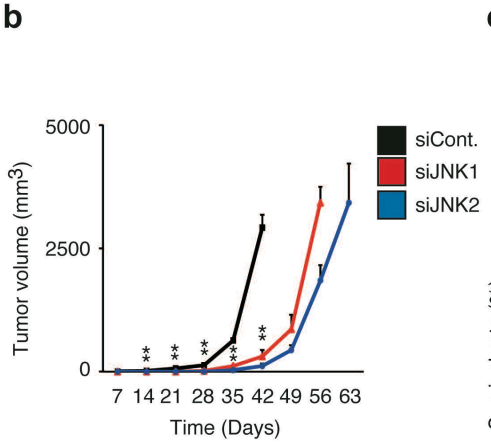
(**b**) Tumourspheres derived directly from a surgical specimen of another glioblastoma patient were dissociated, cultured in the absence (DMSO) or presence of SP600125 (20 μM) for 3 days, and then subjected to serial sphere formation assays in the absence of SP600125. Representative phase-contrast micrographs of the cells are shown (*left*). Values in the graph represent mean ± s.d. of triplicate cultures (*right*). $*P < 0.05$

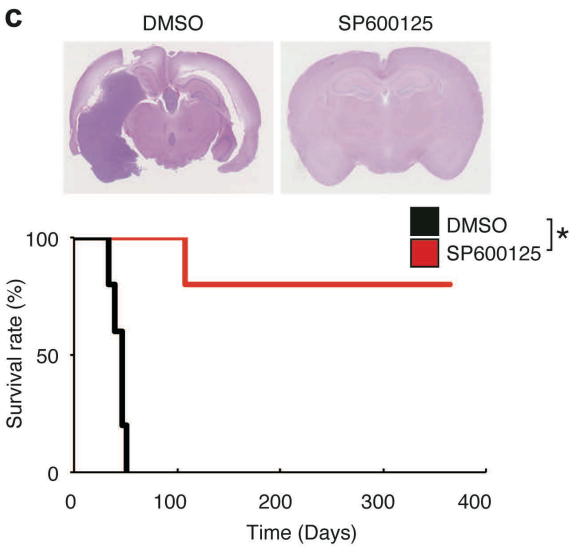
Note that tumourspheres in (**a**) and (**b**) ultimately failed to be established as stem-like glioblastoma cell lines.

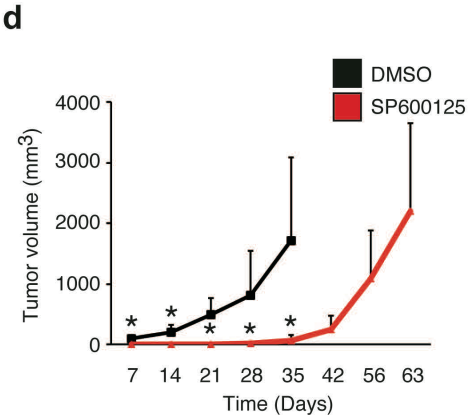
Supplementary Figure 9

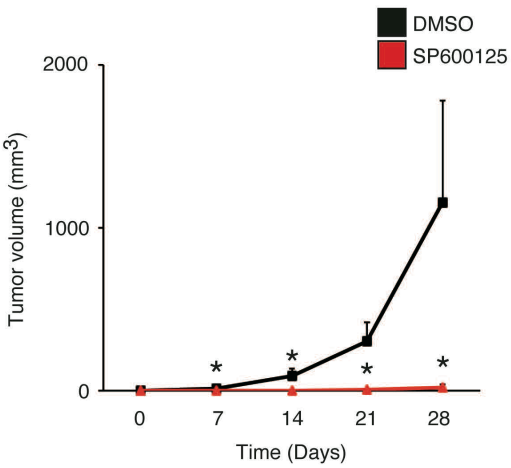


Days	Day7	Day14	Day21	Day28	Day35	Dy42
DMSO	0	0	62.5	126	288	364.5
DMSO	0	0	18	108	245	245
DMSO	0	0	32	180	700	700
DMSO	0	0	75	384	1267.5	1568
DMSO	0	0	32	64	352	320
SP600125	0	0	0	0	0	0
SP600125	0	0	0	0	0	0
SP600125	0	0	0	0	0	0
SP600125	0	0	0	48	162	112.5
SP600125	0	0	0	0	0	0









Supplementary Figure 9

Transient JNK inhibition suppresses tumour-initiating potential of stem-like U87GS cells in vitro and in vivo.

(**a**) Mice were implanted subcutaneously with stem-like U87GS cells (1×10^5 cells) pretreated with SP600125 (20 μM) or the control vehicle (DMSO) for 3 days (5 mice per group). Tumour volume (mm^3) measured at the indicated time points is presented in the graph as mean tumour volume ± s.d. (*left*) and in the table (*right*). **P* < 0.05

(**b**) U87GS cells (1×10^5) transiently transfected with siRNAs against JNK1 or JNK2, or with a control siRNA were implanted subcutaneously into the right flank of mice 3 days after transfection (3 mice per group). Data were then collected and analysed as in (**a**). **P* < 0.05

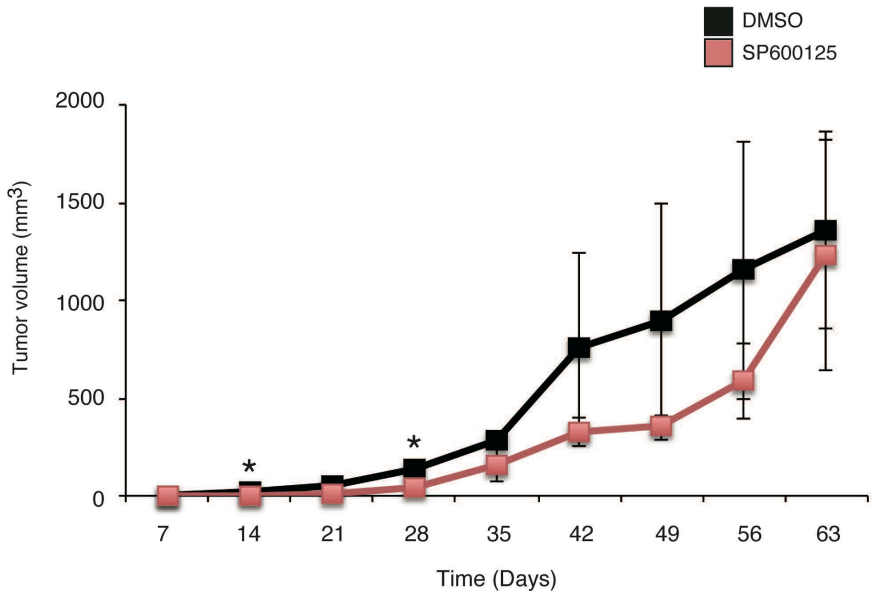
(**c**) A Kaplan-Meier plot (*bottom*) showing survival of mice (5 mice per group) after intracranial implantation of U87GS cells (1×10^4) pretreated with SP600125 (20 μM) or the control vehicle (DMSO) for 3 days. Representative haematoxylin and eosin staining of the brain sections from mice sacrificed at 50 days after implantation is shown (*top*). **P* < 0.05

(**d**) Mice implanted subcutaneously with U87GS (1×10^5 cells) were randomly assigned into control and SP600125 treatment groups (3 mice per group) for intraperitoneal injection of the control vehicle (DMSO) or SP600125 (40 mg/kg/day), respectively, for 5 days starting on the next day of implantation. Tumour volume was measured at the indicated time points (mean ± s.d. of 3 mice). **P* < 0.05

(**e**) Mice implanted subcutaneously with U87GS (1×10^5 cells) were, after tumour formation, randomized into control and SP600125 treatment groups (3 mice per group; tumour volume at randomization, 171 – 288 mm^3) for intraperitoneal injection of the control vehicle (DMSO) or SP600125 (40 mg/kg/day),

respectively, for 5 days. On the next day of the final drug treatment, the mice were sacrificed and dissociated tumour cells (1×10^5 cells per mouse) were transplanted subcutaneously into the right flank of mice (5 mice per group). Tumour volume was measured at the indicated time points (mean ± s.d. of 5 mice). *$P < 0.05$

Supplementary Figure 10



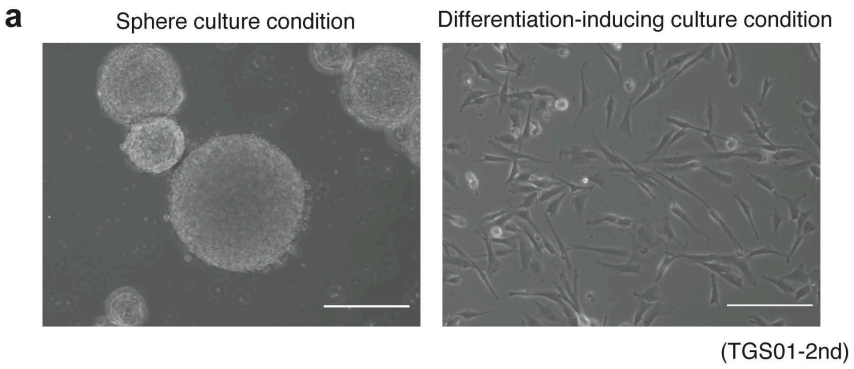
Day	Day7	Day14	Day21	Day28	Day35	Day42	Day49	Day56	Day63
Contol	0	13.5	36	126	288	700	700	847	936
Contol	2	18	30	108	245	1267.5	1568	1912.5	1224
Contol	6	27.5	87.5	180	320	320	405	700	1912.5
SP600125	0	6	13.5	32	180	245	320	364.5	936
SP600125	0	4	6	75	245	384	352	700	1912.5
SP600125	0	4	18	32	64	352	416	700	847

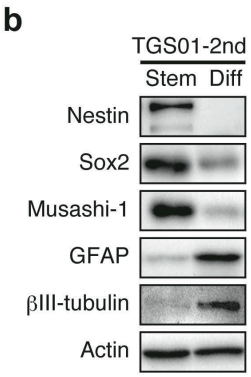
Supplementary Figure 10

Marginal inhibitory effect of JNK inhibition on tumour-initiating potential of serum-cultured U87 cells.

Serum-cultured U87 cells (1×10^6 cells) pretreated with SP600125 (20 μM) or the control vehicle (DMSO) for 3 days were implanted subcutaneously into the right flank of mice (3 mice per group), and tumour volume (mm^3) was measured at the indicated time points. The data are presented in the graph as mean tumour volume ± s.d. of 3 mice (*top*) and in the table (*bottom*). *$P < 0.05$

Supplementary Figure 11





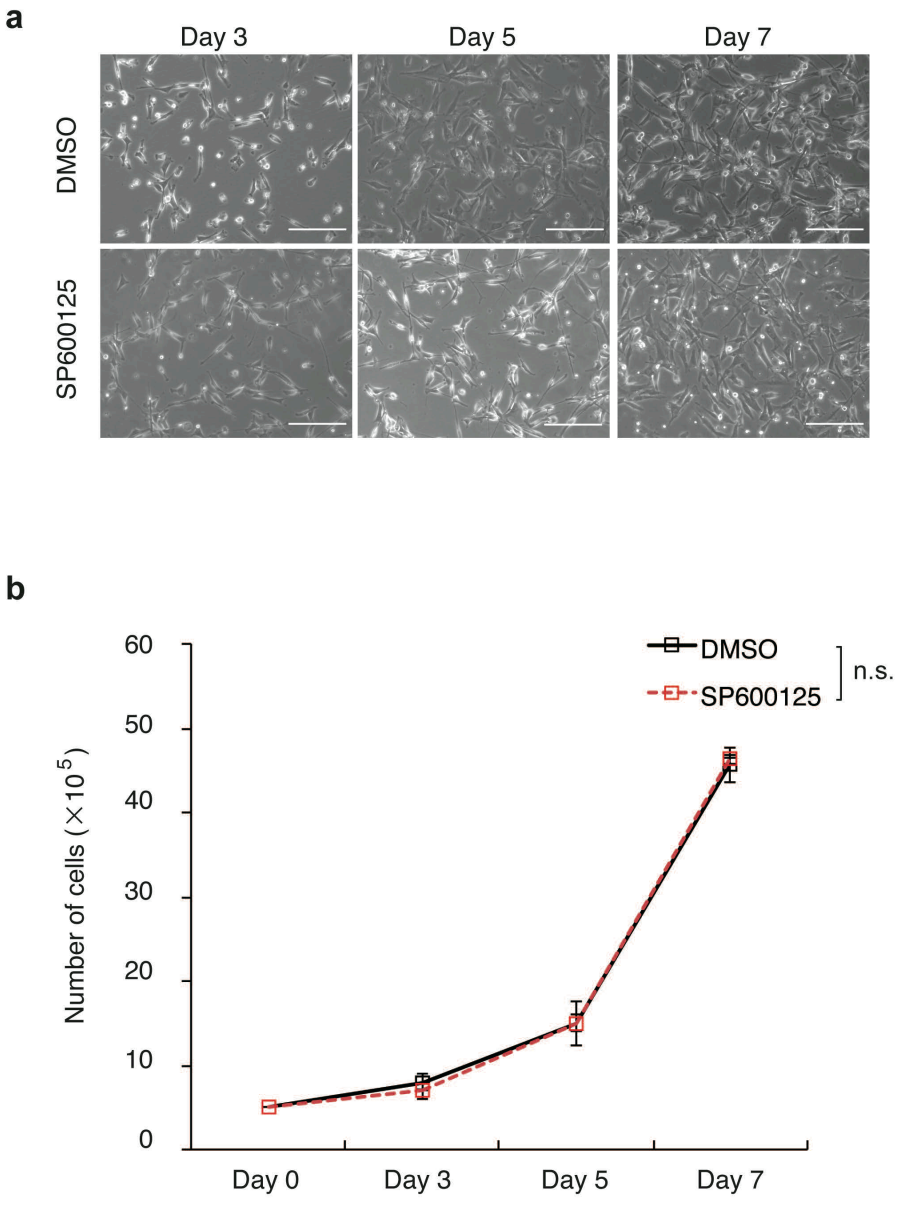
Supplementary Figure 11

Tumour xenografts established from stem-like glioblastoma cells contain stem-like glioblastoma cells with self-renewal capacity and multipotency of differentiation.

(**a**) Stem-like glioblastoma cells isolated from subcutaneous tumours established by implantation of TGS01 cells and maintained under the monolayer stem cell culture condition (TGS01-2nd) were transferred and cultured in the sphere culture condition (*left*) and in the differentiation-inducing culture condition (*right*). Representative phase-contrast photomicrographs of the cells are shown. Scale bars, 200 μm.

(**b**) TGS01-2nd cells maintained under the monolayer stem cell culture condition (Stem) and cultured in the differentiation-inducing culture condition for 1 week (Diff) were subjected to immunoblot analysis for the indicated stem cell and differentiation markers.

Supplementary Figure 12

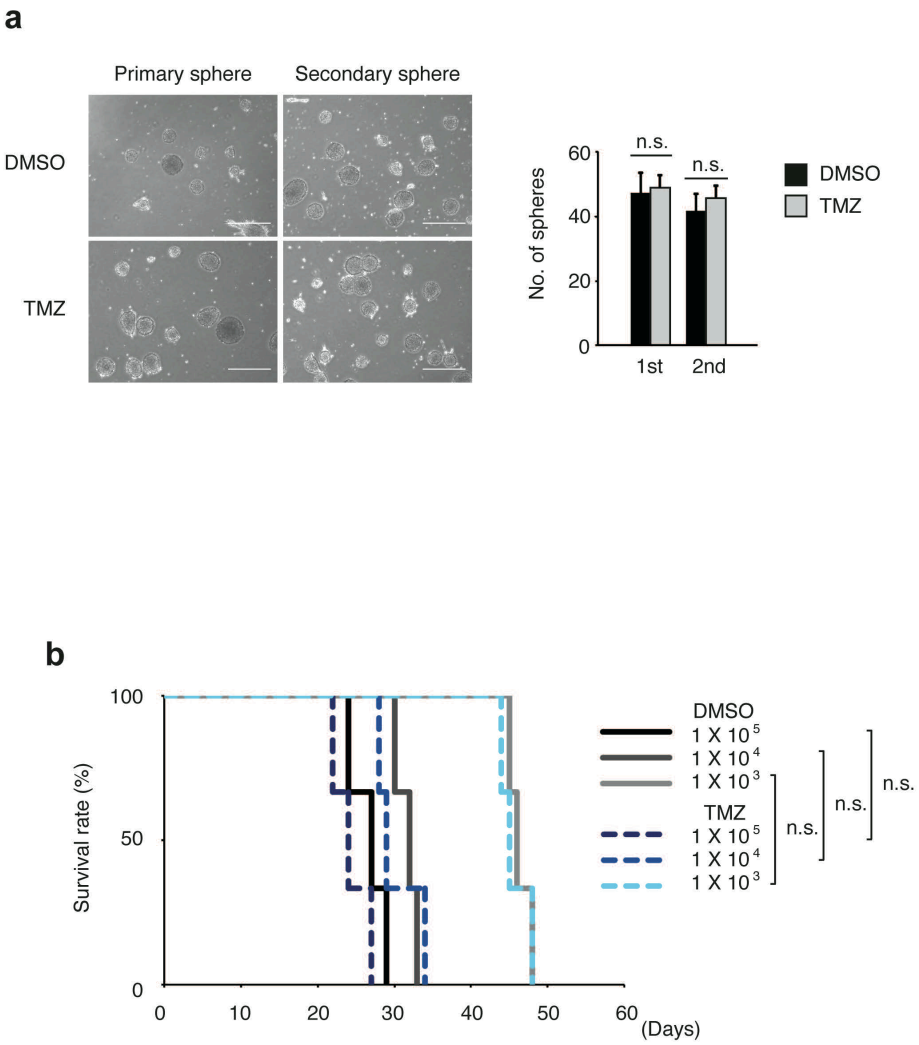


Supplementary Figure 12

Transient inhibition of JNK in vivo, which depletes the self-renewing and tumour-initiating glioblastoma cell populations from established tumours, does not affect the growth of bulk tumour cells under the serum culture condition in vitro.

(**a** - **b**) Mice implanted subcutaneously with stem-like glioblastoma cells (TGS01, 1×10^6 cells) were, after tumour formation, randomized into control and SP600125 treatment groups (3 mice per group; tumour volume at randomization, 171 – 288 mm^3) and administered intraperitoneal injection of the control vehicle (DMSO) or SP600125 (40 mg/kg/day), respectively, for 5 days. On the next day of the final drug treatment (= Day 0), mice were sacrificed and dissociated tumour cells were cultured in the presence of serum (i.e., under the differentiation-inducing culture condition). Phase-contrast photomicrographs (**a**) and growth curves (**b**) of the cells are shown. Scale bars in (**a**), 200 μm. Values in (**b**) represent mean ± s.d. of triplicate cultures. n.s., not statistically significant.

Supplementary Figure 13

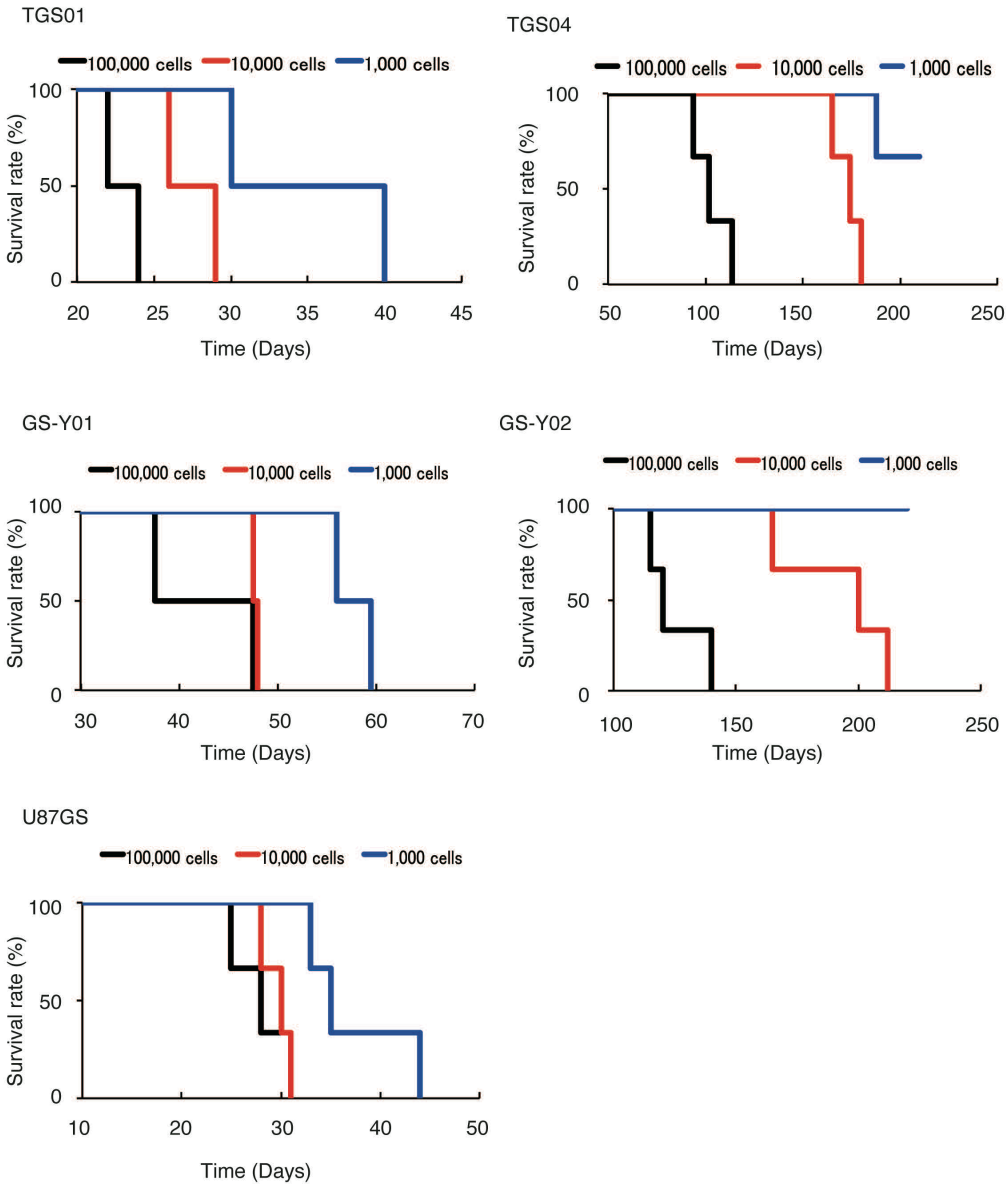


Supplementary Figure 13

Administration of a sublethal dose of temozolomide fails to decrease self-renewing and tumour-initiating glioblastoma cell populations in established tumours in vivo.

(**a** - **b**) Mice implanted subcutaneously with stem-like glioblastoma cells (TGS01, 1×10^6 cells) were, after tumour formation, randomized into control and temozolomide (TMZ) treatment groups (3 mice per group; tumour volume at randomization, 144 – 256 mm^3) and administered intraperitoneal injection of the control vehicle (DMSO) or TMZ (50 mg/kg/day), respectively, for 5 days. On the next day of the final drug treatment, mice were sacrificed and dissociated tumour cells were subjected to serial sphere formation assays (**a**). *Left*, phase-contrast micrographs of the spheres (scale bars, 200 μm). *Right*, number of the spheres formed (mean ± s.d. of triplicate cultures derived from a single tumour of each treatment group). Essentially identical results were obtained regarding the remaining 2 tumours of each group. Alternatively, serial dilutions of the dissociated tumour cells were transplanted intracranially into mice (**b**), and survival was evaluated by Kaplan-Meier analysis (3 mice per group). Note that a higher dose of TMZ (e.g., 60 mg/kg/day) frequently caused death of mice relatively soon after drug administration in the experimental condition. n.s., not statistically significant.

Supplementary Figure 14



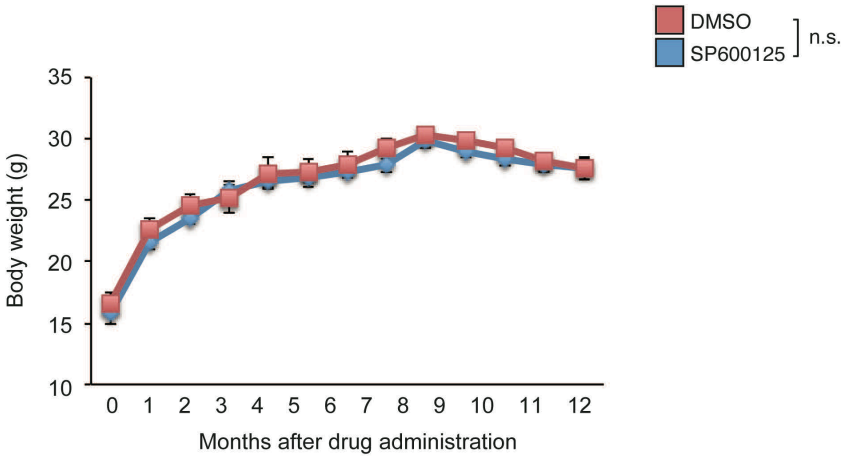
Supplementary Figure 14

Relationship between number of implanted cells and mouse survival in the intracranial xenograft model.

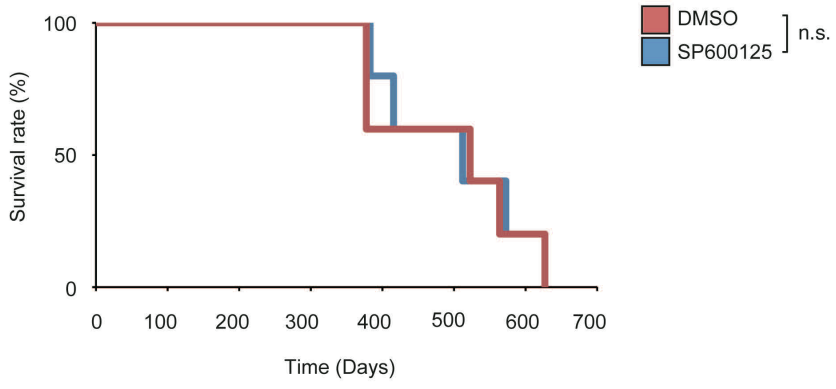
Kaplan-Meier plots showing survival duration of mice implanted intracranially with serial dilutions of the indicated stem-like glioblastoma cells.

Supplementary Figure 15

a



b

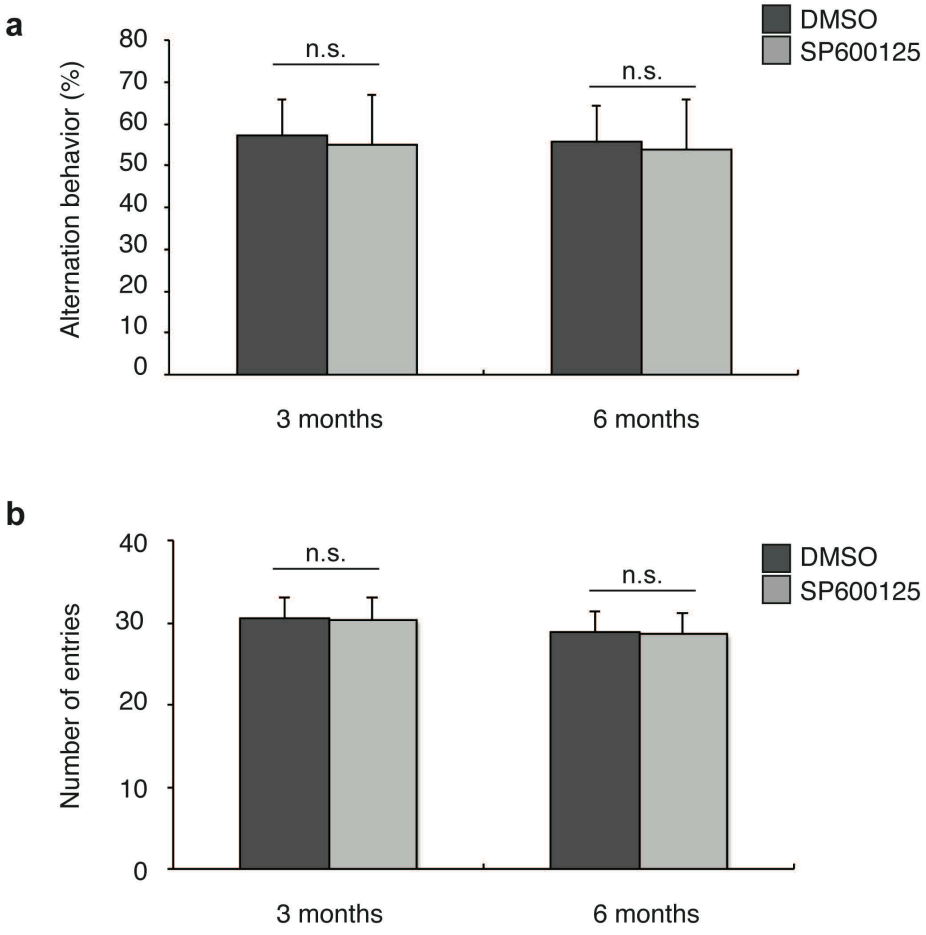


Supplementary Figure 15

Effect of systemic SP600125 administration on mouse body weight and survival.

(**a** - **b**) Two groups of mice (5 per each group) were treated exactly as those in Fig. 5 (i.e., daily intraperitoneal administration of SP600125 [40 mg/kg/day] or the control vehicle [DMSO] for 10 consecutive days) except for intracranial implantation of stem-like glioblastoma cells. Body weight of the mice was monitored on a monthly basis (**a**) and survival was assessed by Kaplan-Meier analysis (**b**). Values in (**a**) represent mean ± s.d. of 5 mice of each group. n.s., not statistically significant.

Supplementary Figure 16

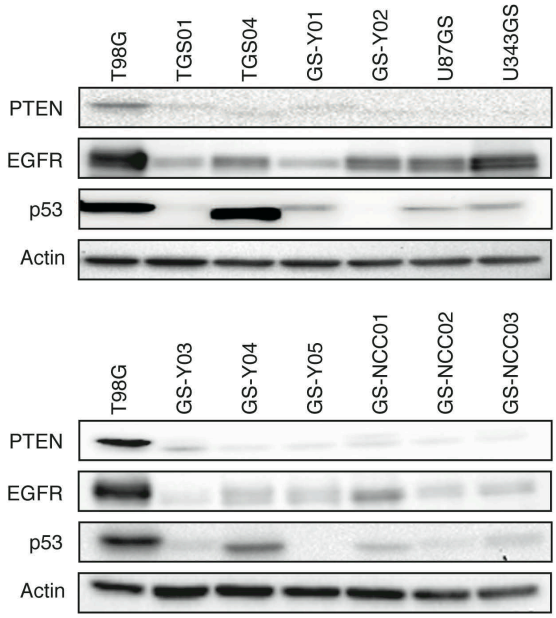


Supplementary Figure 16

Effect of systemic SP600125 administration on brain function as assessed by Y-maze test.

(**a** - **b**) Mice (5 per each group) treated with either SP600125 or the control vehicle (DMSO) in Supplementary Fig. 15 were subjected to the Y-maze test at 3 and 6 months after drug treatment had concluded. The percentage of alternation behaviour (**a**) and the total number of arm entries during the session (**b**), an indicator of locomotor activity, are shown. Values represent mean ± s.d. of 5 mice of each group. n.s., not statistically significant.

Supplementary Figure 17



Supplementary Figure 17

Expression of PTEN, EGFR, and p53 in stem-like glioblastoma cells used in this study.

Expression of PTEN, EGFR (truncated, variant III mutant), and p53 in stem-like glioblastoma cells used in this study was determined by immunoblot analysis. T98G, a serum-cultured, conventional human glioblastoma cell line, was used as a control for expression of PTEN, variant III mutant of EGFR, and mutated p53.