Supplementary Information for:

Targeting JNK for therapeutic depletion of stem-like glioblastoma cells

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#These authors contributed equally to this work
Supplementary Figure 1

(a) Images showing cell morphology for different cell lines under different conditions.

(b) Western blot analysis showing protein expression levels for different markers in various cell lines.

- Nestin
- Sox2
- Musashi-1
- GFAP
- βIII-tubulin
- Actin

Comparison between TGS01, TGS04, GS-Y01, GS-Y02, U87GS, and U343GS cell lines in both Sph and Diff conditions.
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

a

**Primary sphere**

Cell viability (%)  

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b

**Primary sphere**

Cell viability (%)  

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**Secondary sphere**

Cell viability (%)  

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c

**Primary sphere**

Cell viability (%)  

<table>
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<th>siJNK2</th>
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**Secondary sphere**

Cell viability (%)  

<table>
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d

**Primary sphere**

Cell viability (%)  

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**Secondary sphere**

Cell viability (%)  

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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. 2a and Fig. 2e was examined using the dye exclusion method. Values represent mean ± s.d. from 3 experiments. n.s., not statistically significant.
Supplementary Figure 3

a

SP600125 (μM) 0 40
FOXO1 Actin
(TGS01)

SP600125 (μM) 0 20
FOXO1 Actin
(GS-Y01)

b

Cytoplasmic Nuclear
SP600125 (40 μM) - + - +
FOXO1 α-Tubulin PARP
(TGS01)

SP600125 (20 μM) - + - +
FOXO1 α-Tubulin PARP
(GS-Y01)

c

FOXO1 Hoechst Merge
DMSO (GS-Y01)

SP600125 (20 μM)

(d)

siRNA Cont. FOXO1 Cont. FOXO1
SP600125 (40 μM) - + - +
FOXO1 GFAP βIII-tubulin Actin
(TGS01)

SP600125 (20 μM) - + - +
FOXO1 GFAP βIII-tubulin Actin
(GS-Y01)
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 \mu M for TGS01 and 20 \mu M for the others) for 3 days and then subjected to immunoblot analysis for the expression of FOXO family members.
### Supplementary Figure 5

<table>
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<table>
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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

(a) DMSO and SP600125 images showing primary and secondary spheres.

(b) Western blot analysis of SP600125 at 0, 10, and 20 µM showing proteins like Nestin, Sox2, Bmi1, Musashi-1, GFAP, βIII-tubulin, and Actin.

(c) Western blot analysis with SP600125 at 0, 10, and 20 µM.

(d) Immunofluorescence images showing Sox2, Musashi-1, Nestin, CD133, GFAP, and βIII-tubulin under DMSO and SP600125.

(e) Western blot analysis showing JNK1 and JNK2 knockdown effects across siCont, siJNK1, and siJNK2 conditions.

(f) Western blot analysis of Nestin, Sox2, Bmi1, Musashi-1, GFAP, and βIII-tubulin under JNK1 and JNK2 knockdown conditions.

(g) Western blot analysis with JNK1 and JNK2 knockdown conditions.
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

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 - e) 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

(a) Western blot analysis of CD133, Nestin, Sox2, Bmi1, GFAP, and Actin in cells treated with different concentrations of SP600125 (μM).

(b) Comparison of the number of spheres formed by DMSO and SP600125 (20 μM) treatment in the first and second cycles.
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

a

DMSO  SP600125

Tumor volume (mm³)

Time (Days)

b

siCont.  siJNK1  siJNK2

Tumor volume (mm³)

Time (Days)

c

DMSO  SP600125

Survival rate (%)

Time (Days)

d

DMSO  SP600125

Tumor volume (mm³)

Time (Days)

e

DMSO  SP600125

Tumor volume (mm³)

Time (Days)
**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 \((1 \times 10^5 \text{ cells per mouse})\) 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

![Graph showing tumor volume over time for DMSO and SP600125 treatments](https://example.com/graph.png)

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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

(a) Sphere culture condition vs. Differentiation-inducing culture condition

(TGS01-2nd)

(b) Western blot analysis of different cell states

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<thead>
<tr>
<th>Gene</th>
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<th>Diff</th>
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<tr>
<td>Actin</td>
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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 (TGS01-2<sup>nd</sup>) 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) TGS01-2<sup>nd</sup> 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

(a) Images of cells on Day 3, Day 5, and Day 7 for DMSO and SP600125 treatments.

(b) Graph showing the number of cells (×10^5) over days. The comparison between DMSO and SP600125 treatments shows no significant difference (n.s.).
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, $1 \times 10^6$ cells) were, after tumour formation, randomized into control and SP600125 treatment groups (3 mice per group; tumour volume at randomization, $171 – 288 \text{ mm}^3$) 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 $\pm$ s.d. of triplicate cultures. n.s., not statistically significant.
Supplementary Figure 13

a

Primary sphere  Secondary sphere

DMSO

TMZ

No. of spheres

n.s.  n.s.

1st  2nd

DMSO  TMZ

b

Survival rate (%)

DMSO

1 X 10^5

1 X 10^3

1 X 10^1

1 X 10^1

TMZ

n.s.

n.s.

(Days)
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, $1 \times 10^6$ 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

TGS01

GS-Y01

U87GS

TGS04

GS-Y02

Survival rate (%)

Survival rate (%)

Survival rate (%)

Time (Days)

Time (Days)

Time (Days)

Time (Days)
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

![Graph showing body weight over time (0 to 12 months) with labels for DMSO and SP800125, and indication of n.s. (not significant).]

b

![Graph showing survival rate over time (0 to 700 days) with labels for DMSO and SP800125, and indication of n.s. (not significant).]
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

(a) Alternation behavior (%)

(b) Number of entries

DMSO  SP600125

3 months  6 months

n.s.
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

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.