Quiescent human glioblastoma stem cells drive tumor initiation, expansion, and recurrence following chemotherapy

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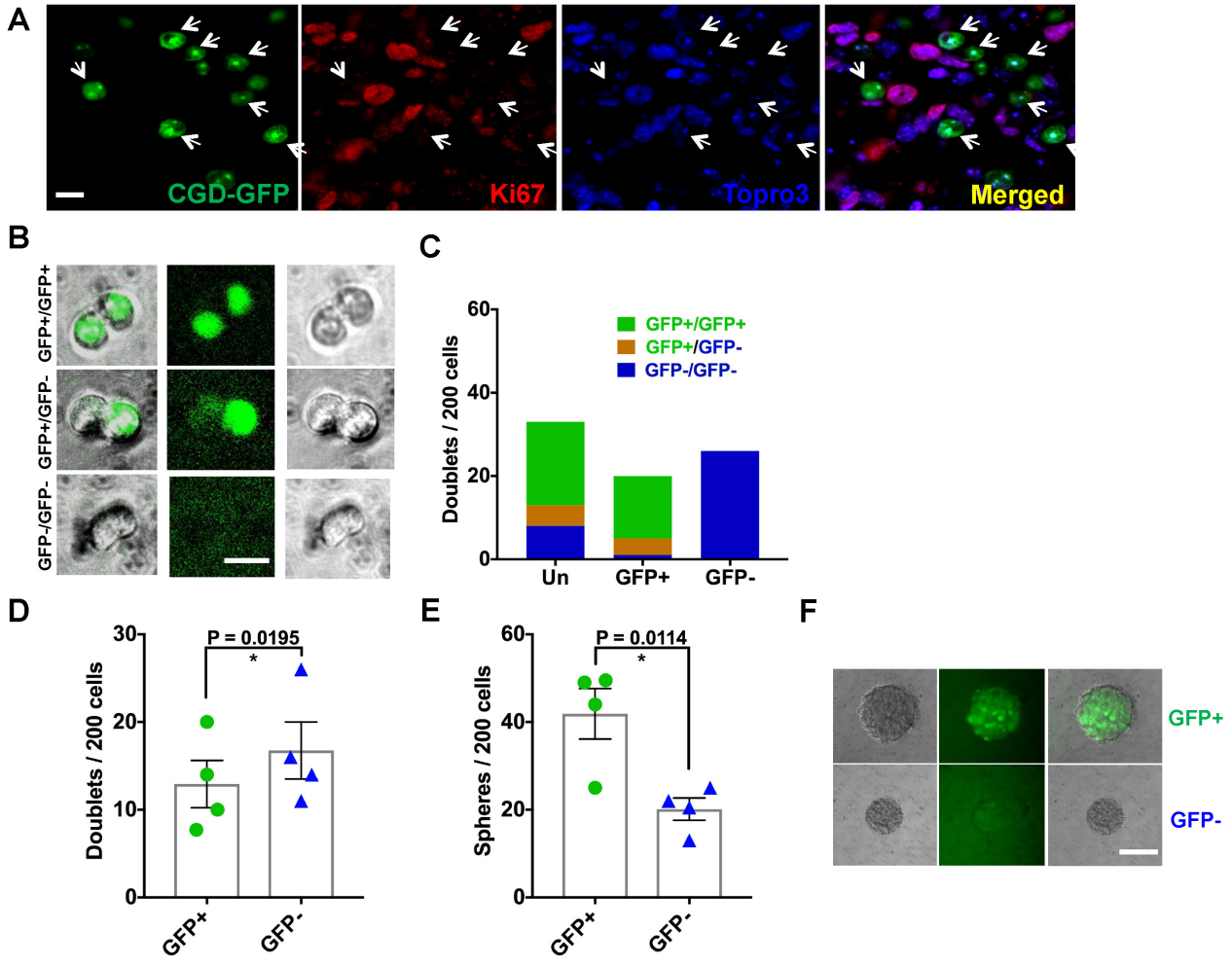


Figure S1. *CGD*-GFP labels quiescent stem-like GBM cells. Related to Figure 1. **(A)** Immunofluorescence (IF) images of a spontaneous mouse GBM. Note the GFP+ cells (arrow heads) are rare and remain Ki67- throughout the tumor. Scale bar: 20μm. **(B)** Representative images of sorted low density cultured GBM GFP+ and GFP- dividing cells. Note GFP+ cells can give rise to GFP+/+ or GFP+/- doublets. **(C)** Representative cell division analysis outcomes derived from cultured spontaneous mouse GBM cells that were: unsorted (Un); GFP+ sorted; or GFP- sorted. **(D)** Doublet formation assay for sorted tumor GFP+ and GFP- cells at 18-24 hours and; **(E)** Sphere formation assay after six days. Paired T test analysis was performed. Mean ± SEM, n = 4 biological replicate tumors for each group (each representing 5 technical replicates). **(F)** Representative tumor sphere images from sorted GFP+ and GFP- cells. Scale bar: 100μm.

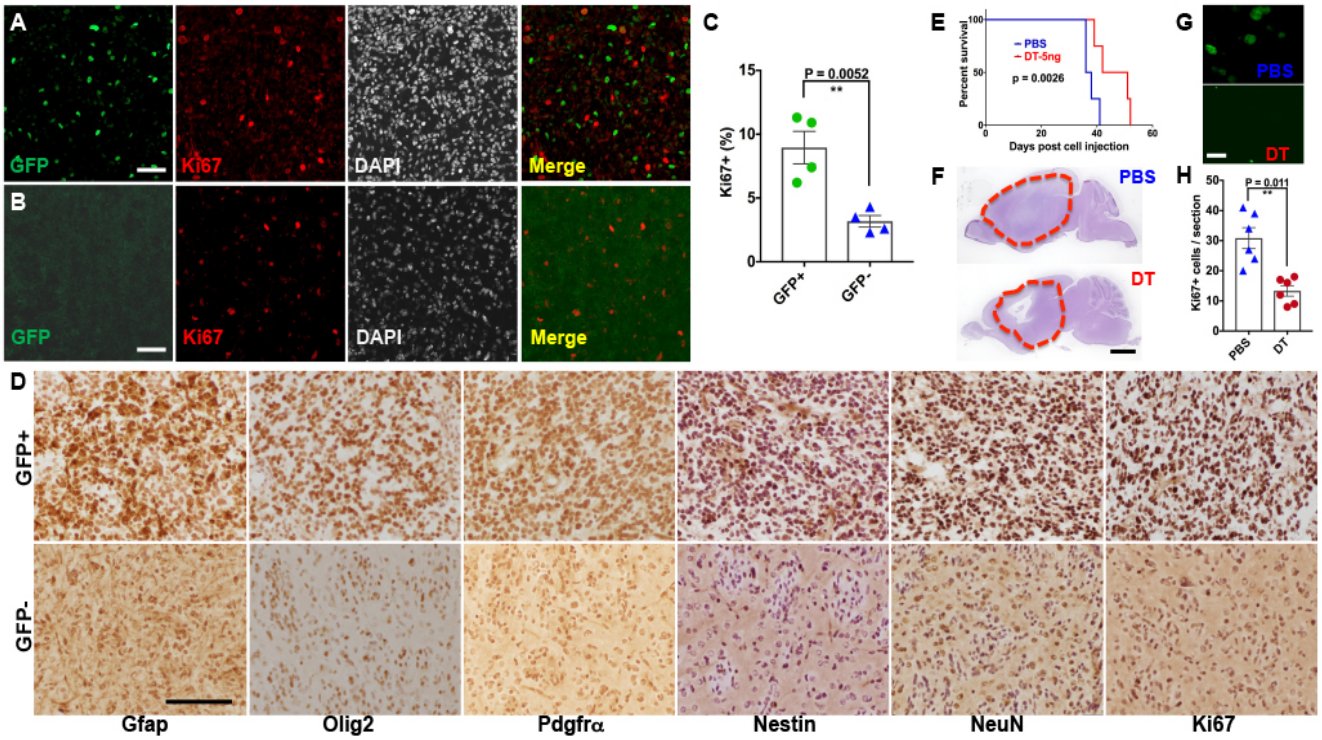
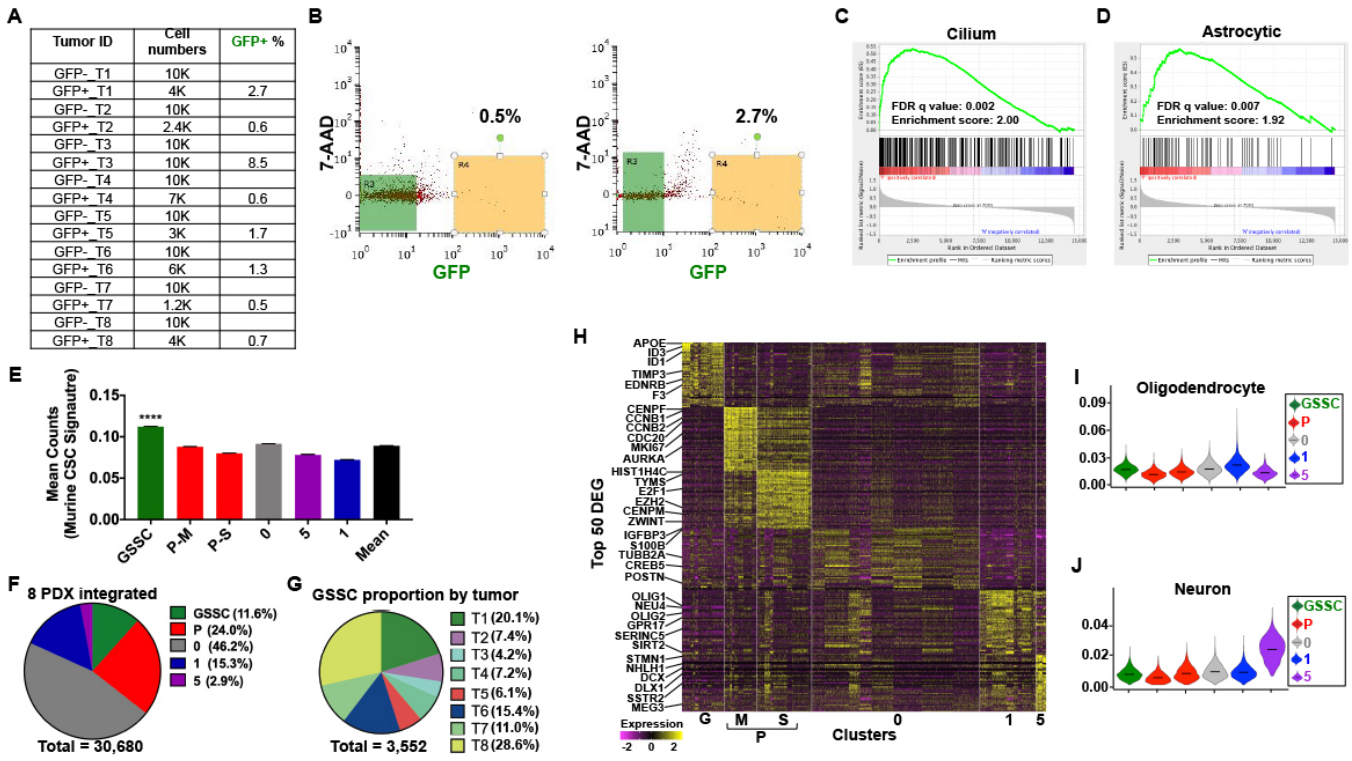


Figure S2. *CGD* transgene GFP labels quiescent, diphtheria toxin sensitive GBM CSC. Related to Figure 2. **(A)** GBM IF images from GFP+ cell transplantation demonstrate mutually exclusive GFP & Ki67. **(B)** Tumors from GFP- cell transplantation are less cell dense (DAPI), lack GFP+ cells, and have limited Ki67+ cells. **(C)** Dot plot indicating elevated Ki67+ tumor cells in GFP+ cell transplanted tumors versus GFP- tumors. Ki67+ cell fractions were quantified relative to total DAPI+ cells. Mean ± SEM, n=4 biological replicate tumors for each group. **(D)** Immunohistochemistry of GBM heterogeneity markers in GFP+ and GFP- cell transplanted tumors. **(E)** A single dose of Diphtheria toxin (DT) significantly prolongs life span of mice transplanted with *CGD* GBM cells as illustrated by survival curves; (**F)** DT reduces tumor volume as shown by H&E staining of GBM containing brain sagittal sections (red circles enclose GBM); **(G)** DT effectively ablates CGD-GFP+ cells as revealed by IF; **(H)** DT treatment reduces Ki67+ cells at end point. n=4 technical replicates for each group. Scale bars in (A), (D), (F) and (G): 40μm, 100μm, 1mm, and 20μm.



Tumor ID	Cell numbers	GFP+ %
GFP-_T1	10K	
GFP+_T1	4K	2.7
GFP-_T2	10K	
GFP+_T2	2.4K	0.6
GFP-_T3	10K	
GFP+_T3	10K	8.5
GFP-_T4	10K	
GFP+_T4	7K	0.6
GFP-_T5	10K	
GFP+_T5	3K	1.7
GFP-_T6	10K	
GFP+_T6	6K	1.3
GFP-_T7	10K	
GFP+_T7	1.2K	0.5
GFP-_T8	10K	
GFP+_T8	4K	0.7

Figure S3. A CGD-GFP+ murine GBM cancer stem cell transcription signature. Related to Figure 3. **(A-B)** Summary of eight pairs of GFP+ and GFP- cells isolated from spontaneous mouse GBM with (B) two examples of FACS sorting gates shown. Collected GFP- and GFP+ cells are shown in gate R3 (green bar) and R4 (orange bar). The two tumors contain 0.5% (left) and 2.7% (right) GFP+ cells. **(C)** Cilium and **(D)** astrocytic signatures are enriched in GFP+ cells, as determined by GSEA analysis. **(E)** Bar plot of the integrated 8 PDX dataset displaying the mean counts for each cluster of the murine CSC signature. The integrated GSSC cluster is marked by 4 asterisks denoting an adjusted p-value<0.0001 for a Kruskal-Wallis ANOVA test with Dunn's multiple comparison test of each cluster compared to all others, indicating that it is significantly higher in mean murine CSC signature than every other cluster. The technical replicates were as follows: N=3552 for GSSC, 4615 for PC-M, 2761 for PC-S, 14164 for cluster 0, 882 for cluster 5, 4706 for cluster 1, and 30680 for the Mean. The error bars represent standard error of the mean. **(F)** Pie charts display the relative proportion of integrated tumor cells enriched for the mouse quiescent CSC signature (GSSC; green), mitotic and S-phase signatures (P; red). Grey indicates remaining unspecified neoplastic cells (clusters 0, 1, & 5). The numbers to the right indicate the relative percentages for each type. The total represents the total cell number for the integrated 8 PDX dataset. **(G)** Pie chart displays the relative proportion of cells for each tumor within the integrated GSSC cluster. The numbers to the right indicate the relative percentages for each tumor. **(H)** Heatmap of the top 50 DEG for each cluster of the 8 PDX integrated dataset showing enrichment of integrated GSSC genes (#3), proliferative genes (#2, #4), and others (see Methods). Rows indicate genes, columns indicate cells. Scale is located at the top right. Selected genes are indicated (see Table S3A-B for a full gene list). G is the integrated GSSC cluster. **(I-J)** Violin plots of the per cell AUCell enrichment scores within the integrated 8 PDX dataset depict enrichment in (I) cluster 1 for oligodendrocyte related, or (J) cluster 5 for neuron related signatures (Table S3C).

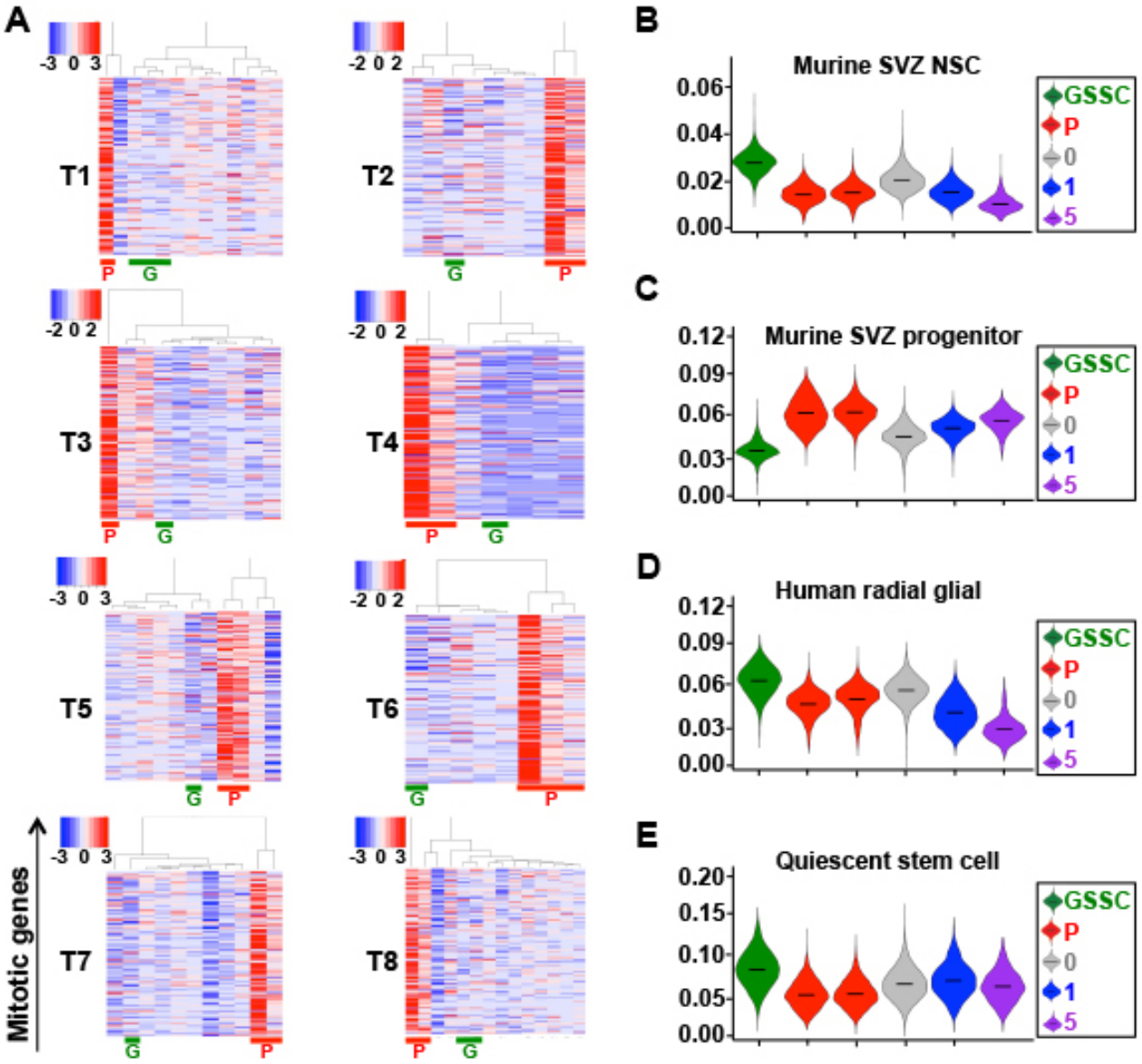


Figure S4. Non-proliferative patient derived GSSC groups enriched for murine CSC and stem cell signatures. Related to Figure 4. **(A)** Heatmaps of individual PDX tumors (1-8) depicting the expression by cell clusters for the mitotic signature genes. G is GSSC clusters. The rows represent gene expression level and the columns are clusters. The colored legend depicts the scale of expression. Note GSSC groups are distinct from the mitotic P groups for all tumors. **(B-C)** Violin plots of the per cell enrichment scores by AUCell within the integrated 8 PDX dataset depict enrichment in (B) GSSC for the wildtype quiescent SVZ NSC, or (C) P for the signature of proliferative neural progenitors (Table S3C). **(D-E)** Violin plot representation of the same analysis as in (B) but for (D) the human radial glial stem cell signature, or (E) the common quiescent stem cell signature (See Table S3C, green = GSSC, red = P).

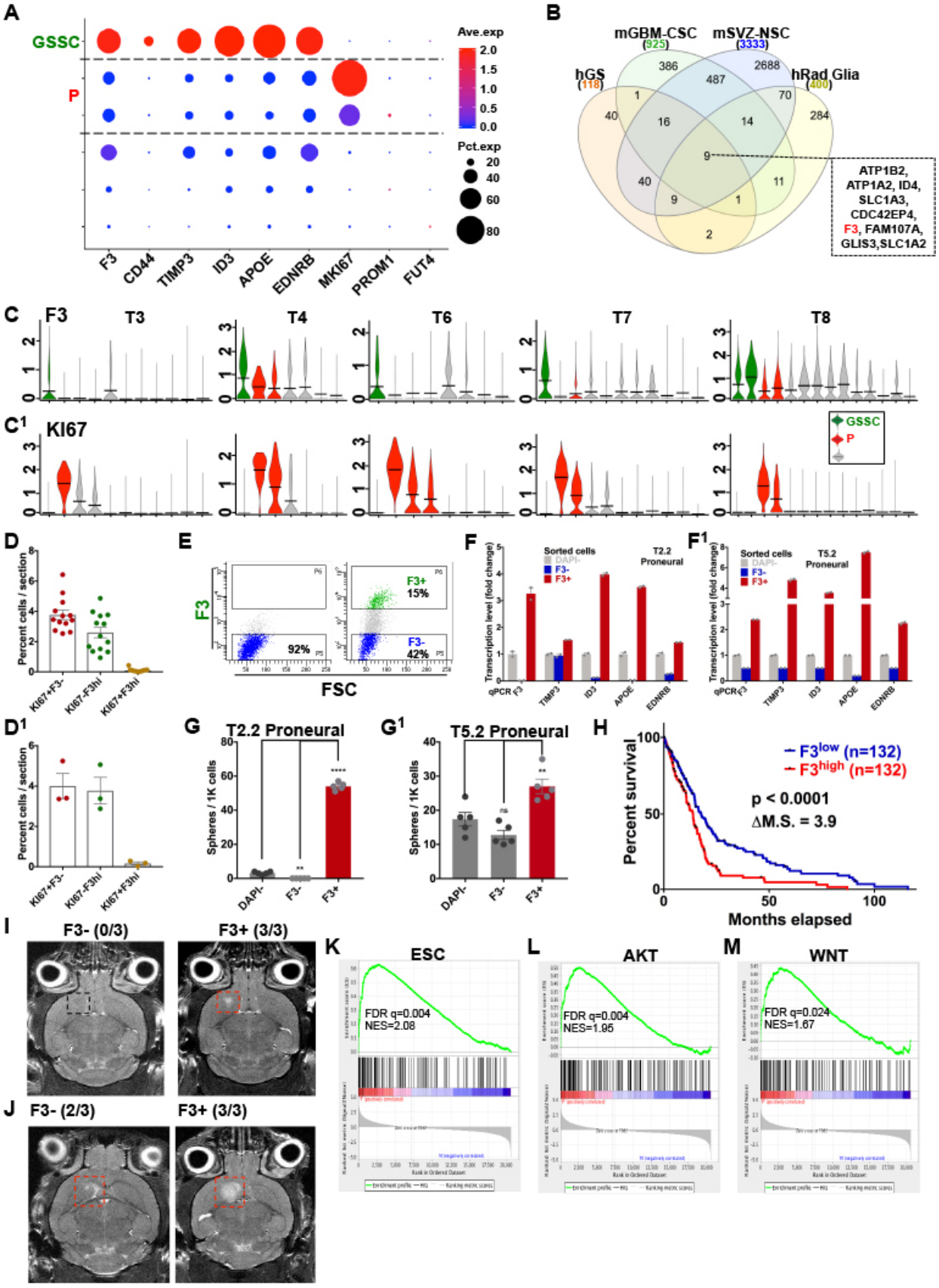

A
GSSC
P
Ave.exp
2.0
1.5
1.0
0.5
0.0
Pct.exp
20
40
60
80
F3
CD44
TIMP3
ID3
APOE
EDNRB
MKI67
PROM1
FUT4
B
mGBM-CSC (925)
mSVZ-NSC (3333)
hGS (118)
hRad Glia (400)
386
2688
487
1
70
40
16
14
284
9
40
11
9
1
2
ATP1B2, ATP1A2, ID4, SLC1A3, CDC42EP4, F3, FAM107A, GLIS3,SLC1A2
C
F3
T3
T4
T6
T7
T8
C1
KI67
GSSC
P
D
Percent cells / section
KI67+F3-
KI67-F3hi
KI67+F3hi
E
F3
FSC
F3+ 15%
92%
F3- 42%
F
Sorted cells
DAPI-
F3-
F3+
T2.2 Proneural
Transcription level (fold change)
qPCR F3
TIMP3
ID3
APOE
EDNRB
F1
T5.2 Proneural
D1
G
T2.2 Proneural
Spheres / 1K cells
DAPI-
F3-
F3+
G1
T5.2 Proneural
H
F3low (n=132)
F3high (n=132)
p < 0.0001
ΔM.S. = 3.9
Percent survival
Months elapsed
I
F3- (0/3)
F3+ (3/3)
J
F3- (2/3)
F3+ (3/3)
K
ESC
FDR q=0.004
NES=2.08
L
AKT
FDR q=0.004
NES=1.95
M
WNT
FDR q=0.024
NES=1.67

Figure S5. F3 antibody as a tool to isolate 118-GS expressing GBM cells. Related to Figure 5. **(A)** Dot plot depicts mRNA enrichment of six signature genes in the human integrated GSSC population, one proliferative marker (KI67) enriched in the P population, and two genes (PROM1 and FUT4) previously used as CSC markers. Circle color indicates average graded gene expression within each unsupervised GBM cell cluster. Circle size represents relative number of cells within each cell cluster expressing the gene (see side legend). **(B)** Venn diagram illustrates nine overlapping genes among the human 118 gene (hGS) signature with a murine GBM-CSC signature, a murine SVZ quiescent stem cell signature, and a human radial glia stem cell signature. Gene numbers are listed in parentheses. See also Table S3C. **(C-C^1)** Five additional GBM PDX unsupervised cell cluster violin plots depicting elevated (C) F3 receptor gene expression in GS designated cell clusters and (C^1) KI67 mRNA in the proliferative clusters. Horizontal black bars indicate mean values. **(D-D^1)** Bar graphs show IF quantitative analysis for F3 protein expression mutually exclusive from KI67+ cells in (D) 13 primary and (D^1) 3 recurrent GBM PDX. Mean ± SEM, n=13 or 3 biological replicate tumors. **(E)** Representative F3 FACS analysis for a GBM PDX sample. **(F-F^1)** qRT-PCR results validate enrichment of 118-GS signature genes in F3-sorted cells from two additional PDX samples T2 and T5. n=2 technical replicates for each group. **(G-G^1)** Sphere formation assays of F3 sorted cells from two additional PDX samples T2 and T5. n=5 technical replicates for each group. **(H)** TCGA GBM patient survival curve illustrates F3 high gene expression tracks with reduced patient survival post prognosis. ΔM.S. = Median survival difference. **(I-J)** MRI of whole mouse brains (I) At day 60 only F3+ sorted cells exhibit tumors image enhancement and (J) At day 78 F3+ cells demonstrate significantly increased enhancement compared to F3- cell tumors. Ratios in parenthesis indicate fraction of detected tumors. Tumors are outlined by red dash box, while corresponding area without tumor lesions are indicated by black dash box. **(K-M)** (K) Embryonic stem cell (ESC), (L) AKT, and (M) WNT signatures are enriched in F3+ cells, as determined by GSEA analysis.

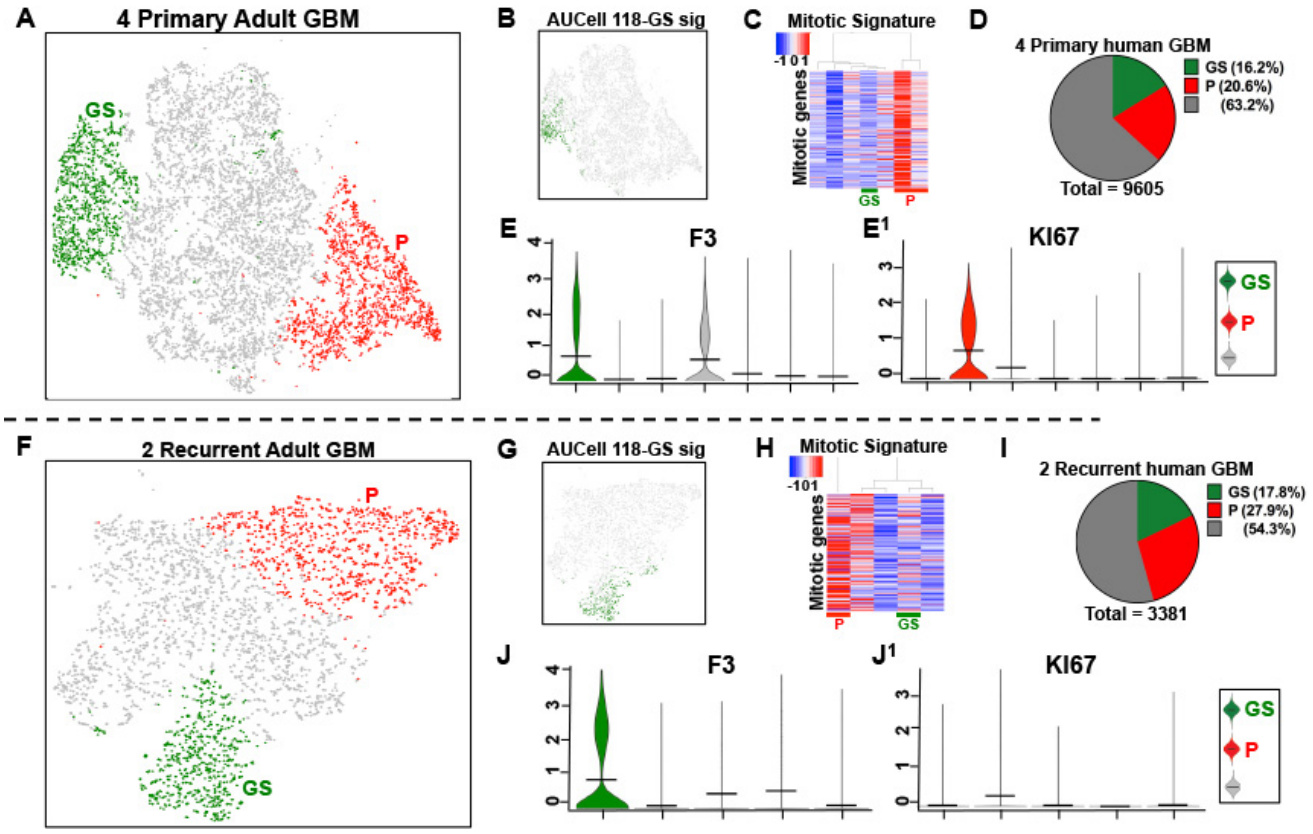


Figure S6. Presegregated non-proliferative GS clusters in primary and recurrent GBM. Related to Figure 6. **(A)** tSNE projection of integrated dataset of four primary human GBM. Green indicates cell clusters enriched for the 118-GS signature (GS). Red indicates cell clusters enriched for the mitotic signature (P). Grey labels other unspecified neoplastic cell clusters. **(B)** tSNE plot of the four primary human GBM datasets with cells that surpass the AUCell enrichment threshold for expression of the 118-GS signature marked in green (predominately in GS) and all other cells in grey. **(C)** Heatmap of the mitotic signature expressed in these clusters. Each row is a gene in the signature, each column is a cluster. The color scale is at the top left. **(D)** Pie chart of the proportions of cell types in the primary human GBM. The numbers in parenthesis indicate the relative percentages for each type within the neoplastic cells. **(E-E^1)** Violin plots depicting elevated (E) F3 receptor gene expression in GS cell clusters and (E^1) KI67 mRNA in the proliferative clusters. Horizontal black bars indicate mean values. **(F-J^1)** The same analyses as in (A-E^1) for two integrated recurrent human GBM dataset (green = GS and red = P).

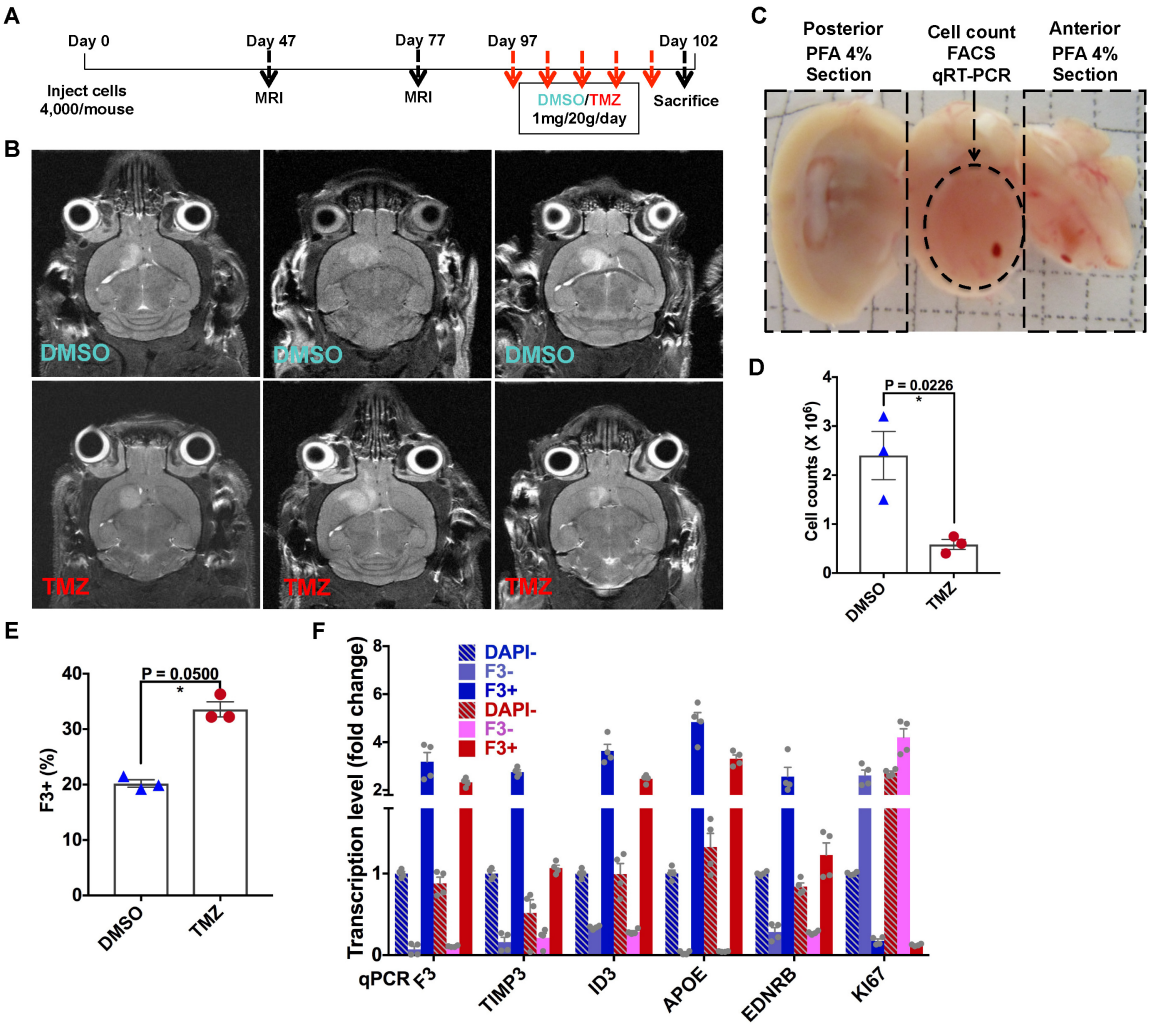


Figure S7. PDX tumor growth following TMZ treatment. Related to Figure 7. **(A)** Diagram of the experimental procedure and schedule for PDX generation, MRI imaging, and DMSO/TMZ treatment. **(B)** Representative whole brain MRI images captured 77 days after GBM cell inoculation. **(C)** Representative images depict the tumor sample harvest and designation. Anterior and posterior regions were used for IF images in Fig. 8A-B. Middle regions were used for analyses in panels D-F. **(D)** Dot plot indicates the total number of tumor cells collected and counted from each tumor after mouse cell depletion (see Methods). n=3 biological replicates for each condition. **(E)** Bar graph demonstrates enrichment of F3+ cell compartment in PDX after TMZ treatment as compared to DMSO. n=3 biological replicates for each condition. **(F)** qRT-PCR data validate retained expression of select GS signature genes (F3, ID3, and APOE) in F3+ cells after TMZ (Red) treatment commensurate to DMSO (Blue). Expression normalized to DAPI- (sorted viable cells) from DMSO group. n=4 technical replicates for each group.