SUPPLEMENTAL MATERIAL

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Gene signatures of quiescent glioblastoma cells reveal mesenchymal shift and interactions with niche microenvironment

Supplemental Data File

File_S1_PMT.gmx

Gene set matrix file (.gmx) with gene sets that were used to analyze proneural-mesenchymal transition (PMT) by gene set enrichment analysis (GSEA)

Supplemental Tables

Table S1 List of DEG in qGBM.xlsx

Lists of differentially expressed genes (DEGs) between GFP^{high} (quiescent) and GFP^{low} (proliferative) cells in organoids of GBM cell line SD3. DEGs from 2 and 4 week -Dox chase organoids are shown (filtered for protein coding genes). Also listed are the common DEGs of both chase paradigms.

Table S2 Genes in top modules.xlsx

List of genes comprising the top 15 MEGENA co-expression modules that were enriched for qGBM DEGs.

Table S3 Functional annotation of top modules.xlsx

Functional annotations for MEGENA co-expression modules that are significantly enriched for qGBM DEGs.

Table S4 Hub genes of c1_21 c1_360 Pubmed sorted.xlsx

List of top hub genes for the modules c1_21 and c1_360 showing the number of Pubmed citations for a given query term (e.g., ‘glioma.pubmed’ means a query of the gene name and “glioma”), as well as status of a gene as significant qGBM DEG (0=false, 1=true).

Supplemental Figures

Figures S1 – S6 on following pages.

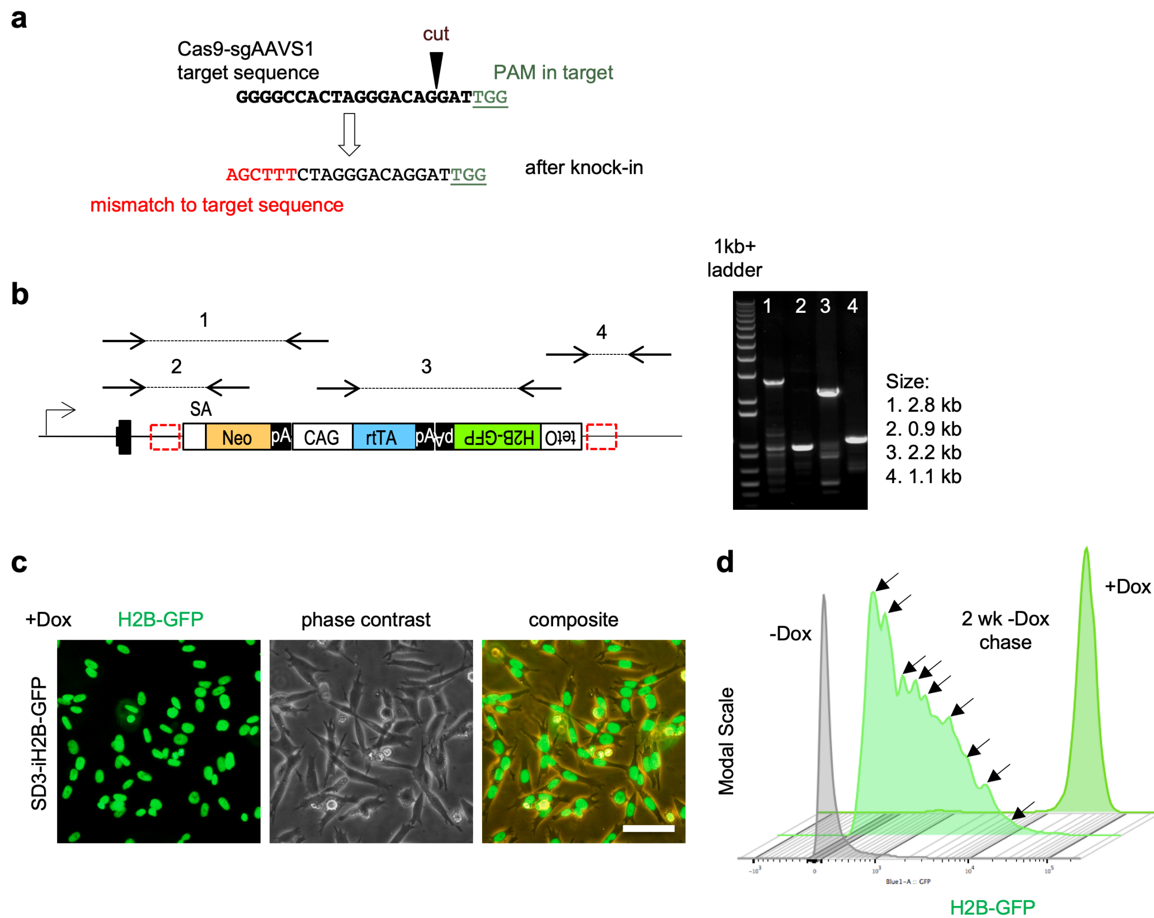


Figure S1. Targeted knock-in of iH2B-GFP reporter into patient-derived GBM cells

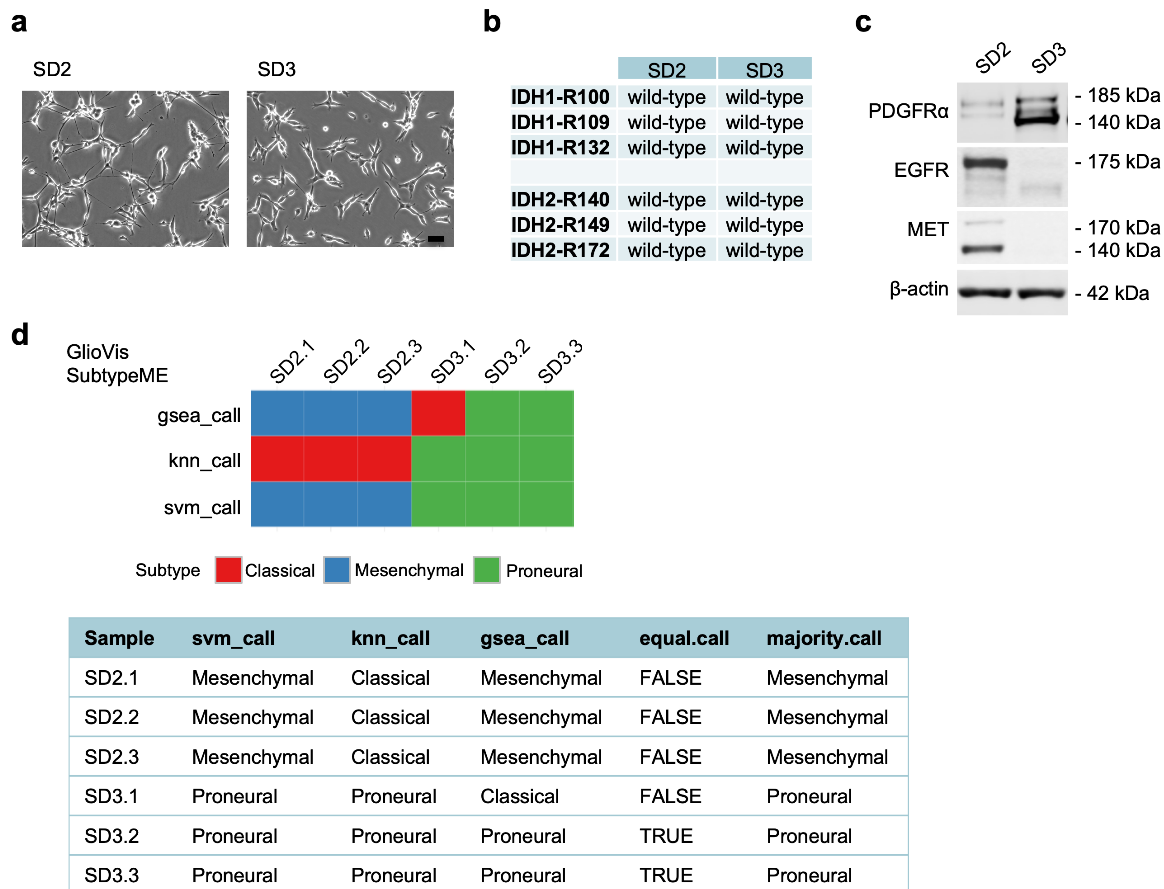
a) CRISPR/Cas9 sgRNA target sequence for AAVS1 locus that was used for knock-in of iH2B-GFP reporter cassette. After knock-in, the CRISPR/Cas9 AAVS1 sgRNA target sequence is disrupted.

b) Confirmation of correct iH2B-GFP reporter knock-in by genomic PCR. The PCR products with four different primer pairs were of predicted size, as shown by gel electrophoresis.

c) Fluorescence and phase contrast images show that SD3-iH2B-GFP cells stably express H2B-GFP when continuously cultured with 1 µg/ml Dox pulse.

d) SD3-iH2B-GFP cells cultured in proliferating conditions on laminin-coated dishes with Dox (+Dox), without Dox (-Dox), or with a Dox pulse/chase paradigm (2 weeks -Dox chase) were analyzed by flow cytometry. Distribution of GFP peaks (arrows) from 2 weeks chase condition indicates that the iH2B-GFP reporter can track up to 9 cell divisions from fully labeled to unlabeled cells.

Scale bar: 50 µm (c).



	SD2	SD3
IDH1-R100	wild-type	wild-type
IDH1-R109	wild-type	wild-type
IDH1-R132	wild-type	wild-type
IDH2-R140	wild-type	wild-type
IDH2-R149	wild-type	wild-type
IDH2-R172	wild-type	wild-type

Sample	svm_call	knn_call	gsea_call	equal.call	majority.call
SD2.1	Mesenchymal	Classical	Mesenchymal	FALSE	Mesenchymal
SD2.2	Mesenchymal	Classical	Mesenchymal	FALSE	Mesenchymal
SD2.3	Mesenchymal	Classical	Mesenchymal	FALSE	Mesenchymal
SD3.1	Proneural	Proneural	Classical	FALSE	Proneural
SD3.2	Proneural	Proneural	Proneural	TRUE	Proneural
SD3.3	Proneural	Proneural	Proneural	TRUE	Proneural

Figure S2. Characterization of SD2 and SD3 GBM cell lines

a) Phase-contrast images of GBM patient-derived cell lines SD2 and SD3, cultured in neural stem cell media on laminin-coated dishes.

b) DNA sequencing indicated wild-type status of key arginine residues in *IDH1* and *IDH2* genes in SD2 and SD3 GBM cells.

c) Western blot analysis showed high expression of EGFR and MET in SD2 GBM cells (lower band at 140 kDa is truncated mutant form) and high expression of PDGF receptor α in SD3 GBM cells. β-actin serves as loading control.

d) GBM cell line SD2 was classified as mesenchymal and SD3 as proneural molecular subtype by the SubtypeMe function of the web platform GlioVis (http://gliovis.bioinfo.cnio.es/). Normalized RNA-Seq gene count data from three replicates of each line (independently cultured cells) was used as input. The SubtypeMe function uses three algorithms for subtype calculation (support-vector machine, SVM; k-nearest neighbors, KNN; single sample gene set enrichment, ssGSEA) and provides a majority call.

Scale bar: 50 µm.

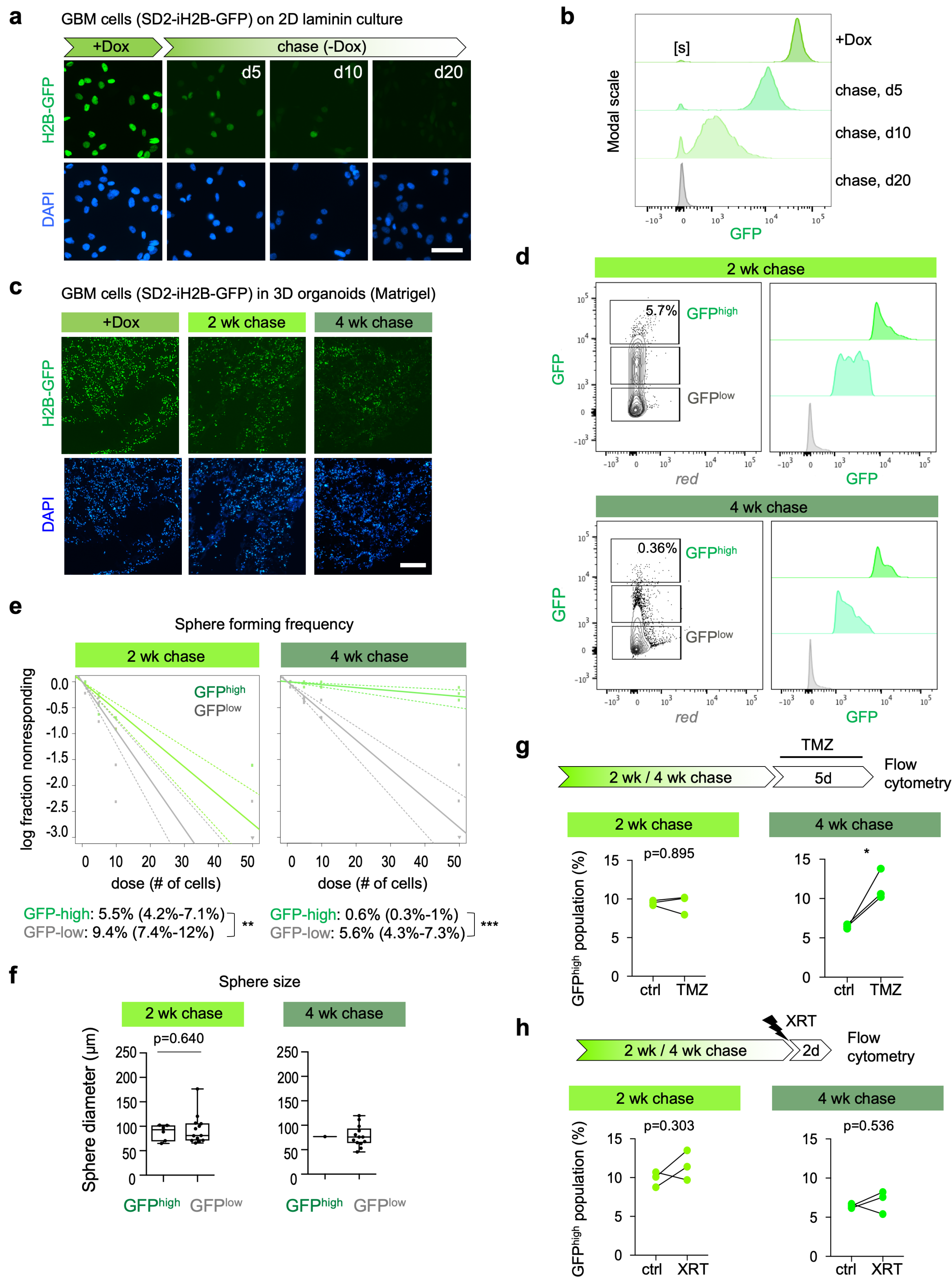


Figure S3. Characteristics of quiescent GBM cells in SD2-iH2B-GFP cell line

a) SD2-iH2B-GFP GBM cells grown as proliferative culture on 2D laminin-coated dishes. In the presence of doxycycline (+Dox), nuclei are labelled with H2B-GFP. Cells dilute H2B-GFP label during -Dox chase periods (5, 10, and 20 days shown) by cell division. DAPI was used for nuclear counter staining.

b) Flow cytometry of SD2-iH2B-GFP cells grown on 2D laminin for the indicated -Dox chase periods. A small population of SD2-iH2B-GFP cells remained GFP-negative even in +Dox conditions (denoted as "[s]"), possibly due to sporadic silencing of transgene. Histograms are normalized on y-axis to modal scale (FlowJo).
c) Fluorescence micrographs of sections of 3D GBM organoids show a declining number of label-retaining SD2 GFP^{high} cells during organoid expansion.
d) SD2 iH2B-GFP GBM organoids were analyzed by FACS after 2 or 4 week -Dox chase. Representative results are shown. After 2 week chase, 5·7% of cells remained GFP^{high}, and after 4 week chase, 0·36% of cells remained GFP^{high}. Three independent experiments (10-12 pooled organoids per experiment) yielded similar results. X-axis shows red auto-fluorescence of cells. Histograms are normalized on y-axis to modal scale (FlowJo).
e) Limiting dilution sphere formation assay with SD2 GFP^{high} and GFP^{low} cells. FACS-sorted cells from GBM organoids after 2 or 4 week -Dox chase were seeded in limiting dilutions in 96 well plates. Sphere formation frequency was quantified after 10 days by ELDA software. Results were generated from three independent experiments. Numbers in parenthesis represent 95% confidence interval. Statistical analysis was performed with ELDA software. ** and *** indicate $p < 0·01$ and $p < 0·001$, respectively.
f) Sphere sizes of gliomaspheres derived from SD2 GFP^{high} or GFP^{low} cells after 20 days of culture, with 10 cells seeded per well. Up to ten spheres per group were measured. Boxplots show 25th, 50th, and 75th percentiles of sphere diameters and whiskers represent minimum and maximum values. Statistical analysis was performed by Mann-Whitney test; *p*-value not available for 4 week chase paradigm (single data point in group GFP^{high}).
g) Drug sensitivity assay for temozolomide (TMZ). Organoids after 2 or 4 week of –Dox chase were treated for 5 days with vehicle or 250 µM TMZ, and fraction of live GFP^{high} cells in organoids was quantified by flow cytometry. Quantification shows increased ratio of GFP^{high} cells in TMZ-treated 4 weeks chase organoids as compared to vehicle treatment (ctrl). Data obtained from three independent experiments (10-12 pooled organoids per experiment). Significance was evaluated by binomial generalized linear mixed effect models. * indicates $p < 0·05$.
h) Radiation sensitivity assay. Organoids after 2 or 4 wk chase were irradiated with one dose of 5 Gy, and 2 days later fraction of live GFP^{high} cells in organoids was quantified. Quantification shows a trend for increased fraction of GFP^{high} cells after XRT-treatment as compared to control (ctrl) in 2 and 4 week chase paradigm. Data obtained from three independent experiments (10-12 pooled organoids per experiment). Significance was evaluated with binomial generalized linear mixed effect models.
Scale bars: 50 µm (a), 200 µm (c).

a **GSEA** HALLMARK gene sets

Positively Enriched in SD3 qGBM

2 wk chase

	Gene set	NES	NOM p-val	FDR q-val
1	EPITHELIAL_MESENCHYMAL_TRANSITION	5.17	<0.001	<0.001
2	TNFA_SIGNALING_VIA_NFKB	4.05	<0.001	<0.001
3	MITOTIC_SPINDLE	4.00	<0.001	<0.001
4	COAGULATION	3.56	<0.001	<0.001
5	MYOGENESIS	3.50	<0.001	<0.001
6	APOPTOSIS	3.18	<0.001	<0.001
7	HYPOXIA	2.88	<0.001	<0.001
8	INFLAMMATORY_RESPONSE	2.55	<0.001	0.001
9	KRAS_SIGNALING_UP	2.46	<0.001	<0.001
10	P53_PATHWAY	2.36	<0.001	0.001
11	UV_RESPONSE_DN	2.31	<0.001	0.001
12	COMPLEMENT	2.31	<0.001	0.001
13	IL2_STAT5_SIGNALING	2.29	<0.001	0.001
14	INTERFERON_ALPHA_RESPONSE	2.26	<0.001	0.001
15	ANGIOGENESIS	2.21	<0.001	0.002
16	KRAS_SIGNALING_DN	2.12	<0.001	0.004
17	APICAL_JUNCTION	2.04	<0.001	0.008
18	ESTROGEN_RESPONSE_EARLY	1.97	0.006	0.011
19	IL6_JAK_STAT3_SIGNALING	1.89	0.01	0.018
20	TGF_BETA_SIGNALING	1.86	0.004	0.02
21	CHOLESTEROL_HOMEOSTASIS	1.72	0.024	0.042
22	UV_RESPONSE_UP	1.68	0.025	0.05
23	INTERFERON_GAMMA_RESPONSE	1.67	0.034	0.049

4 wk chase

	Gene set	NES	NOM p-val	FDR q-val
1	EPITHELIAL_MESENCHYMAL_TRANSITION	4.82	<0.001	<0.001
2	MYOGENESIS	3.74	<0.001	<0.001
3	HYPOXIA	3.35	<0.001	<0.001
4	TNFA_SIGNALING_VIA_NFKB	3.21	<0.001	<0.001
5	COAGULATION	3.13	<0.001	<0.001
6	INFLAMMATORY_RESPONSE	2.91	<0.001	<0.001
7	UV_RESPONSE_DN	2.60	<0.001	<0.001
8	APOPTOSIS	2.42	<0.001	0.001
9	ANGIOGENESIS	2.37	<0.001	0.001
10	APICAL_JUNCTION	2.24	0.004	0.003
11	IL2_STAT5_SIGNALING	2.14	0.002	0.006
12	IL6_JAK_STAT3_SIGNALING	2.13	<0.001	0.005
13	TGF_BETA_SIGNALING	2.00	0.002	0.012
14	INTERFERON_GAMMA_RESPONSE	1.98	0.004	0.013
15	COMPLEMENT	1.86	0.008	0.023
16	PROTEIN_SECRETION	1.83	0.01	0.027
17	KRAS_SIGNALING_UP	1.74	0.027	0.041
18	APICAL_SURFACE	1.72	0.03	0.044
19	ESTROGEN_RESPONSE_LATE	1.67	0.034	0.053

Negatively Enriched in SD3 qGBM

2 wk chase

	Gene set	NES	NOM p-val	FDR q-val
1	MYC_TARGETS_V1	-5.28	<0.001	<0.001
2	OXIDATIVE_PHOSPHORYLATION	-4.85	<0.001	<0.001
3	MYC_TARGETS_V2	-3.86	<0.001	<0.001
4	E2F_TARGETS	-2.28	<0.001	0.002
5	DNA_REPAIR	-2.02	0.006	0.011
6	MTORC1_SIGNALING	-1.76	0.023	0.041
7	UNFOLDED_PROTEIN_RESPONSE	-1.74	0.021	0.039
8	G2M_CHECKPOINT	-1.67	0.038	0.048

4 wk chase

	Gene set	NES	NOM p-val	FDR q-val
1	MYC_TARGETS_V1	-6.96	<0.001	<0.001
2	E2F_TARGETS	-6.08	<0.001	<0.001
3	G2M_CHECKPOINT	-5.32	<0.001	<0.001
4	MYC_TARGETS_V2	-3.99	<0.001	<0.001
5	OXIDATIVE_PHOSPHORYLATION	-3.91	<0.001	<0.001
6	MTORC1_SIGNALING	-3.34	<0.001	<0.001
7	DNA_REPAIR	-2.32	<0.001	0.002
8	UNFOLDED_PROTEIN_RESPONSE	-2.05	0.002	0.008
9	REACTIVE_OXIGEN_SPECIES_PATHWAY	-1.91	0.01	0.016
10	XENOBIOTIC_METABOLISM	-1.61	0.035	0.074

b **GSEA** Proneural-mesenchymal transition related gene sets (File_S1_PMT.gmx)

Positively Enriched in SD3 qGBM

2 wk chase

	Gene set	NES	NOM p-val	FDR q-val
1	PHILLIPS_GLIOBLASTOMA_MESENCHYMAL	4.22	<0.001	<0.001
2	VERHAAK_GLIOBLASTOMA_MESENCHYMAL	4.18	<0.001	<0.001
3	VERHAAK_GLIOBLASTOMA_CLASSICAL	3.75	<0.001	<0.001
4	ANASTASSIOU_MULTICANCER_INVASIVENESS	3.71	<0.001	<0.001
5	SEGERMAN_MULTITHERAPY_RESISTANCE	2.69	<0.001	<0.001

4 wk chase

	Gene set	NES	NOM p-val	FDR q-val
1	VERHAAK_GLIOBLASTOMA_MESENCHYMAL	4.12	<0.001	<0.001
2	PHILLIPS_GLIOBLASTOMA_MESENCHYMAL	3.79	<0.001	<0.001
3	VERHAAK_GLIOBLASTOMA_CLASSICAL	3.34	<0.001	<0.001
4	ANASTASSIOU_MULTICANCER_INVASIVENESS	3.32	<0.001	<0.001
5	SEGERMAN_MULTITHERAPY_RESISTANCE	2.03	<0.001	0.01
6	PHILLIPS_GLIOBLASTOMA_PROLIFERATIVE	1.24	0.21	0.20

Negatively Enriched in SD3 qGBM

2 wk chase

	Gene set	NES	NOM p-val	FDR q-val
1	VERHAAK_GLIOBLASTOMA_PRONEURAL	-3.12	<0.001	<0.001
2	PHILLIPS_GLIOBLASTOMA_PROLIFERATIVE	-1.79	0.02	0.04
3	VERHAAK_GLIOBLASTOMA_NEURAL	-1.44	0.09	0.13
4	PHILLIPS__GLIOBLASTOMA_PRONEURAL	-1.17	0.27	0.26

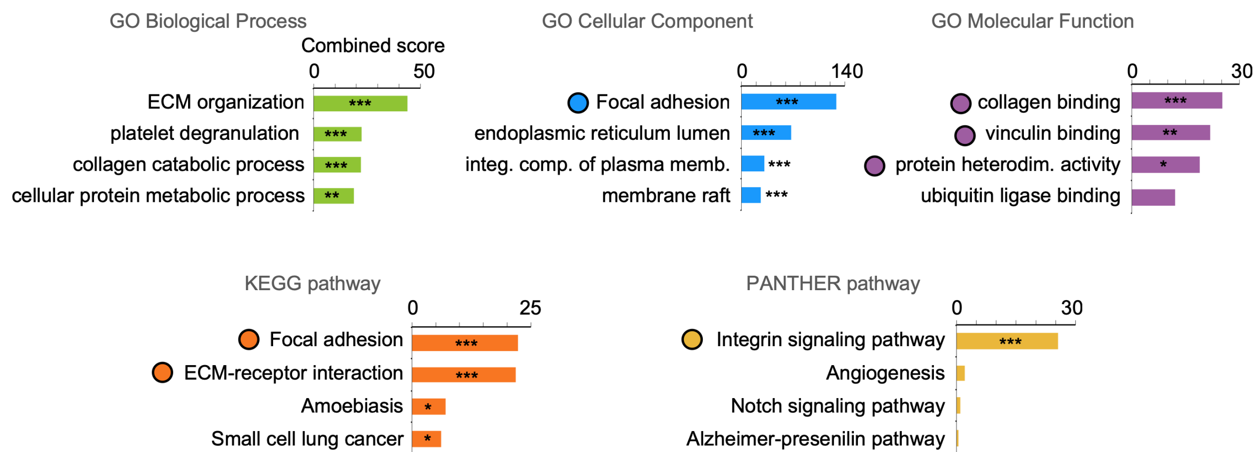
4 wk chase

	Gene set	NES	NOM p-val	FDR q-val
1	PHILLIPS__GLIOBLASTOMA_PRONEURAL	-3.46	<0.001	<0.001
2	VERHAAK_GLIOBLASTOMA_PRONEURAL	-2.37	<0.001	<0.001
3	VERHAAK_GLIOBLASTOMA_NEURAL	-1.36	0.12	0.12

Figure S4. Gene sets enrichment analysis of quiescent SD3-iH2B-GFP cells

a) Tabular results of GSEA analysis (Hallmark gene sets; cut-off: false discovery rate (FDR) adjusted *q*-value < 0·05) for SD3 qGBM cells. The GSEA analysis was applied using all detectable genes ranked by fold expression changes in GFP^{high} vs. GFP^{low} cells at 2 or 4 week -Dox chase stages. The top four positively and top two negatively enriched gene sets of 4 week chase paradigm are color coded in both paradigms for comparison.

b) Tabular results of GSEA analysis for SD3 qGBM cells for gene sets related to proneural-mesenchymal transition process in GBM. The corresponding gene set file is included as supplemental data file File_S1_PMT.gmx (see Methods for description).



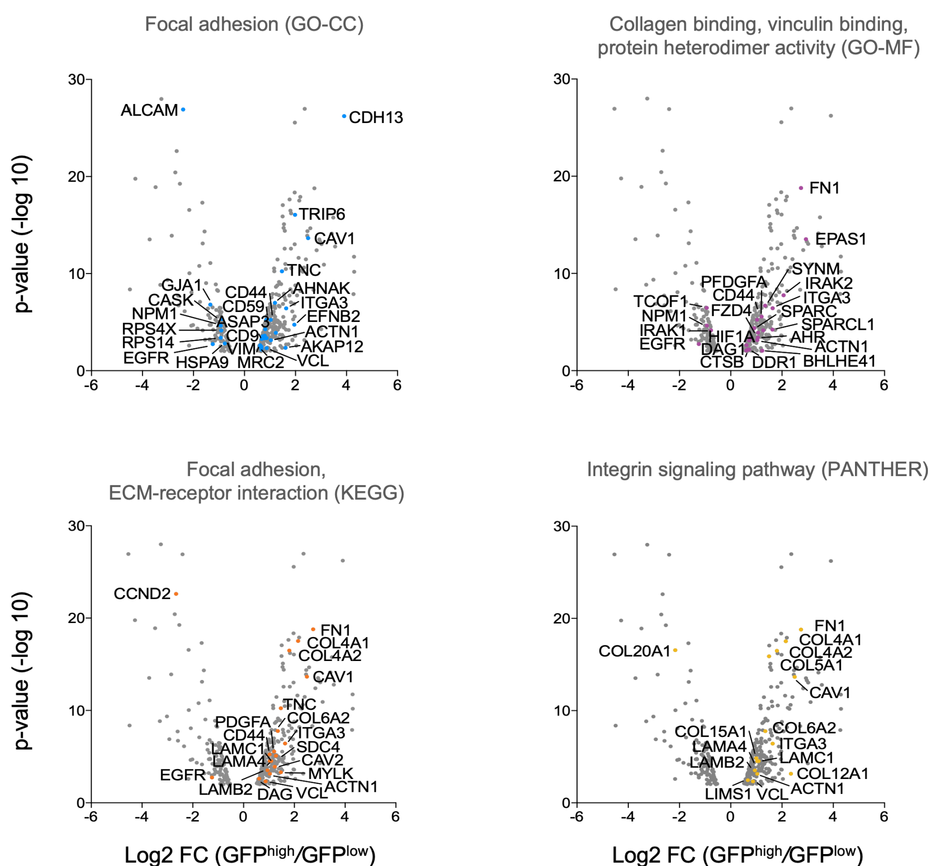


Figure S5. Enriched Gene Ontologies and Pathways in SD3-iH2B-GFP qGBM cells

a) ENRICHR analysis of 345 common DEGs in SD3 qGBM cells was performed for gene ontologies (GO) and KEGG and Panther pathways. The top 4 results by combined score for each category are shown. X-axis indicates combined score as calculated by ENRICHR (adjusted *p*-value multiplied by z-score). *, ** and *** indicate $p < 0{\cdot}05$, $p < 0{\cdot}01$, and $p < 0{\cdot}001$ respectively.

b) Volcano plot showing significantly regulated genes of the indicated enriched GO terms or Pathways marked by colored dots corresponding to the color in (A), gray dots mark common DEGs in qGBM cells (2 week chase).

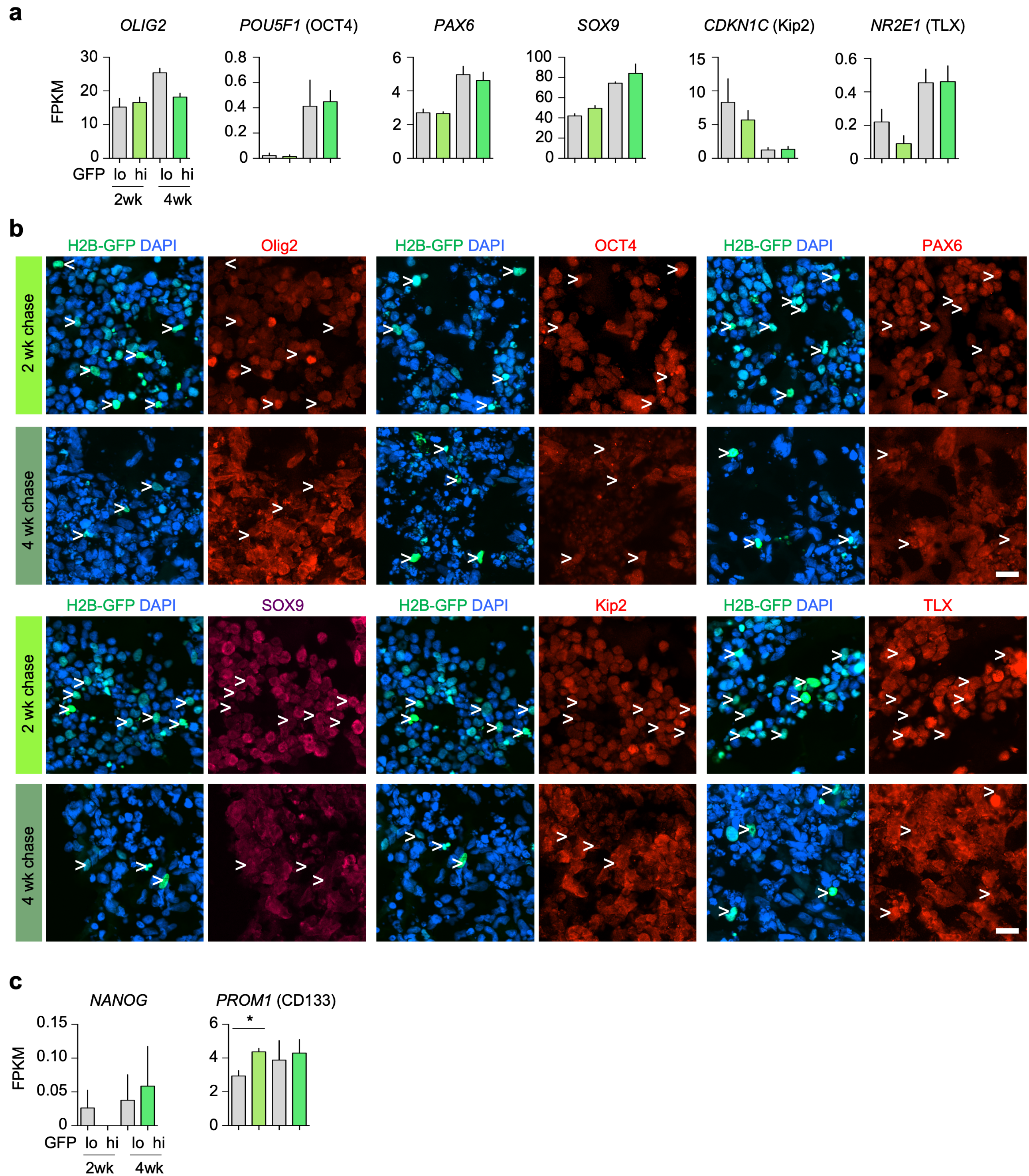


Figure S6. No significant link between quiescence and expression of common stem cell markers in SD3 qGBM cells
a, b) Bar graphs of mean FPKM values (a) and representative IF images of sectioned organoids (b) show expression levels of common stem cells markers in qGBM (H2B-GFP^{high}) and pGBM (H2B-GFP^{low}) at the indicated -Dox chase stages. White arrowheads indicate GFP^{high} cells.
c) Bar graphs showing mean FPKM expression levels of NANOG and PROM1 (CD133) in qGBM vs. pGBM cells at the indicated -Dox chase stages. Proteins for these genes were not detectable by IF on GBM organoids.
Expression data were combined from three independent experiments per paradigm. Statistical analysis was performed using edgeR analysis of read counts, with Benjamini-Hochberg correction. * indicates $p < 0{\cdot}05$, other comparisons did not reach statistical significance. Scale bars: 20 µm (b).

TCGA GBM/LGG patient dataset (GlioVis)
Gene expression levels

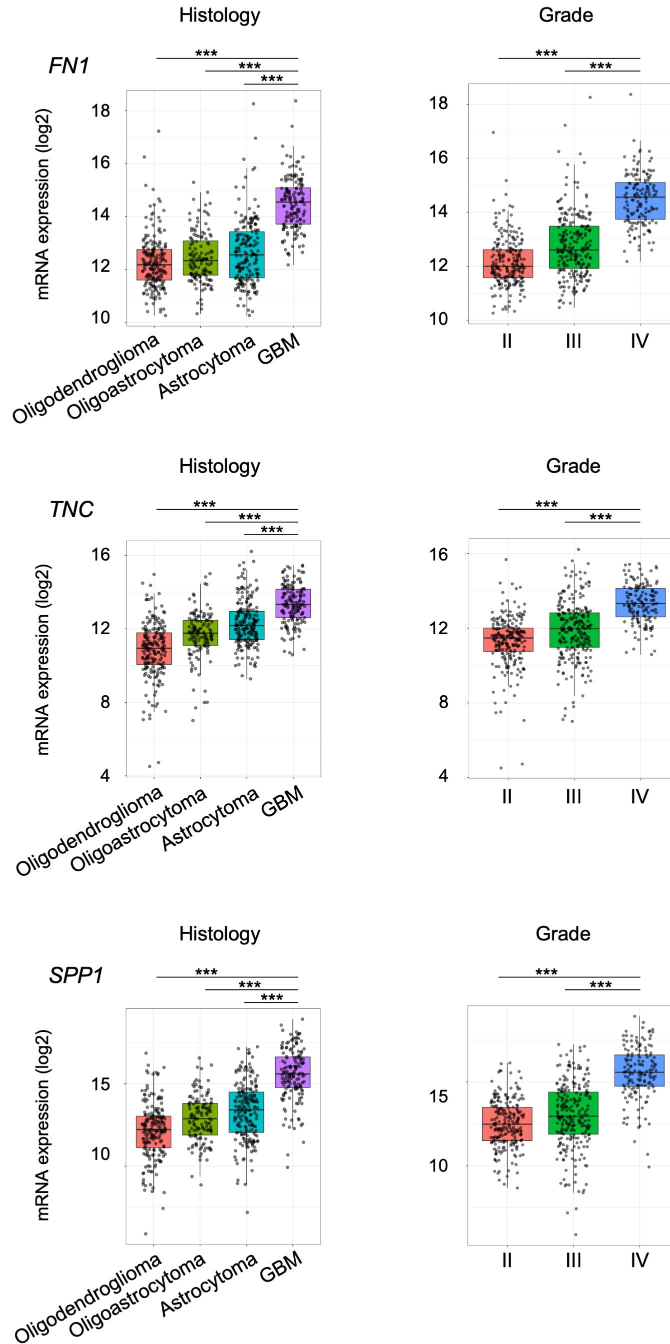


Figure S7. Increased expression of ECM-associated genes with glioma progression

Gene expression values of *FN1*, and *TNC*, and *SPP1* in TCGA GBM-LGG dataset shows that expression of these genes increases with glioma grade (data retrieved from the web platform GlioVis (http://gliovis.bioinfo.cnio.es/). Statistical analysis was performed by GlioVis with pairwise *t*-test, with Bonferroni correction. *** indicates $p < 0{\cdot}001$.