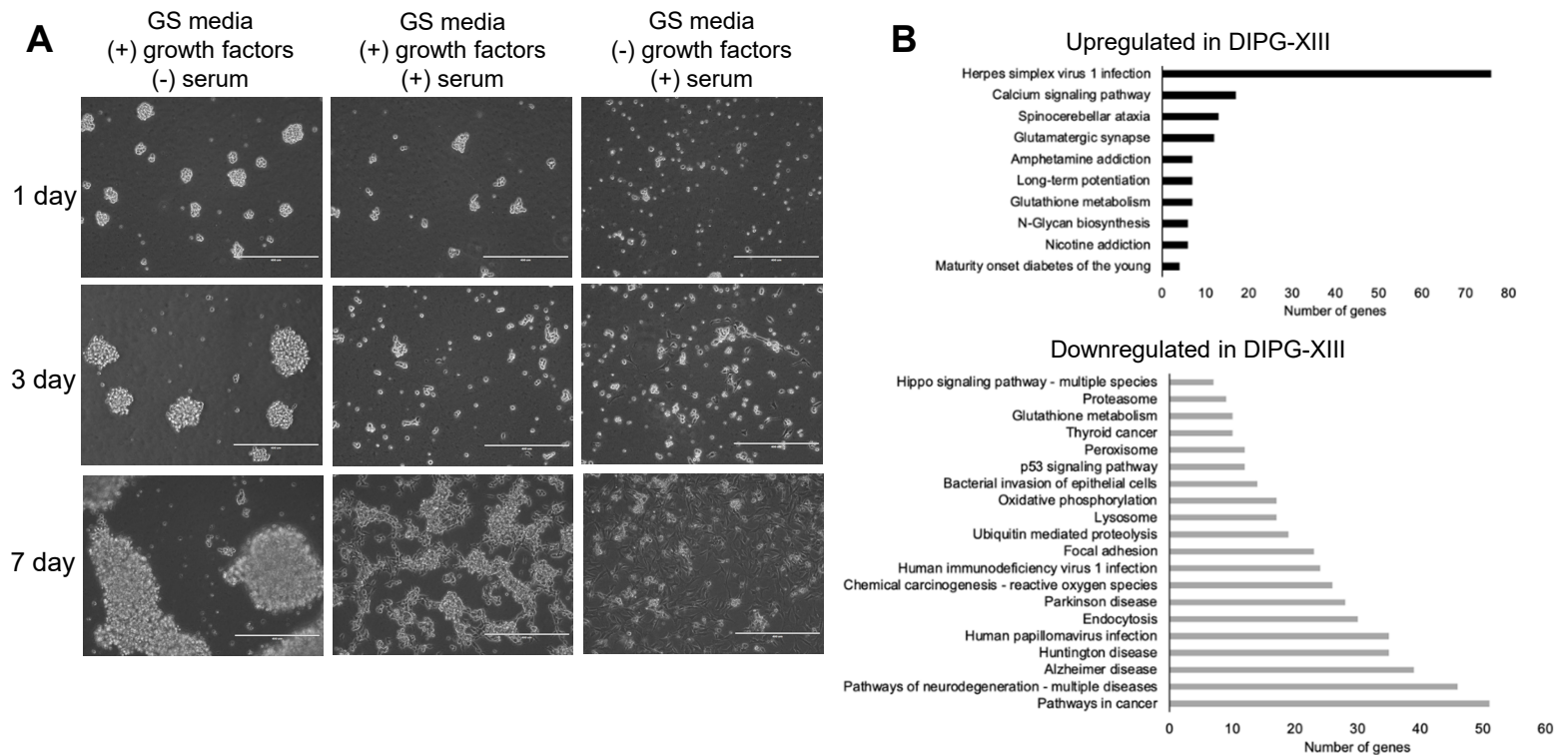
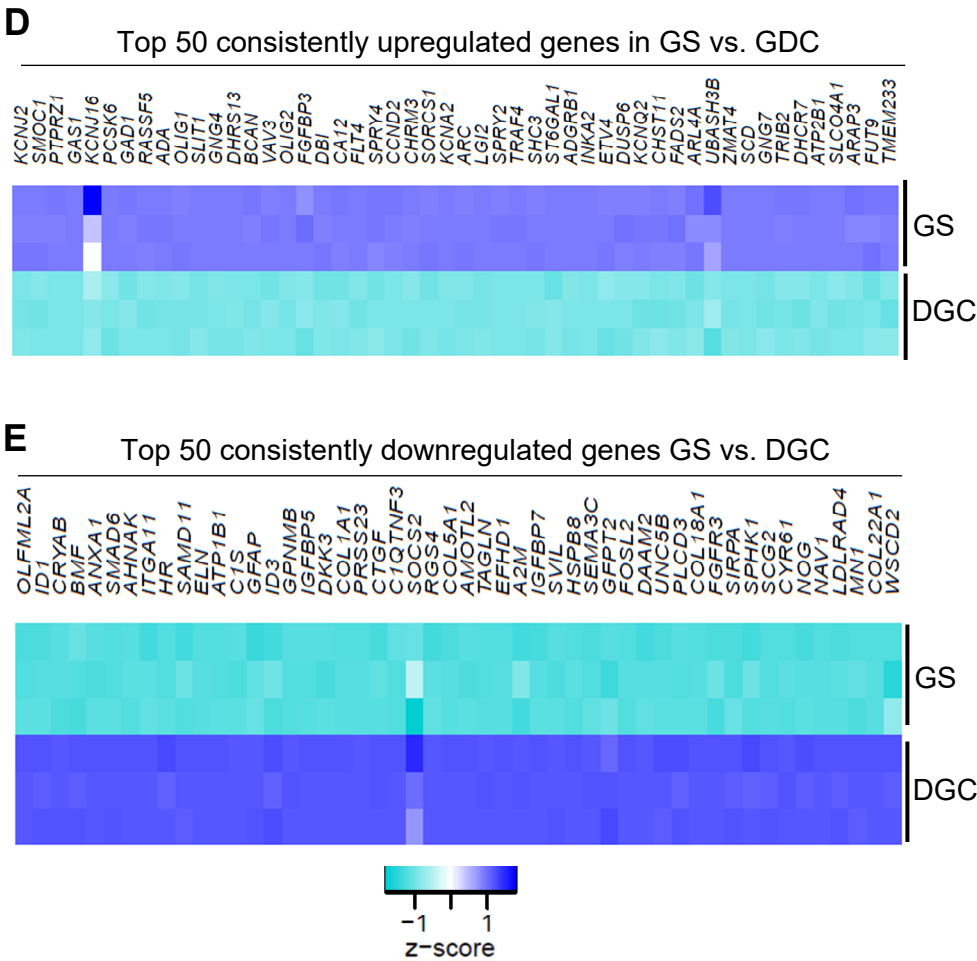
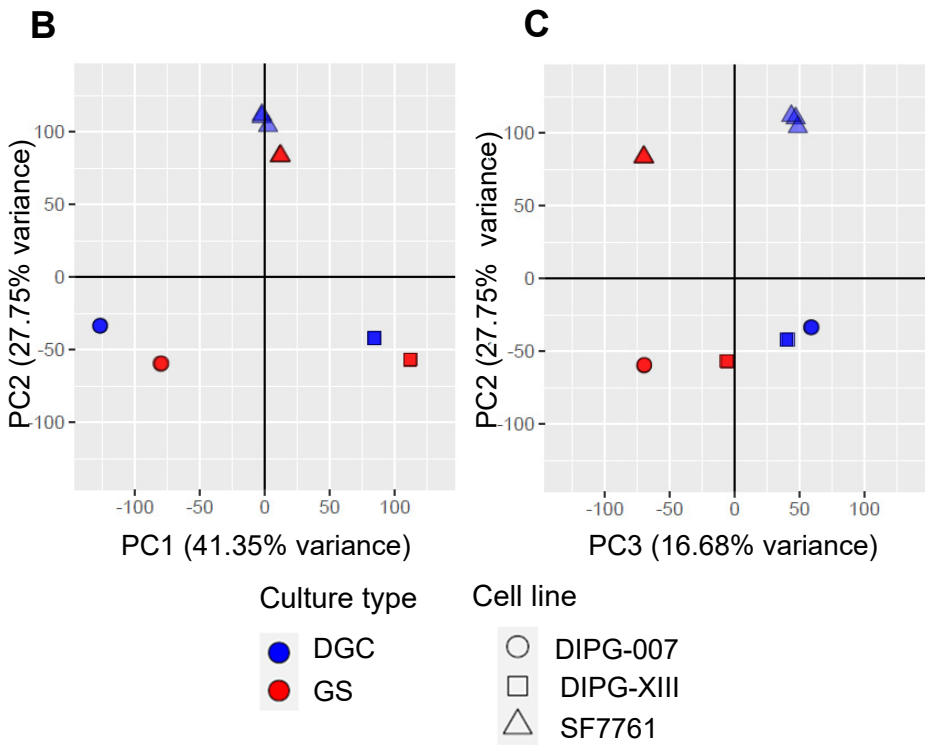
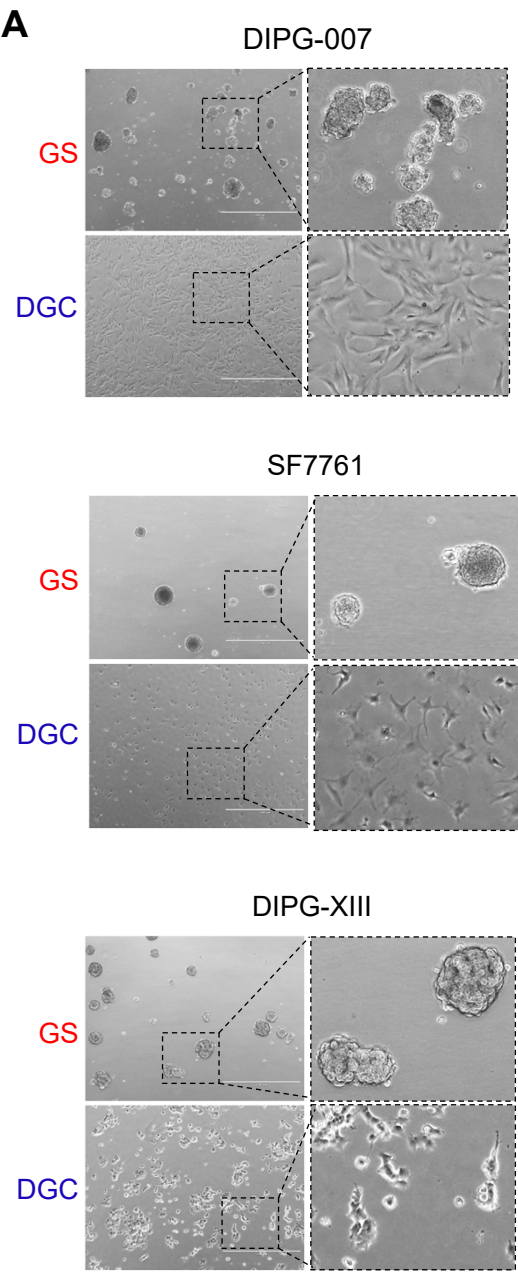
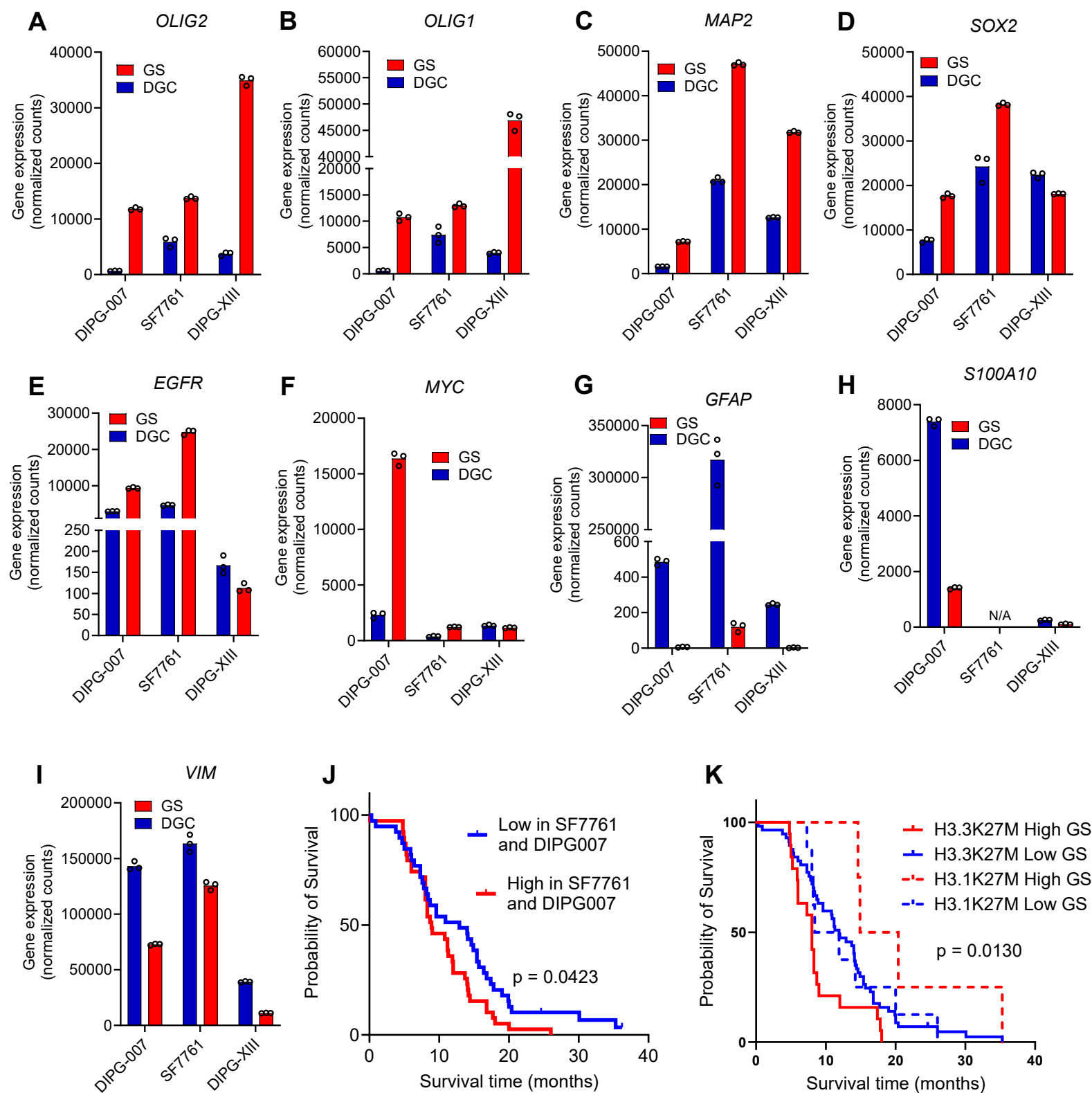




Supplementary Figure 1: Further characterization of the DIPG cultures used in this study. **A)** Brightfield images (10x) of DIPG-007 gliomaspheres (GS) grown on non-tissue culture-treated surfaces in GS media with and without growth factors and with or without 10% fetal bovine serum (FBS) at the indicated time points; scale bars indicate 400um, n=1 **B)** KEGG pathways uniquely upregulated (top) and down-regulated (bottom) in DIPG-XIII GS relative to DGC.



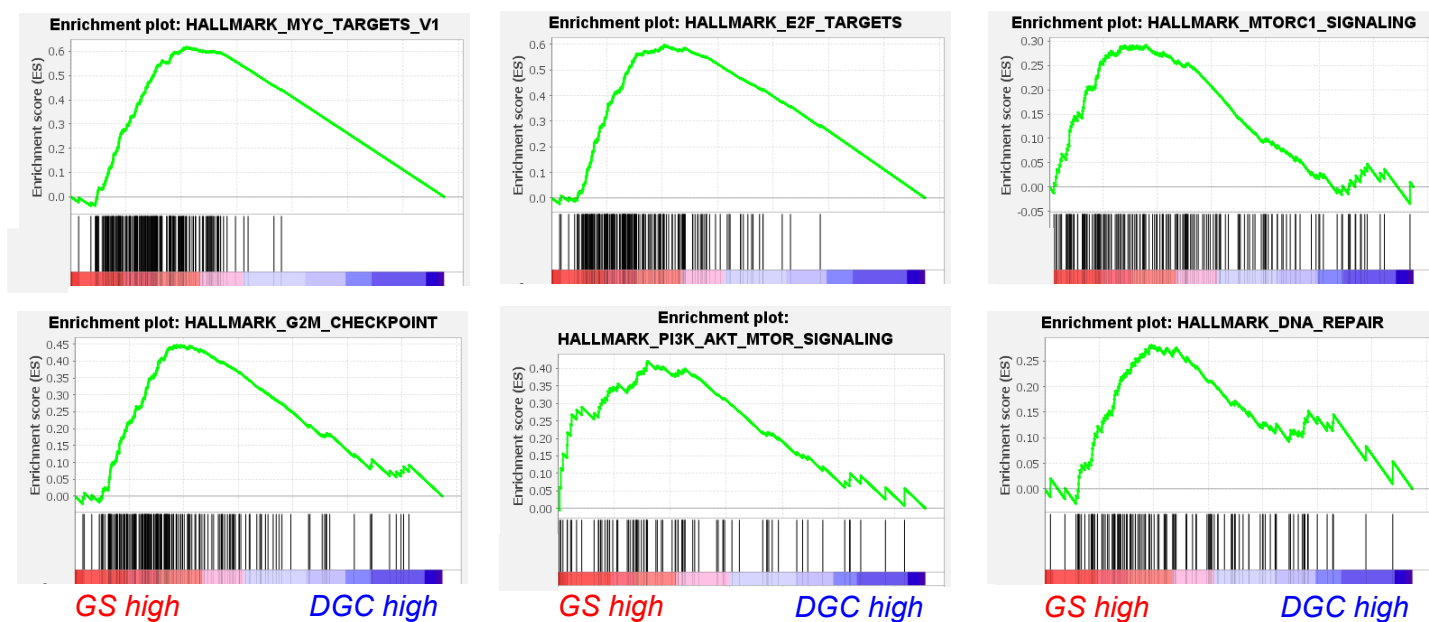
Supplementary Figure 2: Characterization and transcriptomics analysis of DIPG models. **A)** Representative images of DIPG-007, SF7761, and DIPG-XIII, showing morphological differences between gliomaspheres (GS) and differentiated glioma cells (DGC); scale bars indicate 1 mm, n=1. **B, C)** Principal component (PC) analysis of RNA-seq data. **D, E)** Heatmaps indicating the top 50 consistently and significantly upregulated **D)** and downregulated **E)** genes common to all three GS vs. DGC lines. The differential expressed genes between the groups were determined using DESeq2 package in R (Wald test, adjusted $p < 0.05$). Heatmaps were generated using the heatmap.2 function in the gplots package in R. For panels A, B and C, red indicates GS; blue, DGC. For panels D and E, blue represents significantly higher expression; green, significantly lower expression.



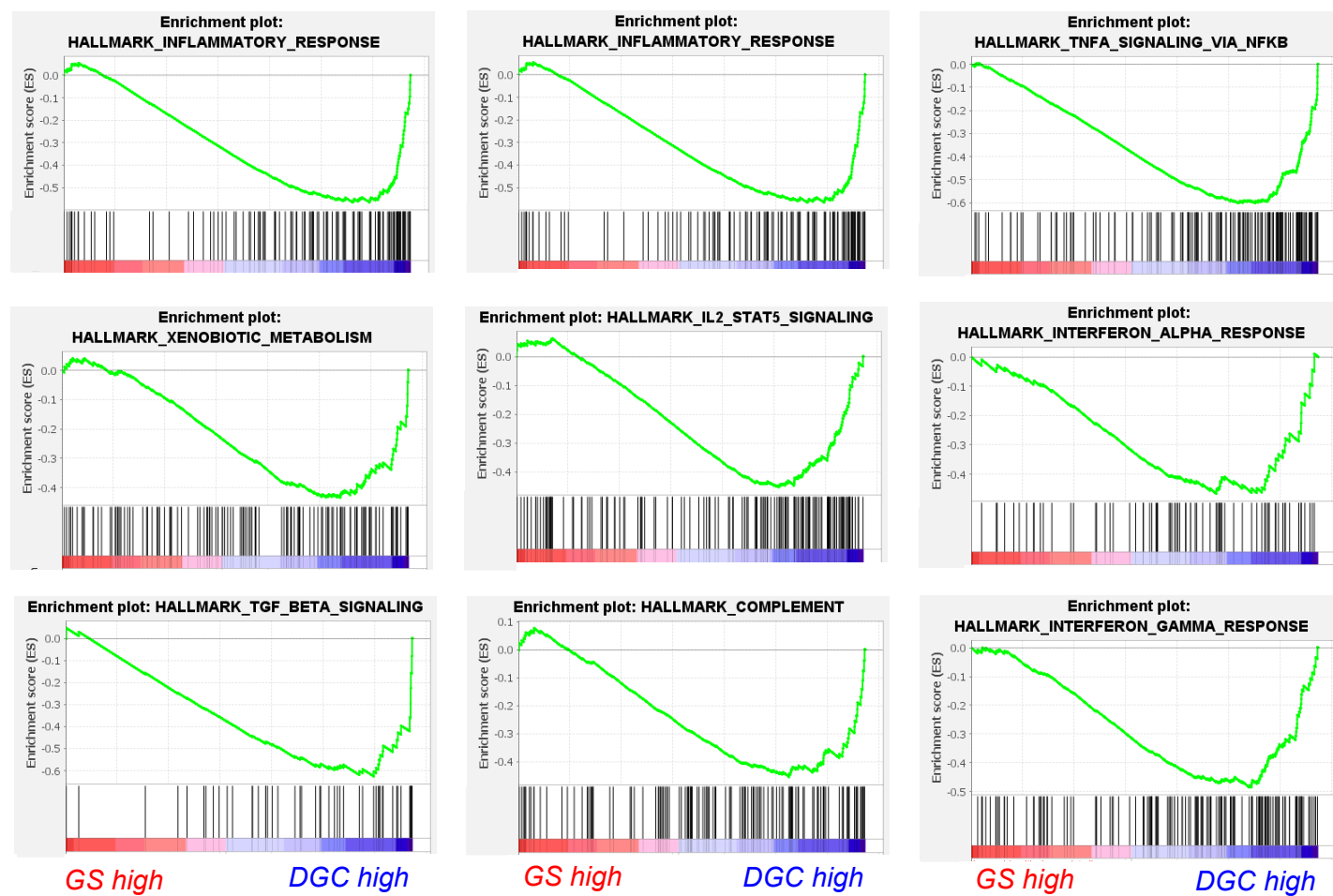
Supplementary Figure 3: Transcriptomics analysis of gene markers in DIPG models.

Relative gene expression of DIPG stemness and differentiation markers in gliomaspheres (GS) vs. differentiated glioma cells (DGC) across DIPG-007, SF7761, and DIPG-XIII. Normalized read counts were subjected to the Wald test (two-sided) and p-values were corrected using the Benjamini-Hochberg method. Genes were significant at adjusted p-value < 0.05. RNA-seq data was generated from three technical replicates, n=1. **A)** oligodendrocyte transcription factor 2 (*OLIG2*), **B)** oligodendrocyte transcription factor 1 (*OLIG1*), **C)** microtubule-associated protein 2 (*MAP2*), **D)** SRY-box transcription factor 2 (*SOX2*), **E)** epidermal growth factor receptor (*EGFR*), **F)** *MYC*, **G)** glial fibrillary acidic protein (*GFAP*), **H)** vimentin (*VIM*), **I)** S100 calcium binding protein A10 (*S100A10*). Statistical comparisons including the p-value, p-adjusted value, and shrunkLFC can be found in the Source data file. **J)** Survival analysis of DIPG/diffuse midline gliomas (DMG) patients based on “GS high” versus “GS low” gene signature from SF7761 and DIPG-007 only. Median survival is 8.90 months versus 12.90 months; Log Rank p=0.0423. **K)** Survival analysis of DIPG/DMG patients based on “GS high” versus “GS low” gene signature in H3.3 versus H3.1. Median survival is 8 months (H3.3K27M High GS), 11.9 months (H3.3K27M Low GS), 17.65 months (H3.1K27M High GS), and 10.15 months (H3.1K27M Low GS); Log Rank p=0.0130. For Kaplan-Meier curves in J-K, n = 78; female = 41, male = 28, unknown = 9. For panels A-I, red indicates GS; blue, DGC; for panels J and K, red indicates “GS high;” blue, “GS low.”

A

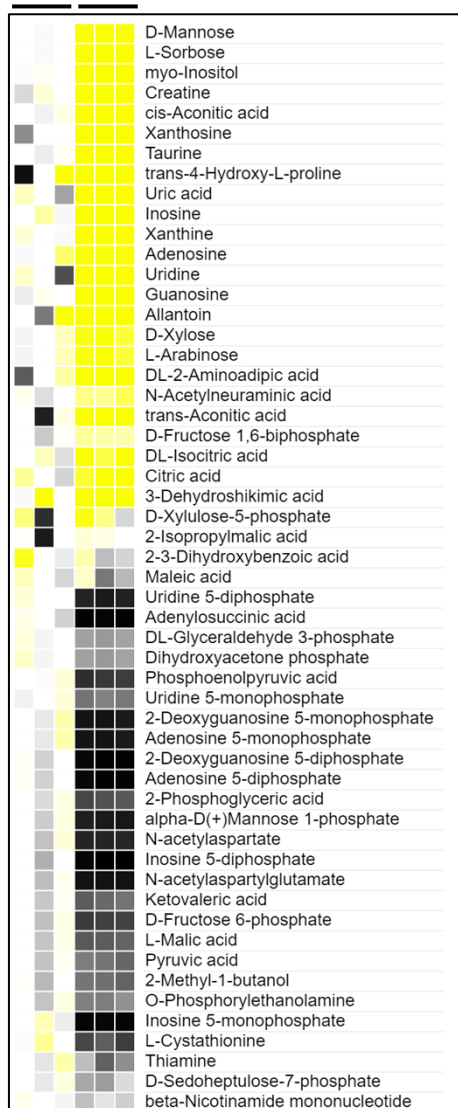


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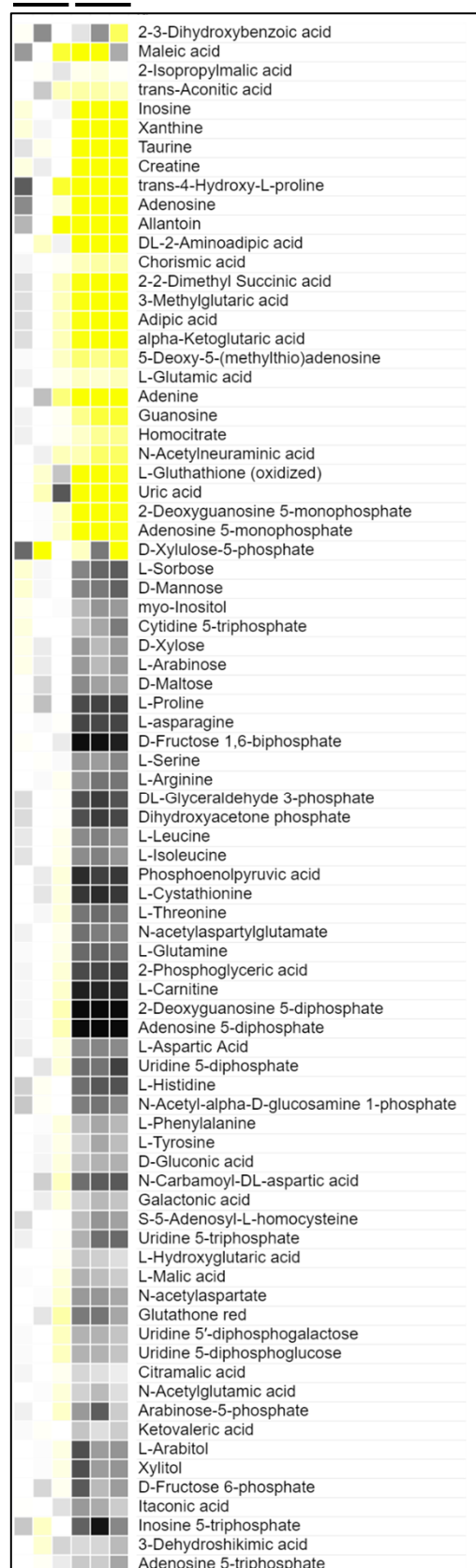


Supplementary Figure 4: Gene set enrichment pathway analysis (GSEA) of DIPG cells. Gene set enrichment analysis (GSEA) indicating pathways that are **A)** upregulated, and **B)** downregulated in DIPG gliomaspheres (GS) vs. differentiated glioma cells (DGC) across all three lines (DIPG-007, SF7761, and DIPG-XIII). GSEA plots show enrichment scores and include values for normalized enrichment score (NES), nominal p value (*P*), and false discovery rate (FDR) q value are derived from GSEA analysis built in within in the software. GSEA plots are derived from RNA-seq analysis generated from three technical replicates, n=1. Red indicates “GS high;” blue, “DGC high.”

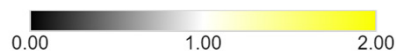
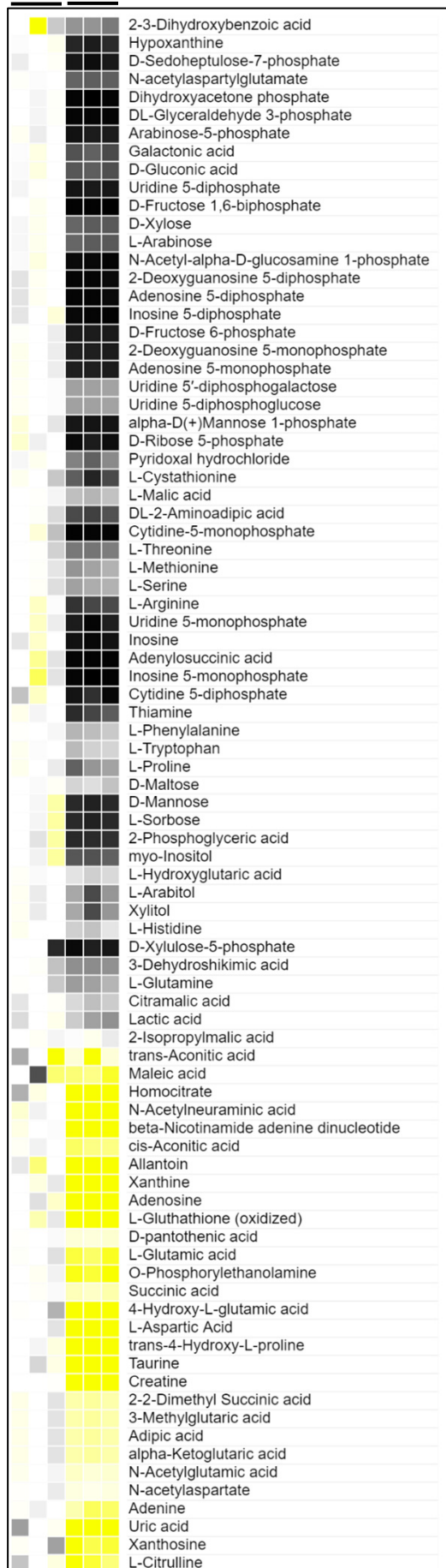
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GS DGC



DIPG-XIII
GS DGC

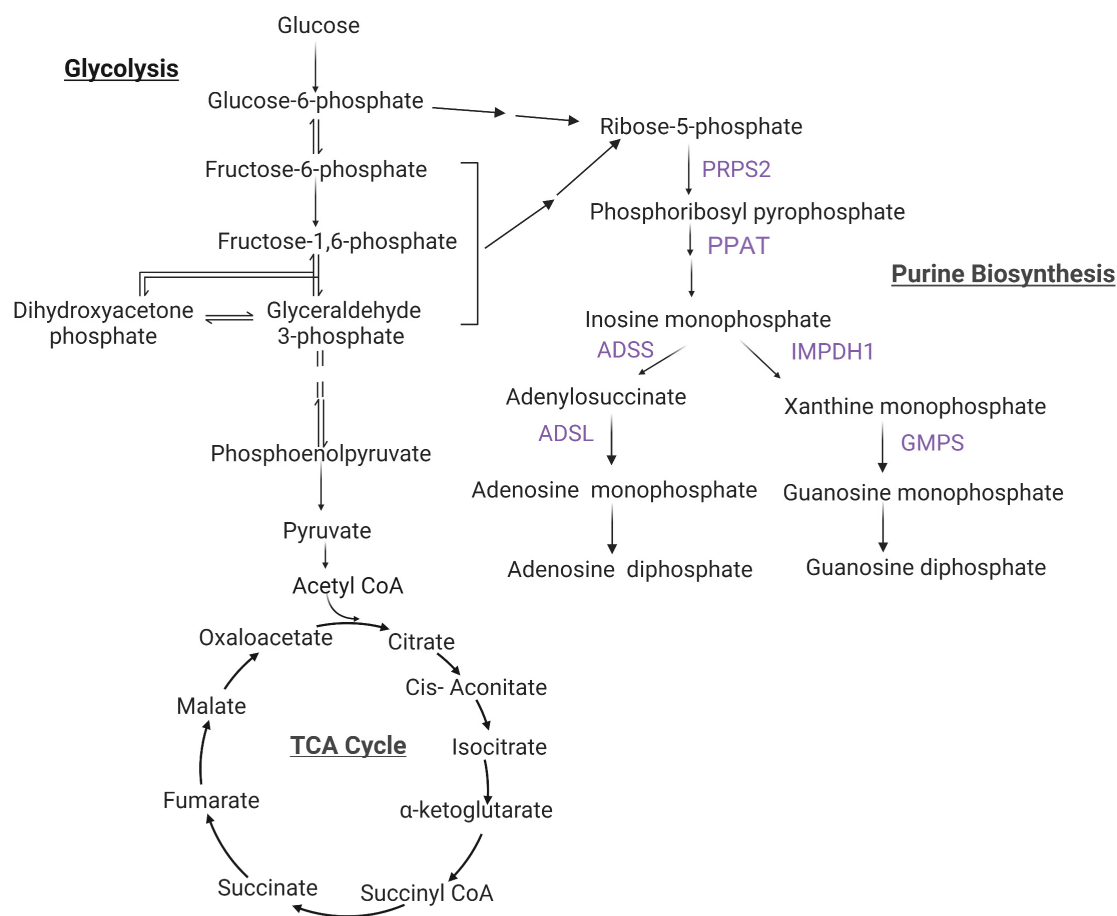
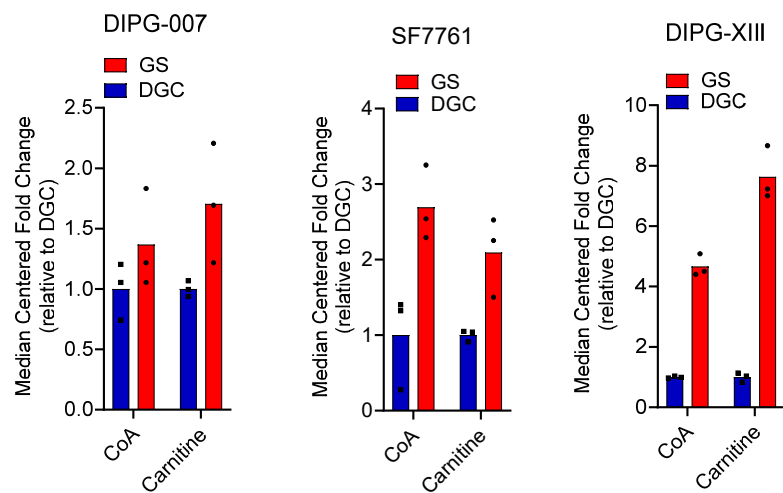
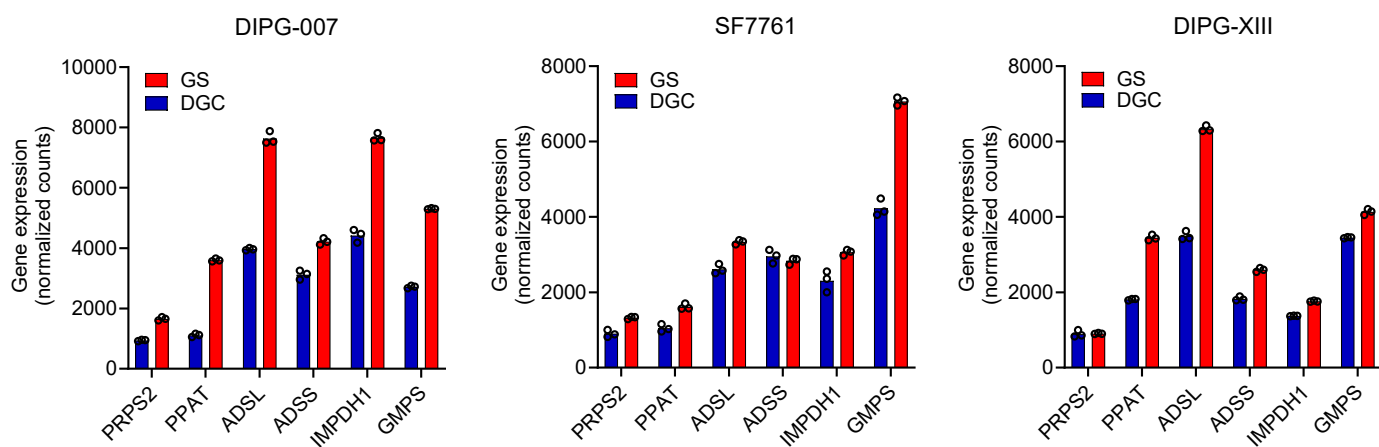


SF7761
GS DGC

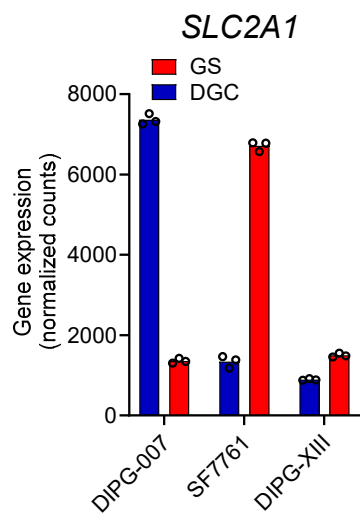
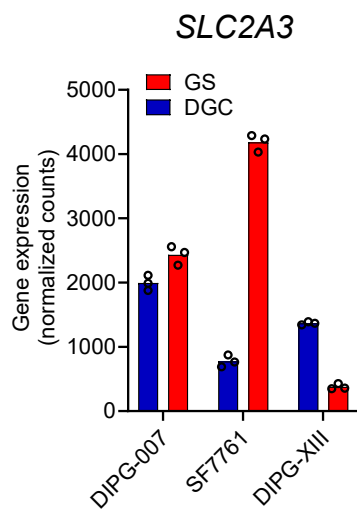
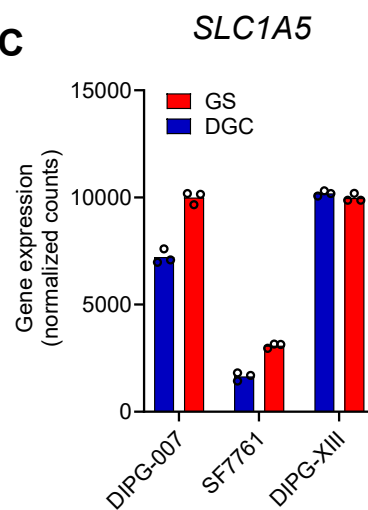
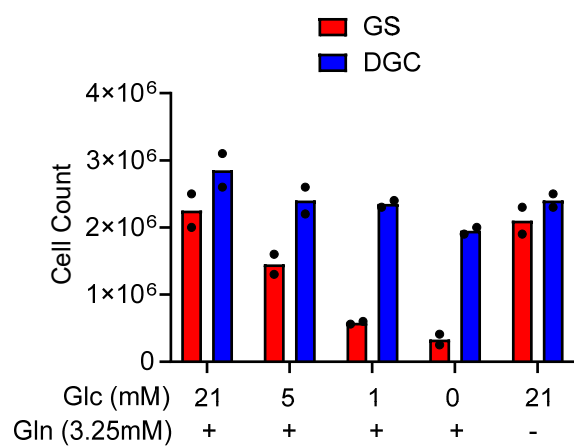
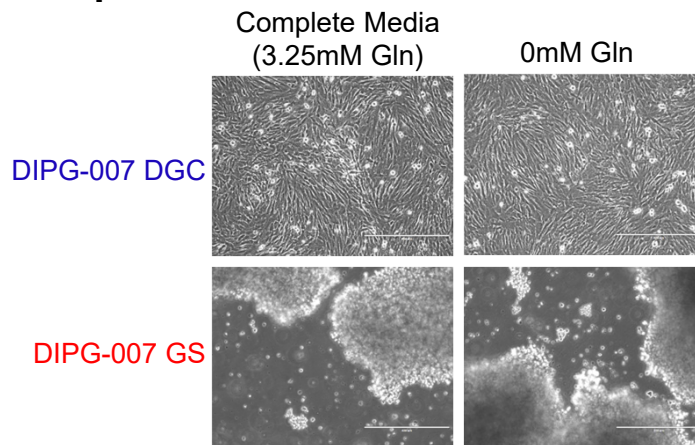
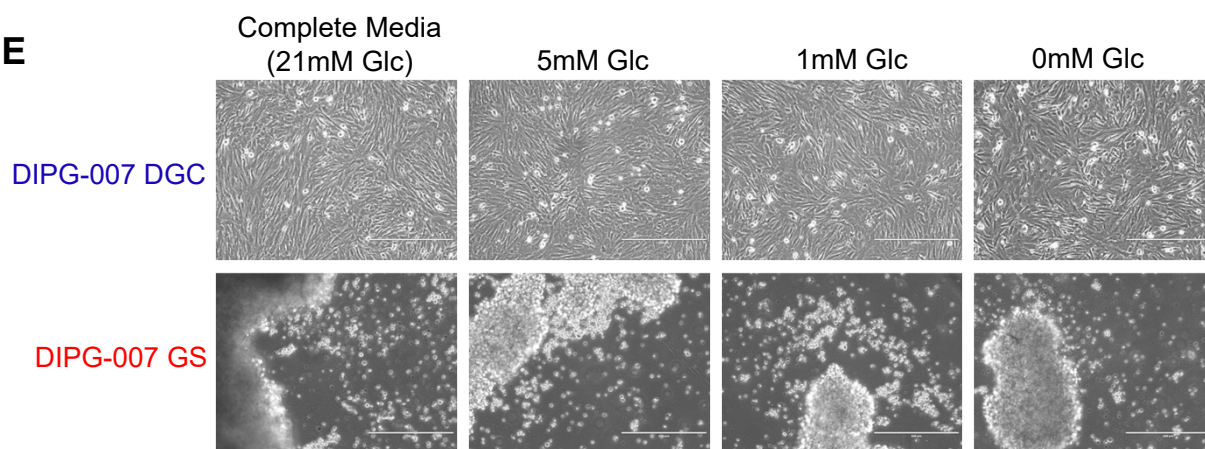


Supplementary Figure 5: Steady-state metabolomics profiling of DIPG cells.

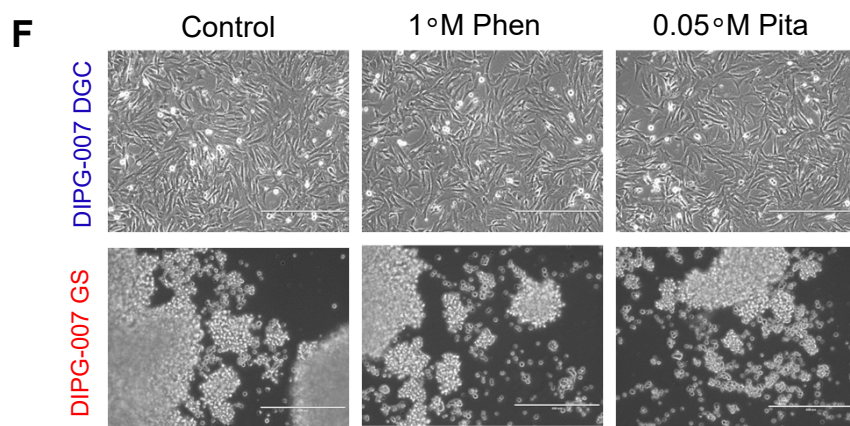
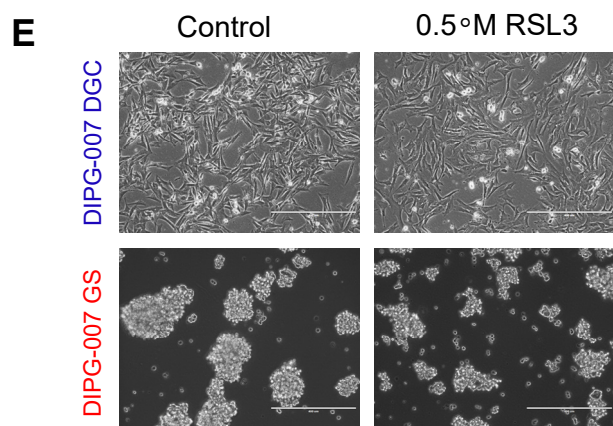
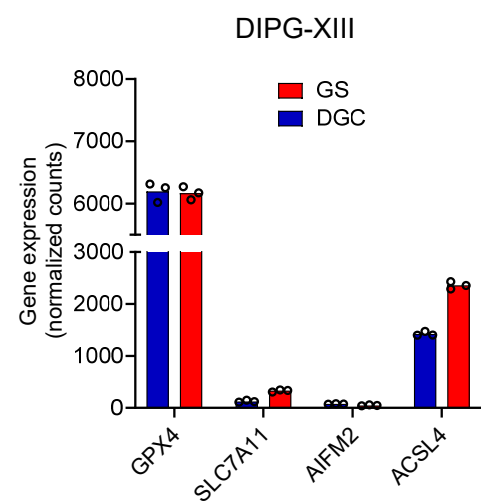
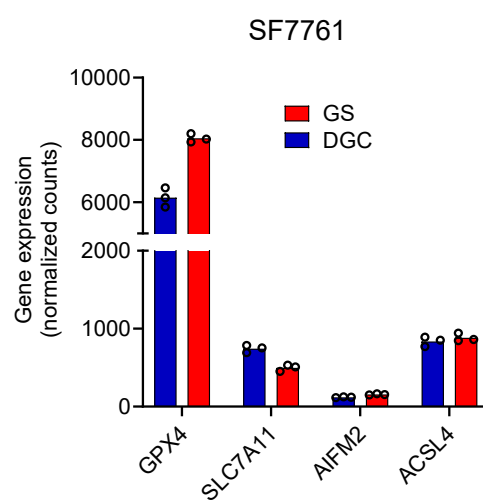
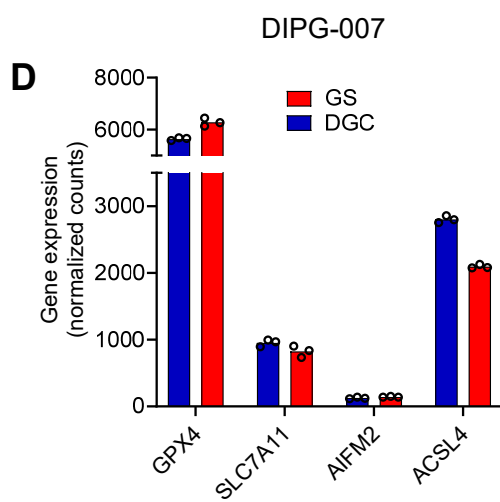
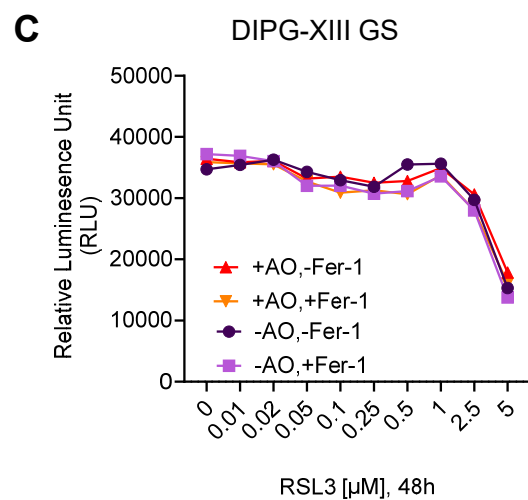
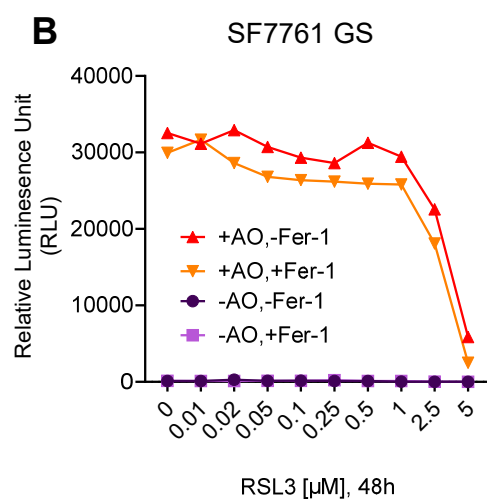
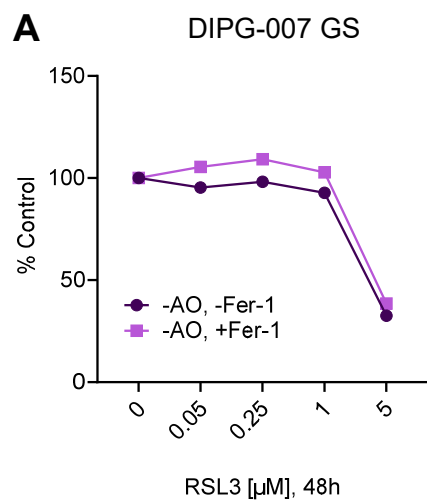
Heatmaps of each individual DIPG cell line pair (DIPG-007, SF7761, and DIPG-XIII) showing significantly altered ($p < 0.01$) and differential abundance of metabolites; metabolites presented are selected from among the 223 detected (see Supplemental Data Table 2). Statistically significant differential metabolites were evaluated using t-test with two-tailed distribution and two-sample unequal variance for independently prepared samples in triplicate. Columns represent independently prepared replicates; rows represent median normalized metabolites within each pair of differentiation models. Each individual cell line was normalized to its gliomasphere (GS) metabolic abundance, and, therefore, yellow indicates highly abundant metabolites while black indicates highly depleted metabolites in differentiated glioma cells (DGC) as compared to GS. Metabolomic profiling data were generated from the average of three replicates samples prepared for 1 experiment.

A**B****C**

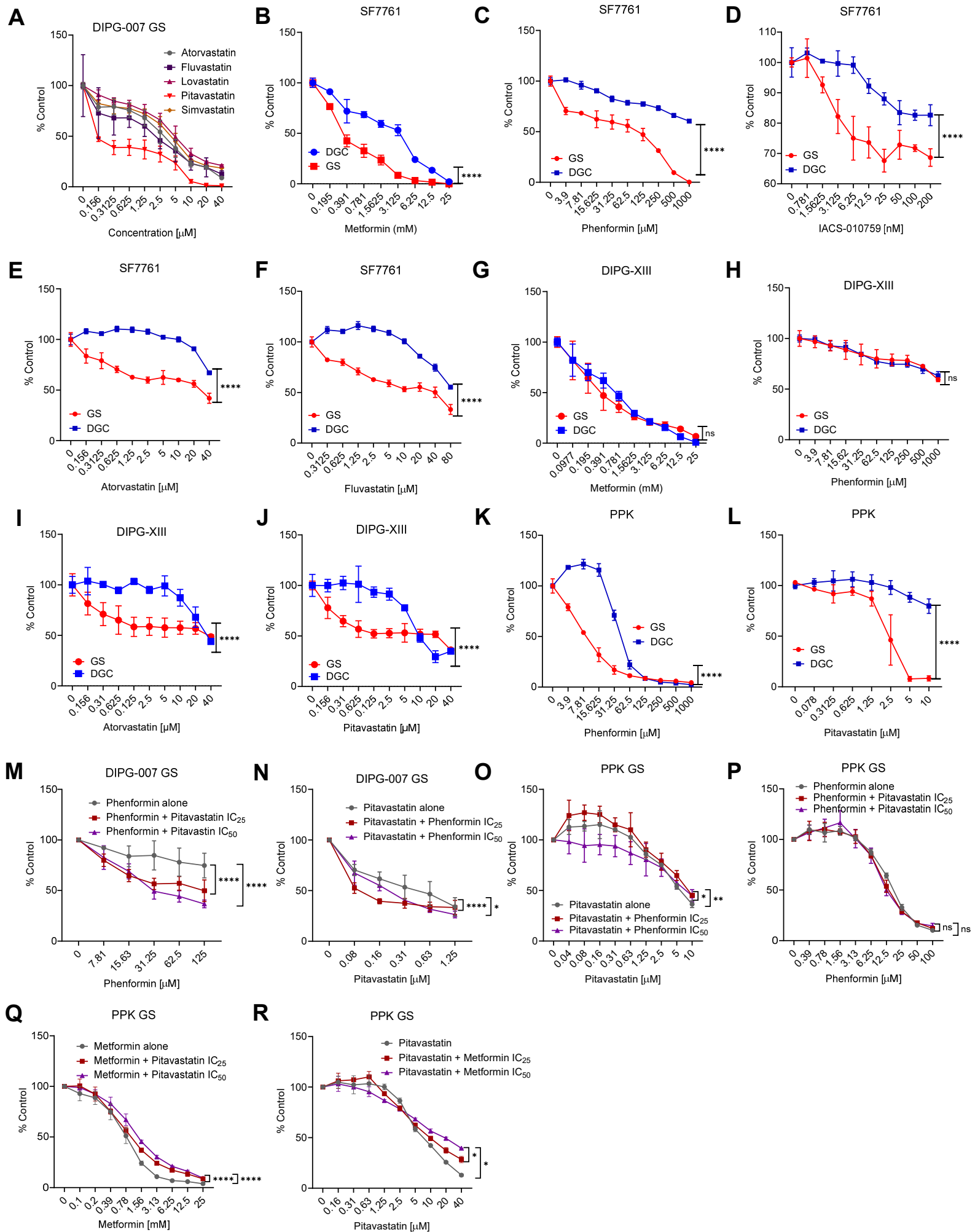
Supplementary Figure 6: Metabolic features of DIPG cells. **A)** Schematic of central metabolic pathways highlighting connections in glycolysis, TCA cycle, and purine biosynthetic pathways. **B)** Levels of CoA and Carnitine in DIPG-007, SF7761, and DIPG-XIII gliomasphere (GS) vs. differentiated glioma cell (DGC) populations. Metabolite levels expressed as median-centered fold change of GS relative to DGC across the three lines from three technical replicates, n=1. **C)** Relative expression of genes (bulk RNA-seq data) encoding enzymes involved in purine biosynthesis in DIPG-007, SF7761, and DIPG-XIII GS and DGC counterparts. Data were generated from three technical replicates, n=1. Normalized read counts were subjected to the Wald test (two-sided). PRPS2, phosphoribosyl pyrophosphate synthetase 2; PPAT, phosphoribosyl pyrophosphate amidotransferase; ADSL, adenylosuccinate lyase; ADSS, adenylosuccinate synthase; IMPDH1, inosine monophosphate dehydrogenase 1; GMPS, guanine monophosphate synthase. For panels B and C, red indicates GS; blue, DGC.

A**B****C****D****F****E**

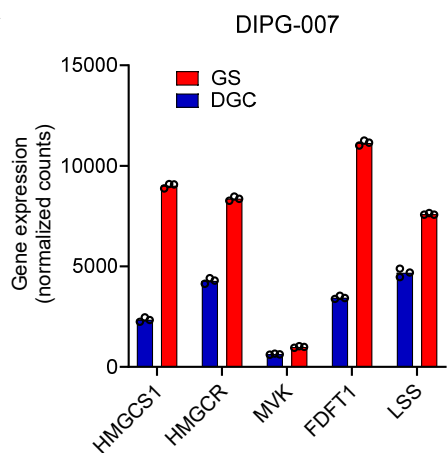
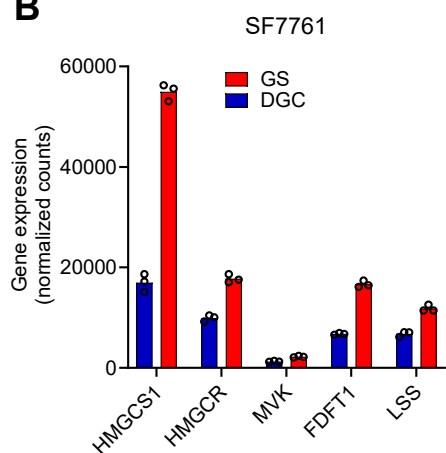
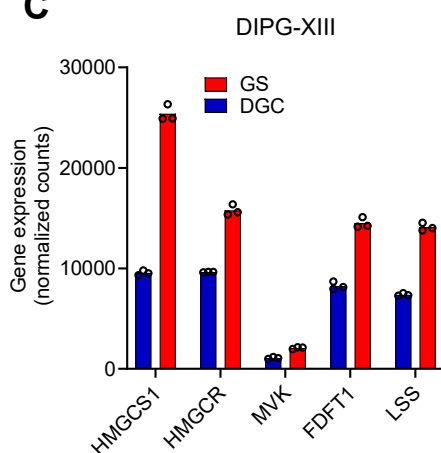
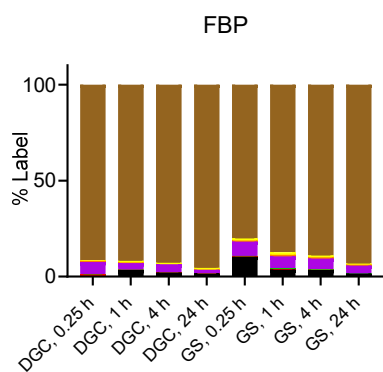
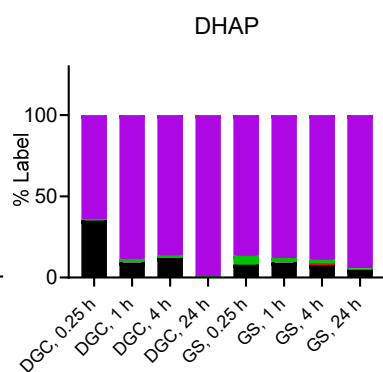
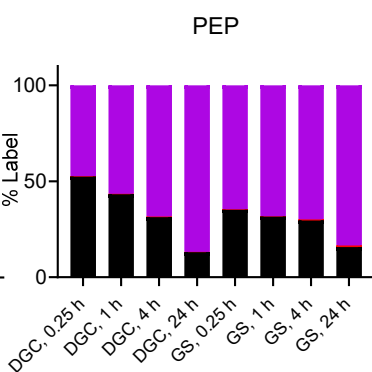
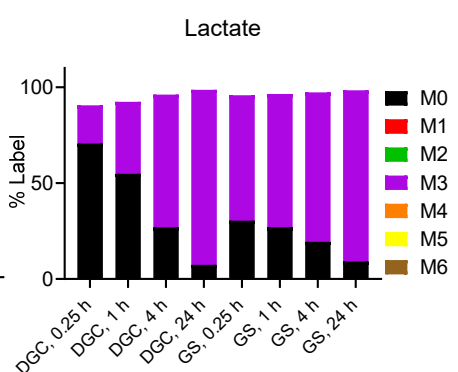
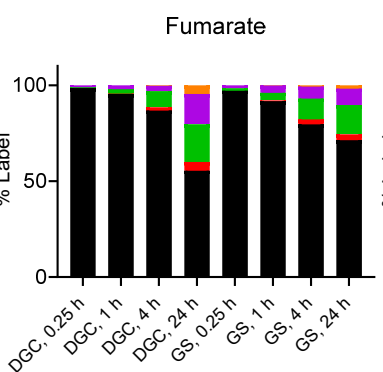
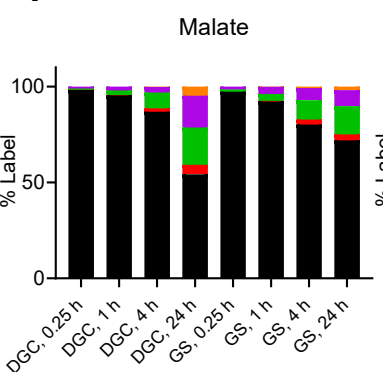
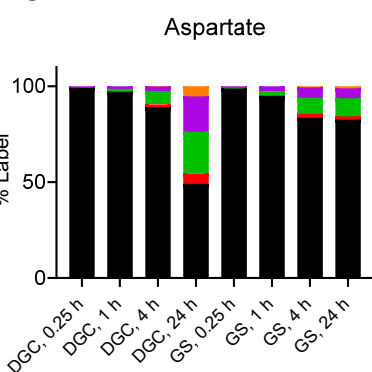
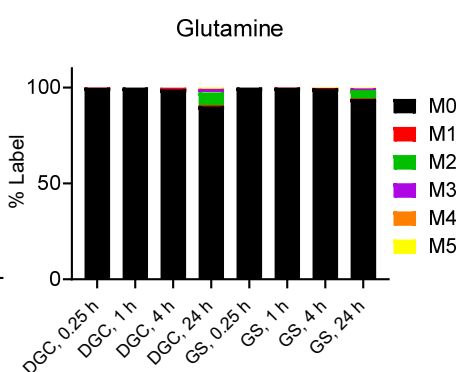
Supplementary Figure 7: Glucose and glutamine utilization in DIPG gliomaspheres (GS) vs differentiated glioma cells (DGC). **A-C)** Relative expression of glucose and glutamine transporters in GS vs. DGC across DIPG-007, SF7761, and DIPG-XIII cell lines. Gene expression normalized counts for solute carrier family 2 member 1 (*SLC2A1*), *SLC2A3* and *SLC1A5* were plotted from bulk transcriptomics analyses of three technical replicates, n=1. **D)** Proliferation of DIPG-007 cells in media with varying glucose (Glc) concentrations and in media with or without glutamine (Gln, 3.25mM) for 7 days was assessed using the Countess II FL Automated Cell Counter (Invitrogen). Results are the average cell count of 2 independent wells, n=1. **E,F)** Representative brightfield images (10x) of the data in (**D**) from two independent wells, n=1; scale bars indicate 400µm. For all panels, red indicates GS; blue, DGC.



Supplementary Figure 8: Ferroptosis properties and differentiation state in DIPG gliomaspheres (GS) and differentiated glioma cells (DGC). RSL3 dose response in the presence or absence of Ferrostatin-1 (Fer-1) in **A)** DIPG-007, **B)** SF7761, and **C)** DIPG-XIII GS cells with (+AO) or without (-AO) antioxidants in the B-27 media supplement. Cell viability was assayed at 3 days post-treatment using the Cell Titer-Glo 3D reagent. Results expressed as percent of control (A) or as relative luminescence units (B,C) representing mean of three technical replicates, n=1. **D)** Relative expression of canonical ferroptosis regulators in GS vs. DGC across DIPG-007, SF7761, and DIPG-XIII. Gene expression normalized counts plotted from the bulk transcriptomics analyses using three technical replicates, n=1. Brightfield images (10x) of DIPG-007 DGC and GS grown in the continuous presence of low-dose **E)** RSL3 (0.5 μ M), **F)** Phenformin (Phen, 1 μ M) or Pitavastatin (Pita, 0.05 μ M) for 7 days to assess differentiation morphology in two independent wells, n=1; scale bars indicate 400 μ m. GPX4, glutathione peroxidase 4; SLC7A11, solute carrier family 7 member 11; AIFM2, apoptosis inducing factor mitochondria associated 2; ACSL4, acyl-CoA synthetase long chain family member. For panels D-F, red indicates GS; blue, DGC.

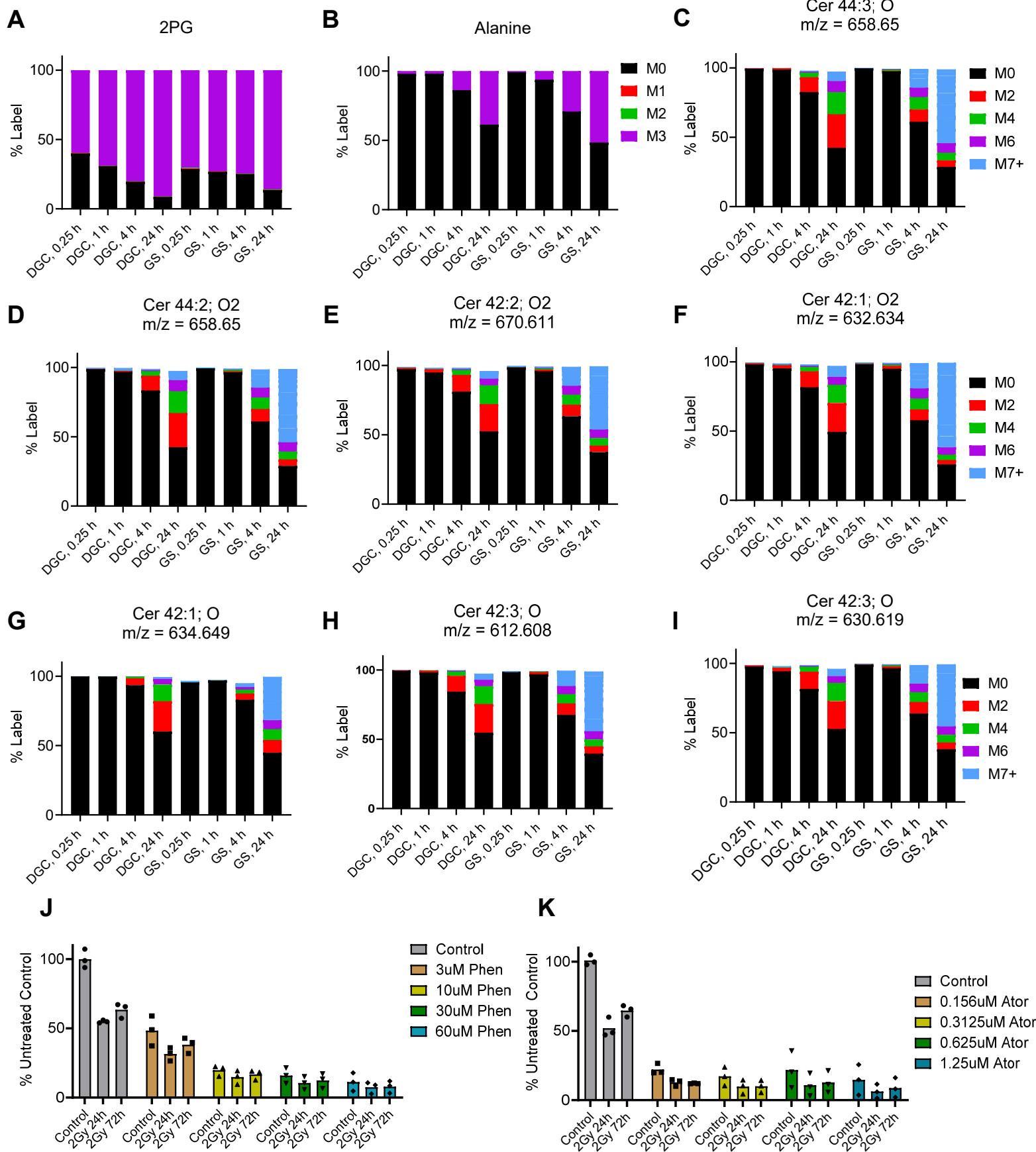


Supplementary Figure 9: *In vitro* targeting of DIPG cells with OXPHOS inhibitors and statins. **A)** Efficacy comparison of various clinical statins (Atorvastatin, Fluvastatin, Lovastatin, Pitavastatin, and Simvastatin) in DIPG-007 gliomaspheres (GS). **B-F)** Dose response curves of SF7761 GS and differentiated glioma cells (DGC) treated with **B)** Metformin, **C)** Phenformin, **D)** IACS-010759, **E)** Atorvastatin, and **F)** Fluvastatin. **G-J)** Dose response curves of DIPG-XIII GS and DGC treated with **G)** Metformin, **H)** Phenformin, **I)** Atorvastatin, and **J)** Pitavastatin. **K,L)** Dose response curves of PPK (murine H3K27M) GS and DGC treated with **K)** Phenformin, and **L)** Pitavastatin. **M-R)** Combination drug treatment studies. **M)** DIPG-007 GS co-treated with IC₂₅ or IC₅₀ of Pitavastatin, relative to vehicle control, across a dose response of Phenformin or **N)** co-treated with the IC₂₅ or IC₅₀ of Phenformin, relative to vehicle control, across a dose response of Pitavastatin. **O)** PPK GS co-treated with the IC₂₅ or IC₅₀ of Phenformin, relative to vehicle control, across a dose response of Pitavastatin or **P)** co-treated with the IC₂₅ or IC₅₀ of Pitavastatin, relative to vehicle control, across a dose response of Phenformin. **Q)** PPK GS co-treated with the IC₂₅ or IC₅₀ of Pitavastatin, relative to vehicle control, across a dose response of Metformin or **R)** co-treated with the IC₂₅ or IC₅₀ of Metformin, relative to vehicle control, across a dose response of Pitavastatin. For all cell lines and treatments, cell viability was assayed using Cell Titer-Glo 2.0 (DGC) or 3D (GS) at 3 days or 7 days (Metformin) post-treatment. All results are expressed as percent of control and mean $\pm$ SD from three biological replicates; ns = not significant, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; by area under proliferation curve test followed by two-tailed Student's t-test (A-L) or One-way ANOVA test (M-R). For panels B-L, red indicates GS; blue, DGC.

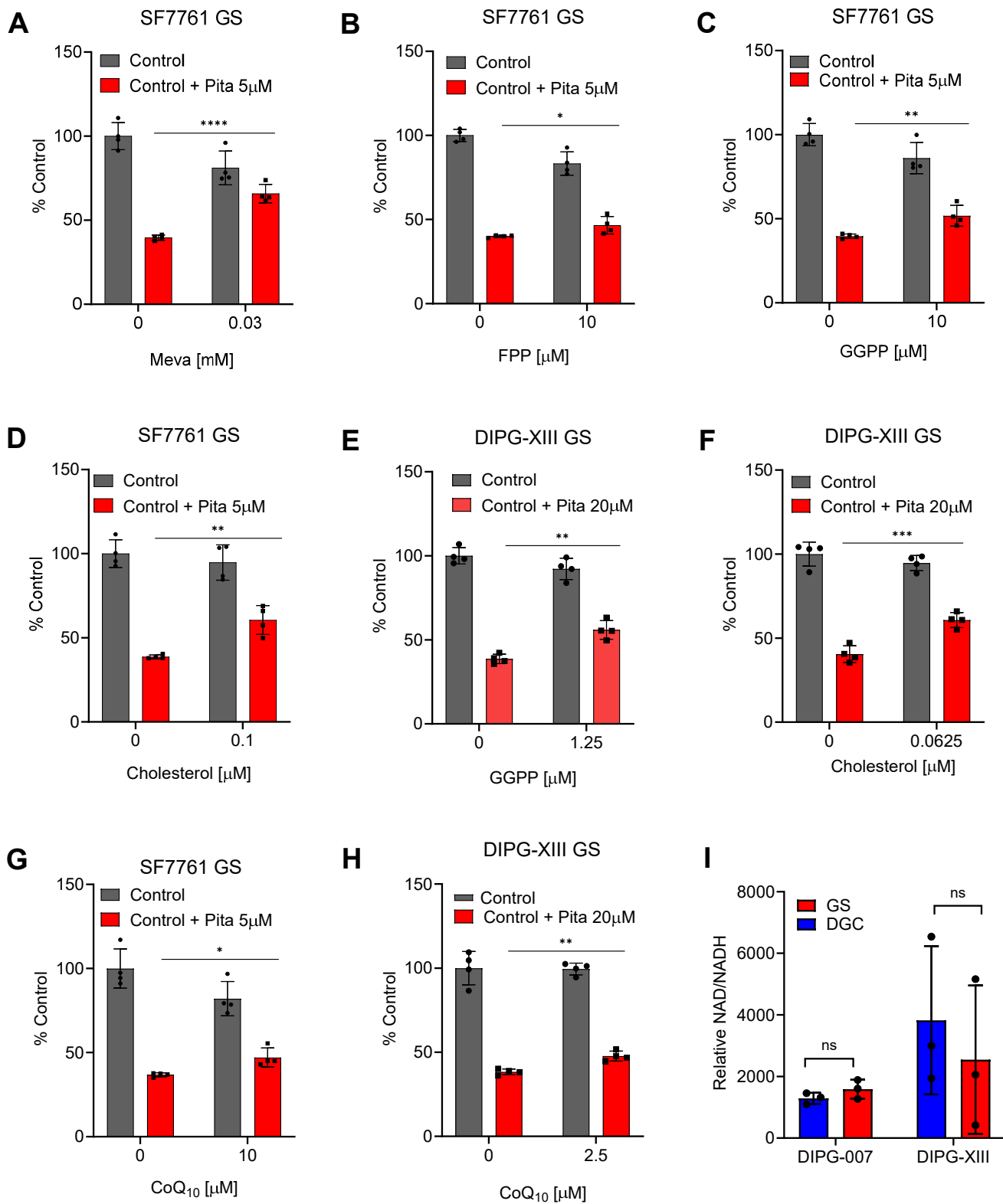
A**B****C****D****E****F****G****H****I****J****K**

Supplementary Figure 10: Sterol biosynthetic pathway gene markers and glucose metabolism in gliomaspheres (GS) and differentiated glioma cells (DGC). A-C)

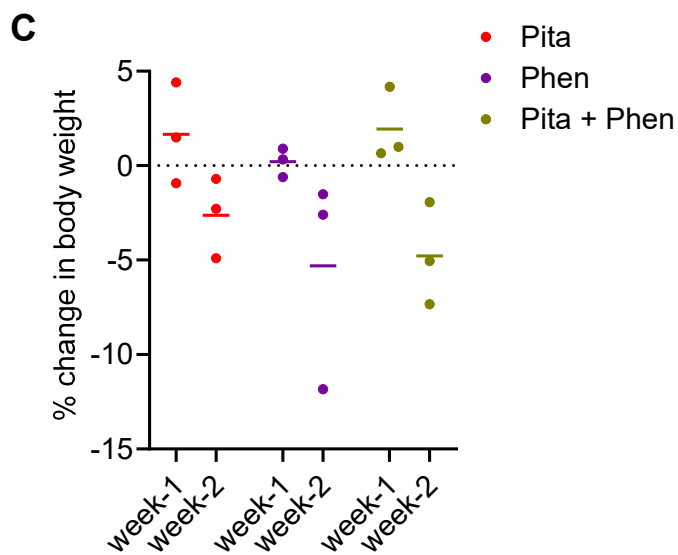
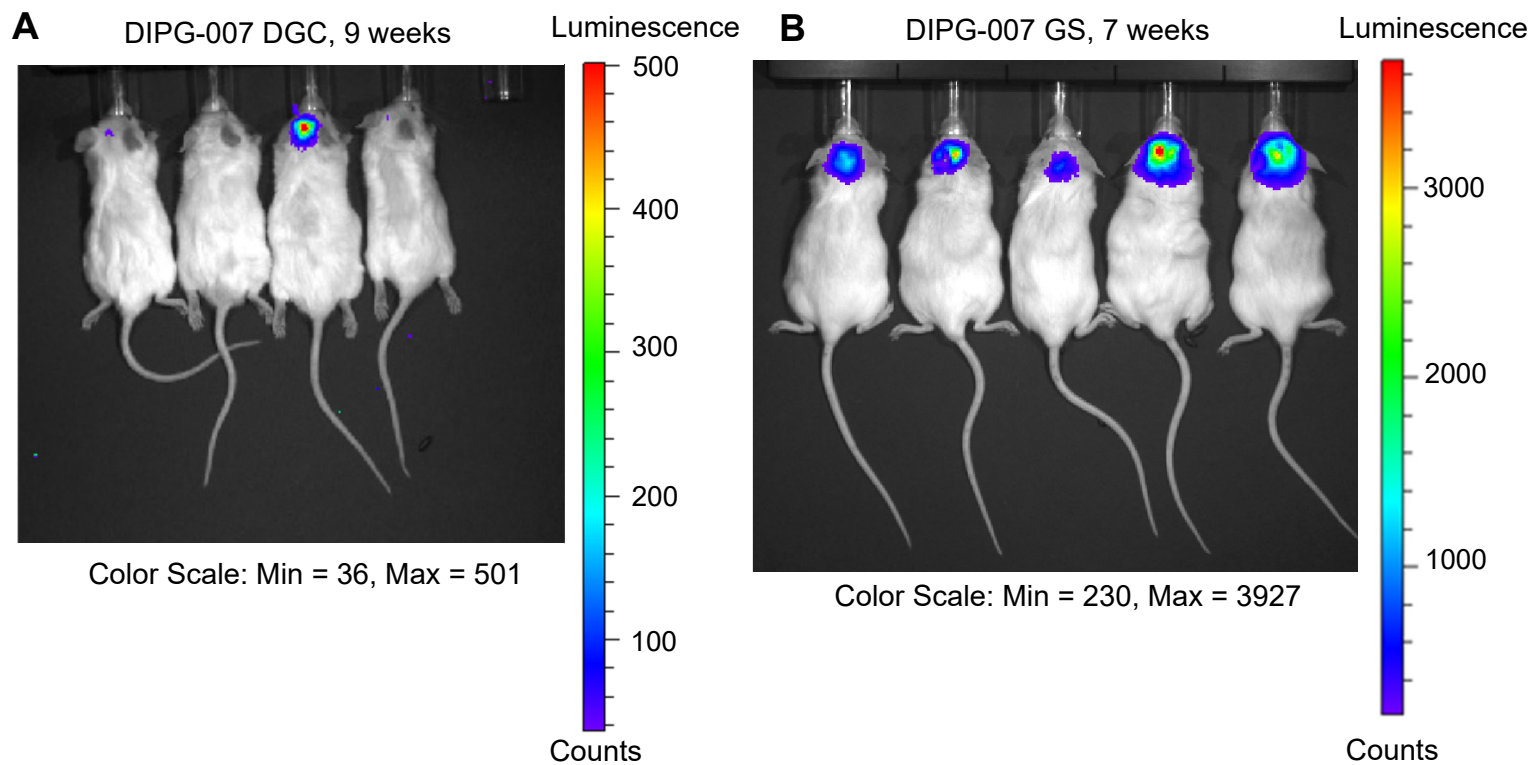
Relative expression of genes encoding enzymes involved in cholesterol biosynthesis in DIPG-007, SF7761, and DIPG-XIII GS and DGC. Gene expression normalized counts plotted from the bulk transcriptomics analysis with three technical replicates, n=1. **D-K)** Metabolomics-based assessment of glucose-derived carbon entry into downstream metabolism in DIPG-007 DGC or GS at the indicated timepoints. Metabolites are presented for **D-G)** glycolysis, **H,I)** the TCA cycle, and **J,K)** non-essential amino acids. Enrichment is presented as mass + the number of carbons labeled in the metabolite, m+n. Data are presented as the fractional enrichment of the pool. Each colored, stacked bar represents each of the isotopologues and the bar height within each group is determined by the mean calculated from three technical replicates, n=1. HMGCS1, 3-hydroxy-3-methylglutaryl-CoA synthase 1; HMGCR, HMG-CoA reductase; MVK, mevalonate kinase; FDFT1, farnesyl-diphosphate farnesyltransferase 1; LSS, lanosterol synthase; FBP, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; PEP, phosphoenolpyruvate. For panels A-C, red indicates GS; blue, DGC.



Supplementary Figure 11: Glucose metabolism and radiation combination studies.
A,B) Metabolomics and **C-I)** lipidomics-based assessment of glucose-derived carbon entry into downstream metabolism in DIPG-007 differentiated glioma cells (DGC) or gliomaspheres (GS) at the indicated timepoints. Metabolites are presented for **A,B)** glycolysis and **C-I)** ceramides generated via *de novo* lipid biosynthesis. Enrichment is presented as mass + the number of carbons labeled in the metabolite, m+n. Data are presented as the fractional enrichment of the pool. Each colored, stacked bar represents each of the isotopologues and the bar height within each group is determined by the mean calculated from three technical replicates, n=1. 2PG, 2-phosphoglycerate; Cer (XX:X;OX), ceramide (carbons:unsaturation;oxygen); m/z, mass/charge. **J,K)** DIPG-007 GS treated with **J)** Phenformin (Phen) or **K)** Atorvastatin (Ator) with or without 2 gray (Gy) radiation treatment at 24 or 72 hours (h). Cell viability was assessed after 7 days using Cell Titer-Glo 3D. Results are expressed as percent of control from three technical replicates, n=1.

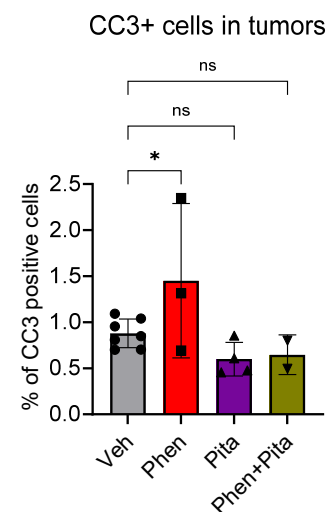
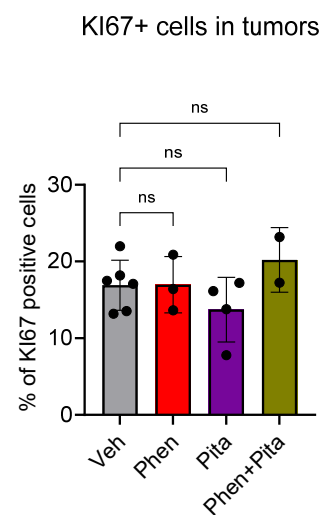
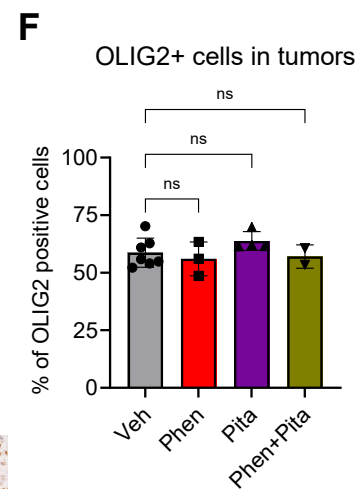
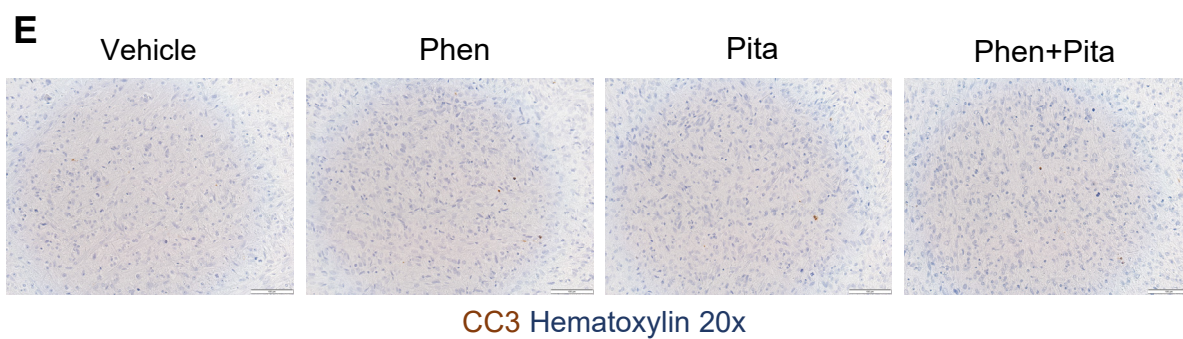
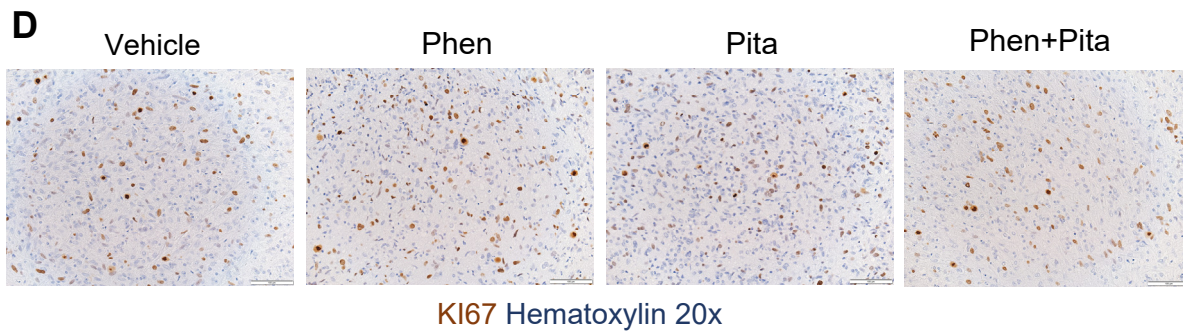
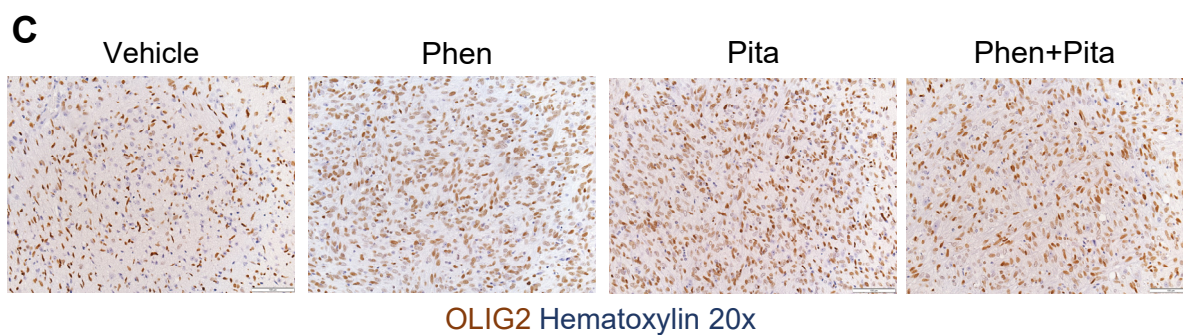
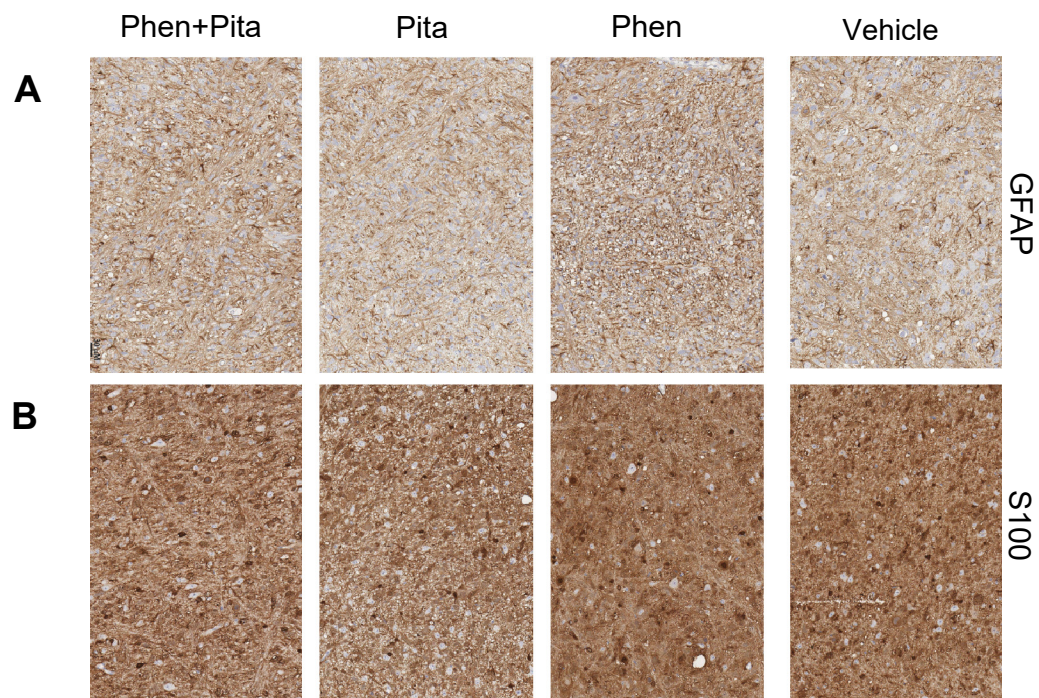


Supplementary Figure 12: Dependency of DIPG gliomaspheres (GS) on cholesterol biosynthesis. A-D) Cell viability of SF7761 GS following treatment with vehicle (0.4% DMSO) or Pitavastatin (Pita) with or without co-treatment with mevalonate (Meva), farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP), or cholesterol, at the indicated concentrations **E,F)** Cell viability of DIPG-XIII GS following treatment with vehicle (0.4% DMSO) or Pita with or without co-treatment with GGPP or cholesterol, at the indicated concentrations **G,H)** Cell viability of **G)** SF7761 GS and **H)** DIPG-XIII GS following treatment with vehicle (0.4% DMSO) or Pita with or without co-treatment with coenzyme Q₁₀ (CoQ₁₀), at the indicated concentrations **A-H)** Cell viability was assayed at 72 hours post-treatment using Cell Titer-Glo 3D with results expressed as percent of vehicle control and error bars representing mean $\pm$ SD from three biological replicates; ns = not significant, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ by Unpaired T-test. **I)** Relative NAD/NADH levels in GS vs. differentiated glioma cell (DGC) DIPG-007 and DIPG-XIII, as determined by mass spectrometry. Results are expressed as mean $\pm$ SD from three biological replicates. For all panels, red indicates GS; blue, DGC.



Supplementary Figure 13: Establishment of tumor growth and treatment parameters.

A) DIPG-007 differentiated glioma cells (DGC) or **B)** gliomaspheres (GS) were orthotopically implanted into the pons of NSG mice. At 9- and 7-weeks post implantation, respectively, tumors were visualized using bioluminescence imaging (BLI). Minimum (Min) and maximum (Max) luminescence values are presented below the images, with color scales ranging from 36 (purple) to 501 (red) for DGC and 230 (purple) to 3927 (red) for GS. **C)** Percent change in body weight was assessed using a 2-week dosing study with Pitavastatin (Pita), Phenformin (Phen), or the combination (Pita + Phen) of both drugs to assess safety and tolerability. Three mice were included in each arm. Mice were dosed on day 1 (Pita, 10mg/kg; Phen 50mg/kg) and day 8 (Pita, 20mg/kg; Phen, 100mg/kg). Each point represents one mouse, n=1.



Supplementary Figure 14: Histological analysis of DIPG-007 gliomasphere (GS) tumors. Representative images of Figure 7 endpoint tumors that were fixed and stained for the differentiation/stemness markers **A)** glial fibrillary acidic protein (GFAP), **B)** S100, and **C)** oligodendrocyte transcription factor 2 (OLIG2), the proliferation marker **D)** KI67, or the apoptotic marker **E)** cleaved caspase 3 (CC3). Protein expression is indicated in brown and sections were counterstained with hematoxylin in blue. Images for panels A and B were captured at 40X from scanned slides using the Aperio ImageScope, with scale bars indicating 30um, n=3. For OLIG2, KI67 and CC3 (C-E), multiple tumors were stained (scale bars indicate 100um) and quantitated using QuPath software (**F**). Each point represents the average of 3 images per tumor, with error bars representing mean $\pm$ SD; ns = not statistically significant; * $p < 0.05$ by one-way ANOVA statistical test, Tukey's multiple comparisons test.