Science Advances
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Supplementary Materials for

Ribonucleotide reductase regulatory subunit M2 drives glioblastoma TMZ resistance through modulation of dNTP production

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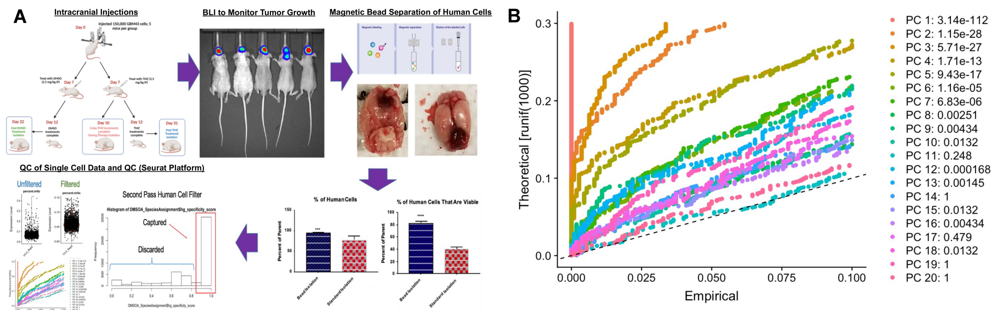
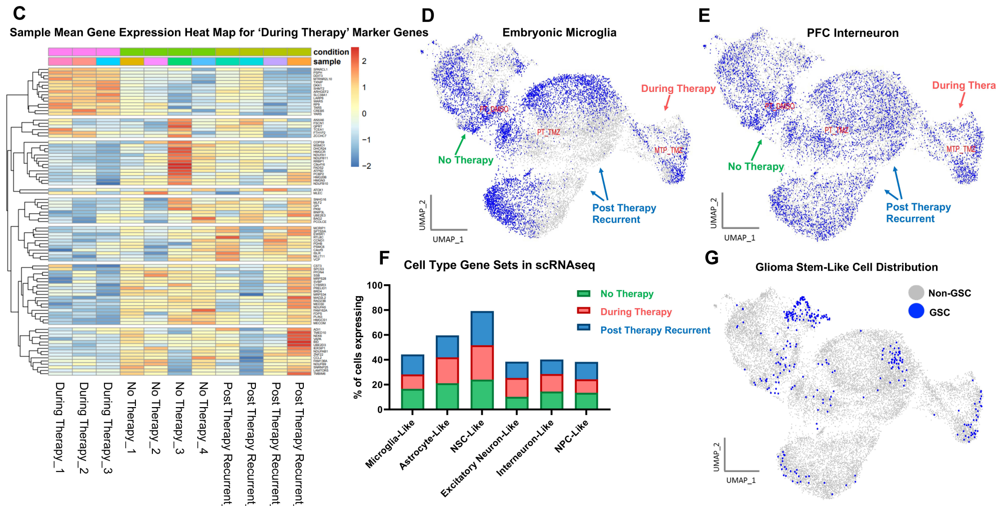
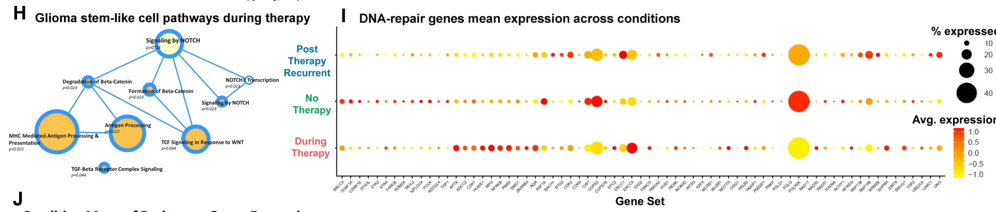
Supplemental Materials



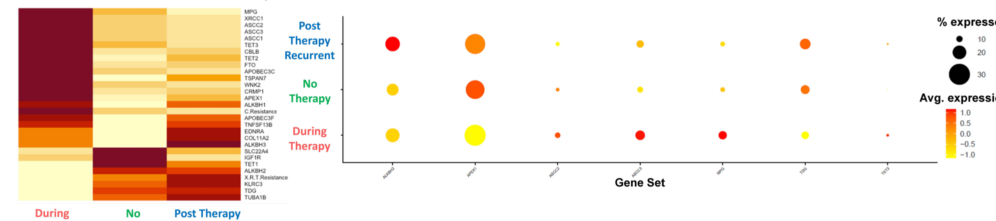
A
Intracranial Injections
BLI to Monitor Tumor Growth
Magnetic Bead Separation of Human Cells
QC of Single Cell Data and QC (Seurat Platform)
Unfiltered
Filtered
Second Pass Human Cell Filter
Captured
Discarded
% of Human Cells
% of Human Cells That Are Viable
B
Theoretical [runif(1000)]
Empirical
PC 1: 3.14e-112
PC 2: 1.15e-28
PC 3: 5.71e-27
PC 4: 1.71e-13
PC 5: 9.43e-17
PC 6: 1.16e-05
PC 7: 6.83e-06
PC 8: 0.00251
PC 9: 0.00434
PC 10: 0.0132
PC 11: 0.248
PC 12: 0.000168
PC 13: 0.00145
PC 14: 1
PC 15: 0.0132
PC 16: 0.00434
PC 17: 0.479
PC 18: 0.0132
PC 19: 1
PC 20: 1



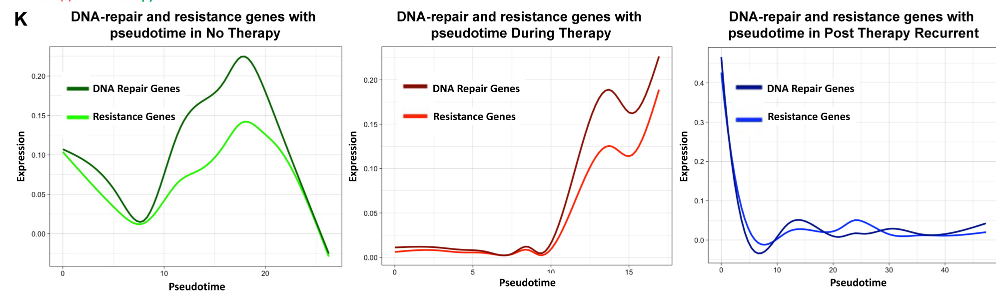
C
Sample Mean Gene Expression Heat Map for 'During Therapy' Marker Genes
condition
sample
During Therapy_1
During Therapy_2
During Therapy_3
No Therapy_1
No Therapy_2
No Therapy_3
No Therapy_4
Post Therapy Recurrent_1
Post Therapy Recurrent_2
Post Therapy Recurrent_3
Post Therapy Recurrent_4
D
Embryonic Microglia
During Therapy
No Therapy
Post Therapy Recurrent
UMAP_1
UMAP_2
E
PFC Interneuron
During Therapy
No Therapy
Post Therapy Recurrent
UMAP_1
UMAP_2
F
Cell Type Gene Sets in scRNAseq
% of cells expressing
No Therapy
During Therapy
Post Therapy Recurrent
Microglia-Like
Astrocyte-Like
NSC-Like
Excitatory Neuron-Like
Interneuron-Like
NPC-Like
G
Glioma Stem-Like Cell Distribution
Non-GSC
GSC
UMAP_1
UMAP_2



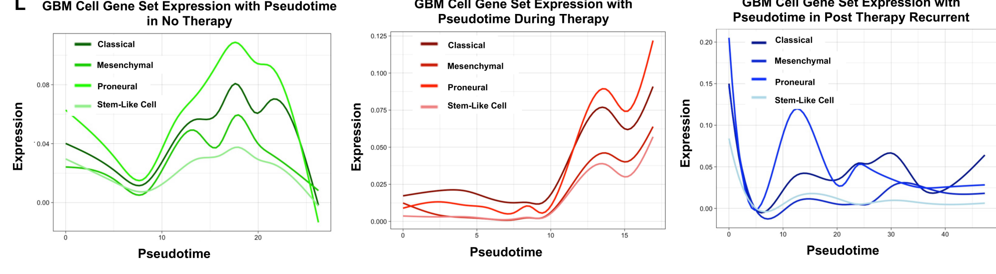
H
Glioma stem-like cell pathways during therapy
Signaling by NOTCH
Degradation of Beta-Catenin
Formation of Beta-Catenin
NOTCH3 Transcription
Signaling by NOTCH
MHC Mediated Antigen Processing & Presentation
Antigen Processing
TCF Signaling in Response to WNT
TGF-Beta Receptor Complex Signaling
I
DNA-repair genes mean expression across conditions
Post Therapy Recurrent
No Therapy
During Therapy
% expressed
Avg. expression
Gene Set



J
Condition Mean of Resistance Genes Expression
MPG
XRCC1
ASCC2
ASCC3
ASCC1
TET3
CBLB
TET2
FTO
APOBEC3C
TSPAN7
WNK2
CRMP1
APEX1
ALKBH1
C.Resistance
APOBEC3F
TNFSF13B
EDN8A
COL11A2
ALKBH3
SLC22A4
IGF1R
TET1
ALKBH2
X.R.T.Resistance
KLRC3
TDG
TUBA1B
During Therapy
No Therapy
Post Therapy Recurrent
% expressed
Avg. expression
Gene Set



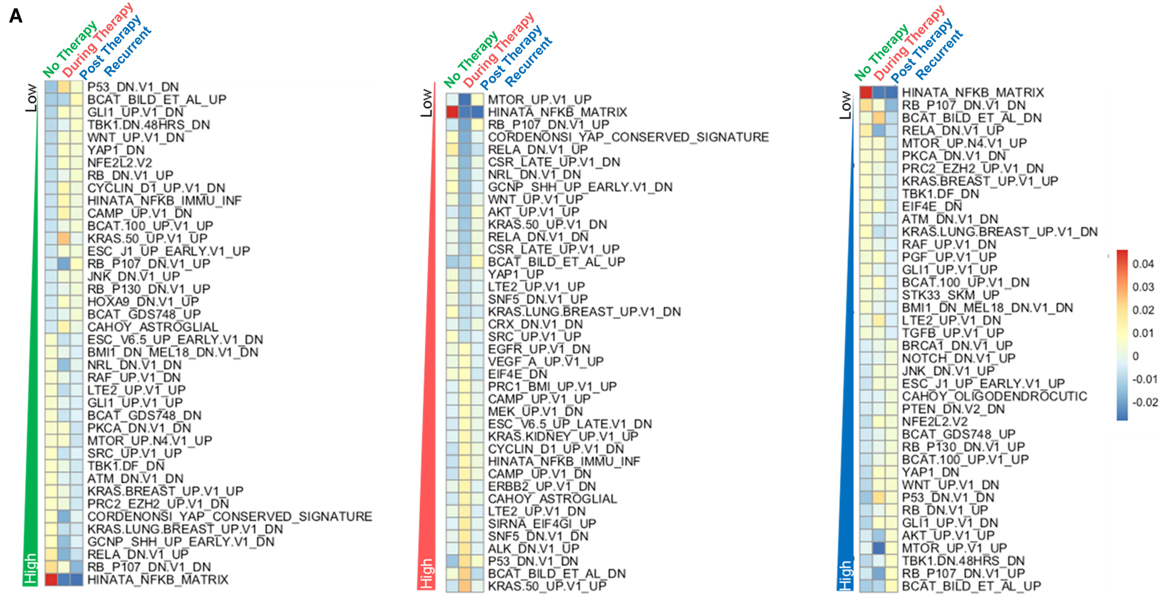
K
DNA-repair and resistance genes with pseudotime in No Therapy
DNA Repair Genes
Resistance Genes
Expression
Pseudotime
DNA-repair and resistance genes with pseudotime During Therapy
DNA Repair Genes
Resistance Genes
Expression
Pseudotime
DNA-repair and resistance genes with pseudotime in Post Therapy Recurrent
DNA Repair Genes
Resistance Genes
Expression
Pseudotime

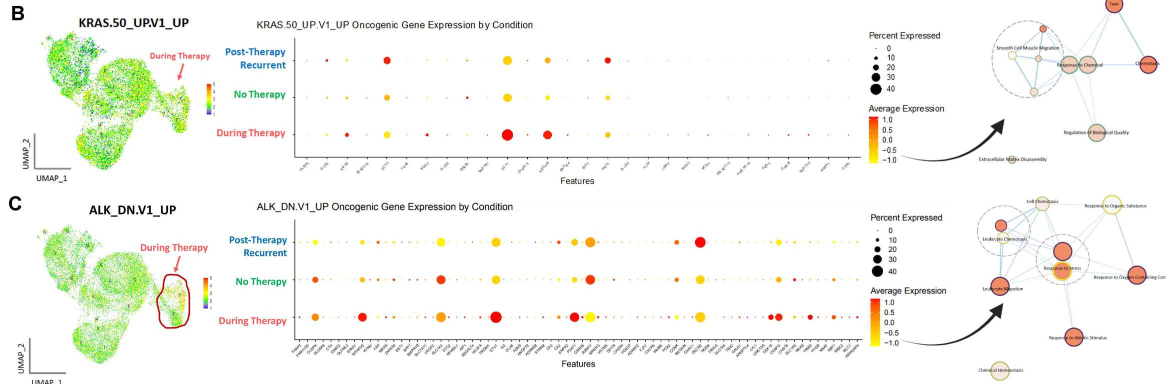


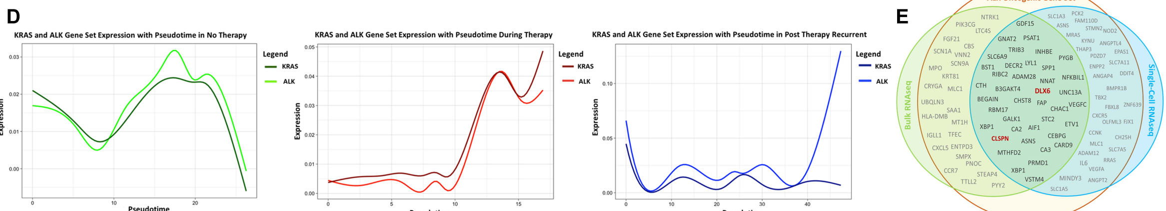
L
GBM Cell Gene Set Expression with Pseudotime in No Therapy
Classical
Mesenchymal
Proneural
Stem-Like Cell
Expression
Pseudotime
GBM Cell Gene Set Expression with Pseudotime During Therapy
Classical
Mesenchymal
Proneural
Stem-Like Cell
Expression
Pseudotime
GBM Cell Gene Set Expression with Pseudotime in Post Therapy Recurrent
Classical
Mesenchymal
Proneural
Stem-Like Cell
Expression
Pseudotime

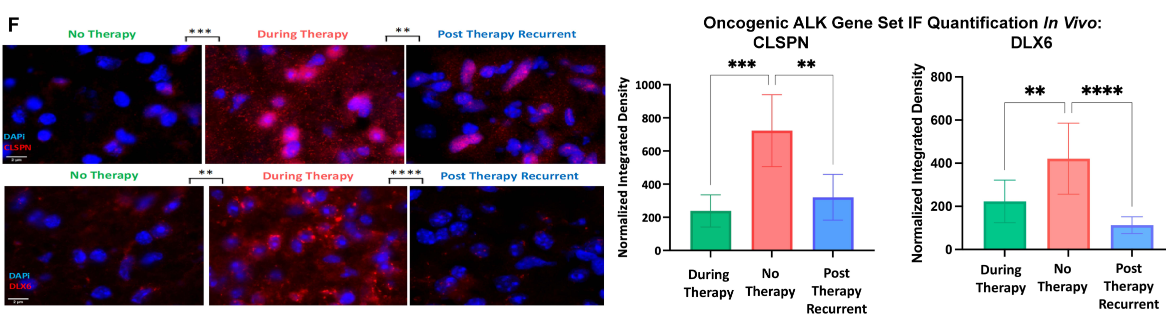
Figure S1: Single-cell RNA sequencing *in vivo* pipeline

(A) Schematic of scRNA-sequencing *in vivo* pipeline performed using droplet-based sequencing of human GBM43 cells. **(B)** Principal Component Analysis and Seurat Analysis clustering of our scRNAseq data. **(C)** Representative heat map of the sample means gene expression for 'during therapy' marker genes distributed across all scRNAseq conditions. **(D-E)** Cell distribution of cell-type gene sets expressed in our scRNAseq data. GSEA sets used: FAN_EMBRYONIC_CTX_MICROGLIA_1 and ZHONG_PFC_C1_DLX5_POS_INTERNEURON. **(F)** Representative bar graphs of GSEA cell-type gene sets expressed in our scRNAseq data. **(G)** Cell distribution of glioma stem-cell marker expression present in our scRNAseq data. GSEA sets used: BEIER_GLIOMA_STEM_CELL_UP. **(H)** Pathway enrichment analysis for top 5 enriched glioma-stem cell like genes in scRNAseq data. GSEA set: BEIER_GLIOMA_STEM_CELL_UP. **(I)** Representative dot plot of DNA repair associated genes separated by group condition expressed in our scRNAseq data. GSEA set: HALLMARK_DNA_REPAIR. **(J)** Representative heat map and dot plot of GSEA Resistance associated genes separated by group condition expressed in our scRNAseq data. GSEA sets: GO_DNA_DEALKYLATION and AMUNDSON_GAMMA_RADIATION_RESISTANCE. **(K)** Monocle3 trajectory inference graphs with pseudotime of DNA Repair and Resistance associated gene set expression in 'no therapy' (left), 'during therapy' (middle), and 'post therapy recurrent' (right). **(L)** Monocle3 trajectory inference graphs with pseudotime of GBM gene set expression in 'no therapy' GBM (left), 'during therapy' (middle), and 'post therapy recurrent' GBM (right). GSEA sets used: VERHAAK_GLIOBLASTOMA_CLASSICAL, VERHAAK_GLIOBLASTOMA_MESENCHYMAL, and VERHAAK_GLIOBLASTOMA_PRONEURAL.









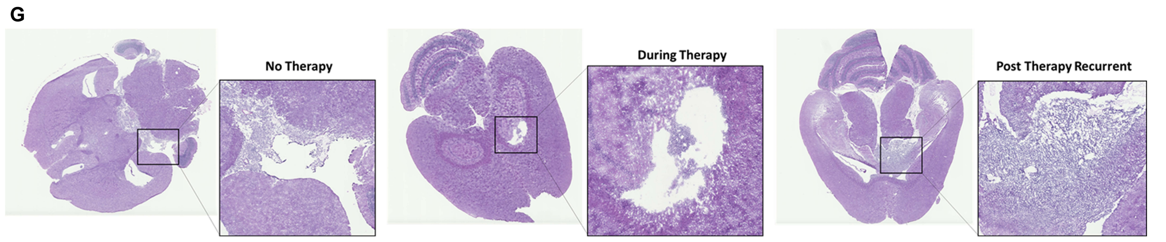


Figure S2: Oncogenic gene set expression in single-cell RNA sequencing data

(**A**) Representative heat maps of oncogenic gene set expressions in: ‘no therapy’ GBM (left), ‘during therapy’ GBM (middle), and ‘post therapy recurrent’ GBM (right). (**B**) Representative feature plot and dot plot of KRAS.50_UP.V1_UP gene set enrichment ‘during therapy’ compared to ‘no therapy’ (*p=0.052*) and ‘post therapy recurrent’ (*p=0.012*). Enriched pathways: Response to Chemical (*p=0.016*), Chemotaxis (*p=0.005*), Regulation of Biological Quality (*p=0.015*). **(C)** Representative feature plot and dot plot of ALK_DN.V1_UP gene set enrichment ‘during therapy’ compared to ‘no therapy’ (*p=4.96e-05*) and ‘post therapy recurrent’ (*p=1.10e-05*). Enriched pathways: Response to External Stimulus and Stress (*p=4.4e-06*) and Response to Organic Substance (*p=2.6e-05*). **(D)** Monocle3 trajectory inference graphs with pseudotime of KRAS and ALK gene set expression in ‘no therapy’ GBM (left), ‘during therapy’ (middle), and ‘post therapy recurrent’ (right). **(E)** Venn Diagram of ALK gene set genes, enriched ‘during therapy’ and in bulk RNAseq analysis comparing TMZ to control. **(F)** IHC of CLSPN (Alexa Fluor 647) and DLX6 (Alexa Fluor 647) in ‘no therapy,’ ‘during therapy,’ and ‘post therapy recurrent’ GBM43 tissue. Representative bar graphs of IHC quantification. **(G)** H&E staining of paired ‘no therapy,’ ‘during therapy,’ and ‘post therapy recurrent’ GBM43 tissue.

*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns, not significant.

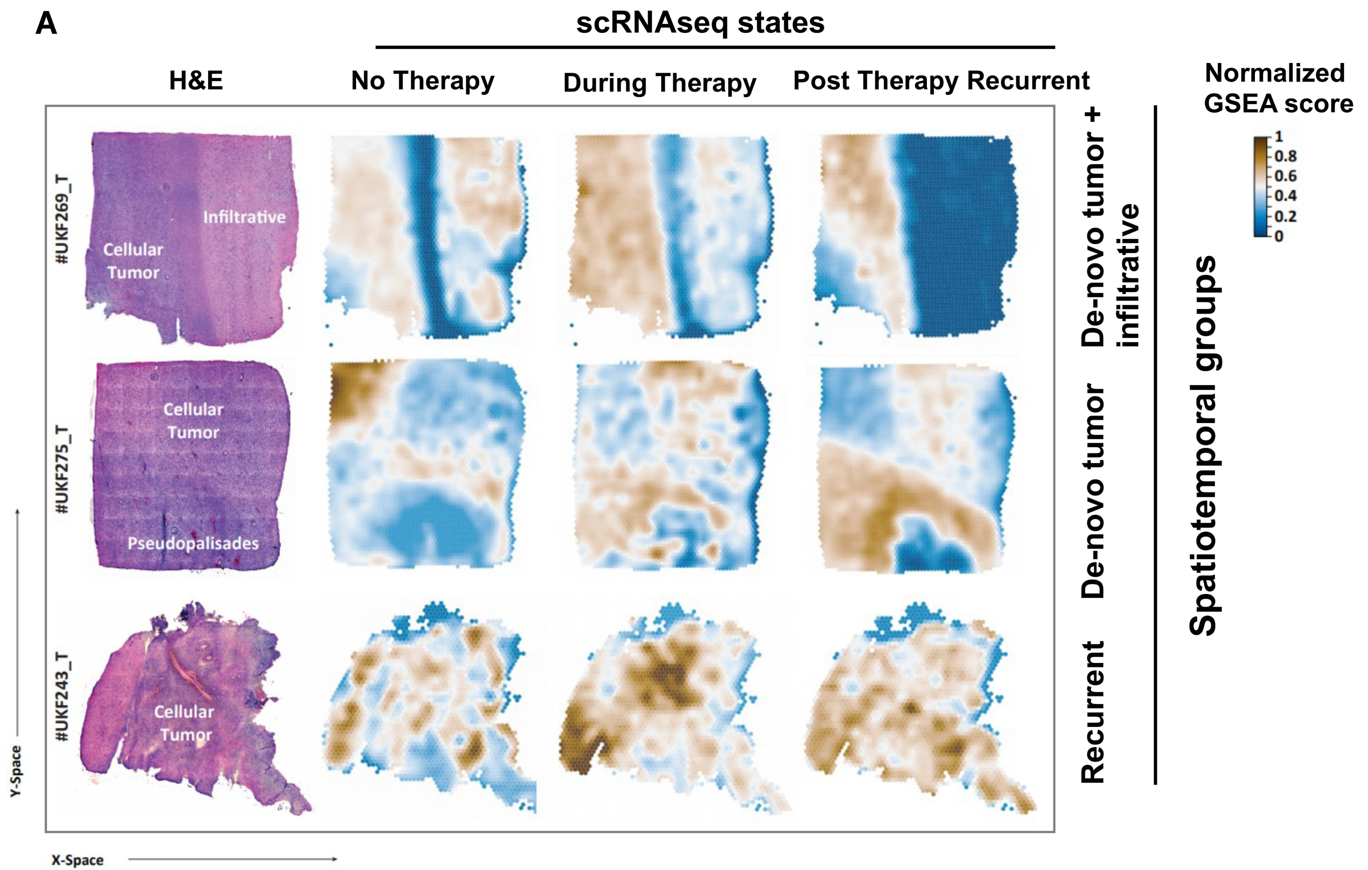
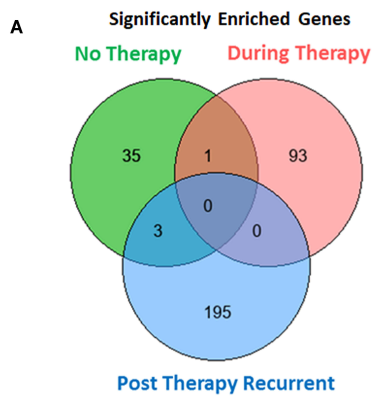
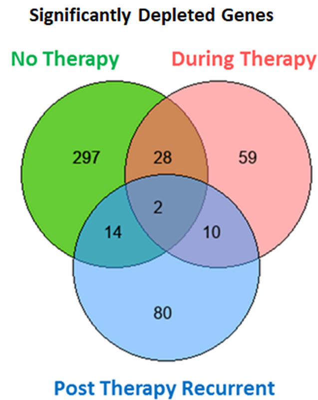
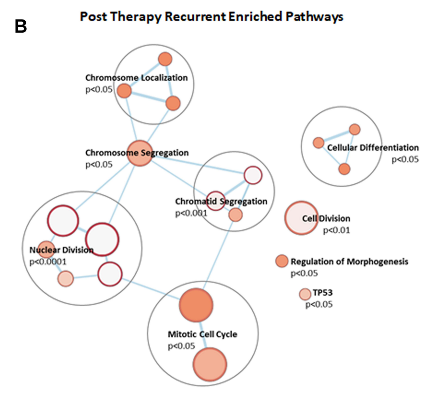


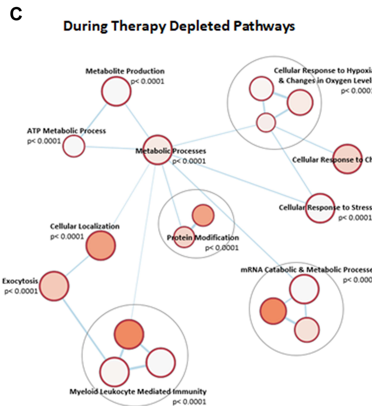
Figure S3: Spatial transcriptomics analysis to compare *in vivo* single-cell transcriptomic signatures from different time points during therapy with patients' GBM tissues

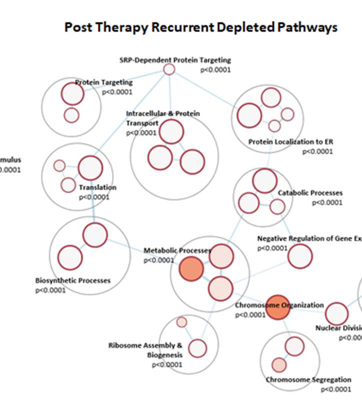
(A) Histological images (H&E) and surface plots of scRNAseq gene set enrichment unique to 'no therapy,' 'during therapy,' and 'post therapy recurrent' signatures. Primary GBM tissue sample used in stRNAseq from De Novo + Infiltrative region, De Novo region, and Recurrent region.

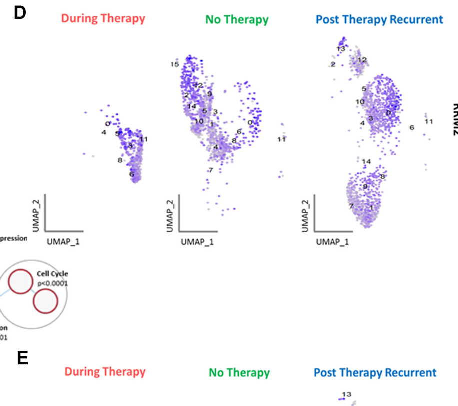


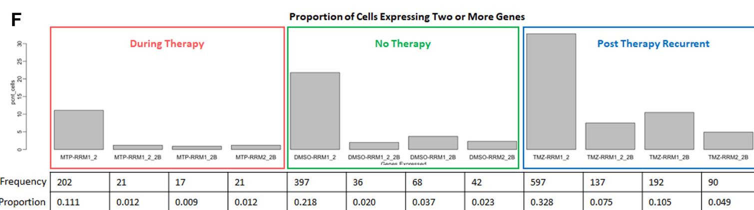




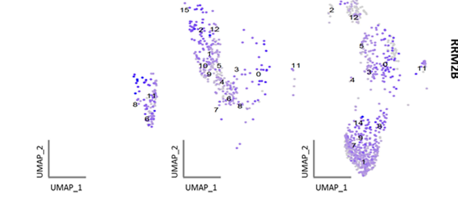


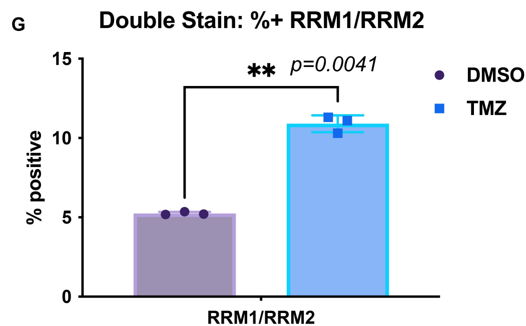


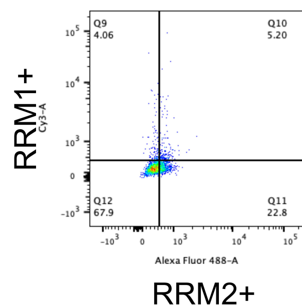


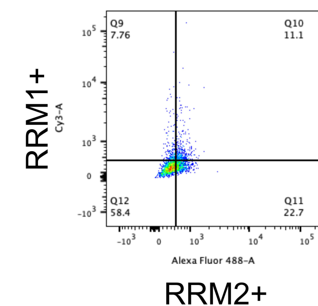


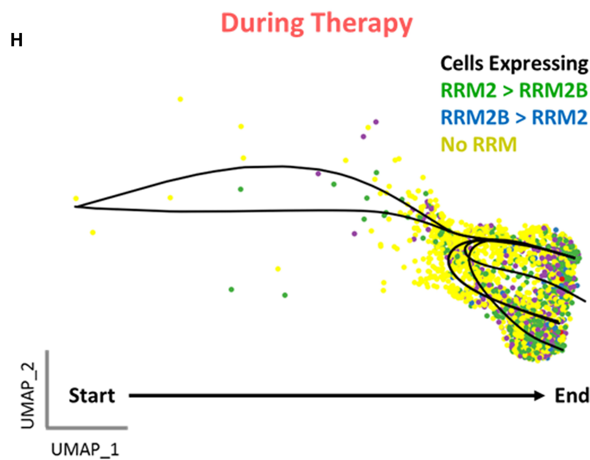
Frequency	202	21	17	21	397	36	68	42	597	137	192	90
Proportion	0.111	0.012	0.009	0.012	0.218	0.020	0.037	0.023	0.328	0.075	0.105	0.049











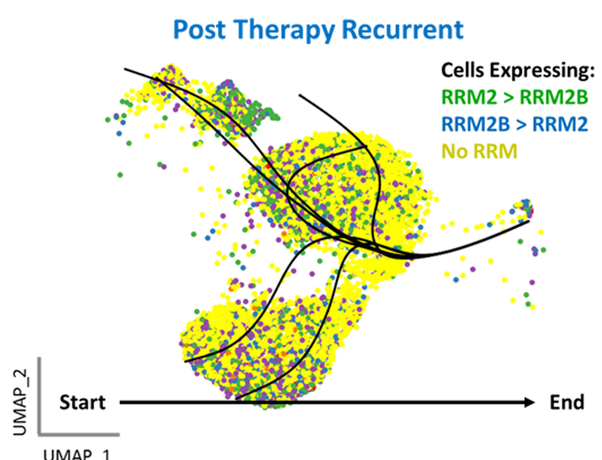


Figure S4: Single-cell RNA sequencing pathway analysis and RNR gene expression patterns

(A) Venn Diagrams of significantly enriched and significantly depleted genes unique to ‘no therapy,’ ‘during therapy,’ and ‘post therapy recurrent’ tumors. **(B-C)** Pathway analysis of genes enriched or depleted in our scRNAseq data. **(D-E)** Seurat cluster data for cells in: ‘during therapy’ (left), ‘no therapy’ (middle), or ‘post therapy recurrent’ (right). Cell distribution mapped for high *RRM2* or *RRM2B* expression. **(F)** scRNAseq analysis of the proportion of genes expressing a combination of *RRM1*, *RRM2*, and *RRM2B*, in ‘during therapy’ (left), ‘no therapy’ (middle), and ‘post therapy recurrent’ (right) scRNAseq conditions. **(G)** Representative flow cytometry bar graphs and gates for intracellular RRM1_RRM2 double positive expression in DMSO or TMZ (50uM). **(H)** Seurat clusters created using only ‘during therapy’ scRNA-seq data (left) or only ‘post therapy recurrent’ scRNA-seq data (right). The lineage progression of cell development from cluster to cluster determined using Slingshot. Progression of the lineages shown using the black line projections from the starting cluster. Cells highlighted by their expression status, with either expression of *RRM2*>*RRM2B*, *RRM2B*>*RRM2*, or no significant RNR gene expression.

A

RRM2 1.2%

RRM2B 0.4%

Gene Expression...

Methylation Status

Mutation Count

IDH1 Mutation

MGMT Status

Genetic Alteration: Missense Mutation (unknown significance), Amplification (unknown significance), No alterations

Gene Expression Subtype: Classical, G-CIMP, Mesenchymal, Neural, Proneural, No data

Methylation Status: CL_1, CL_2, CL_3, CL_4, CL_6, G-CIMP, No data

Mutation Count: 3, 113

IDH1 Mutation: R132G, R132H, WT

MGMT Status: METHYLATED, UNMETHYLATED, No data

B

RRM2 - TCGA_GBM: Agilent-4502A

RRM2B - TCGA_GBM: Agilent-4502A

C

RRM2 - TCGA_GBM: RNA-Seq

RRM2B - TCGA_GBM: RNA-Seq

D

Rembrandt

R = 0.68
p<0.001

RRM2

RRM1

R = -0.16
p<0.001

RRM2B

RRM1

TCGA_GBM: RNAseq

R = 0.57
p<0.001

RRM2

RRM1

R = -0.18
p=0.03

RRM2B

RRM1

TCGA_GBM: Agilent-4502A

R = 0.62
p<0.001

RRM2

RRM1

R = -0.22
p<0.001

RRM2B

RRM1

E

Normal

Glioma: RRM2 Stain

Glioma: RRM2B Stain

F

Fibro, Astro, H1B, NSC, 43, 6, 39, H4, U251

RRM1

RRM2

RRM2B

GAPDH

Figure S5: RNR gene expression in patient datasets

(**A**) Mutation rates of *RRM2* and *RRM2B* genes across 248 patient samples in cBioPortal. Corresponding key on right. **(B)** Representative GlioVis plots of *RRM2* (left) and *RRM2B* (right) expression in different conditions (i.e. GBM subtype, IDH mutant status, etc.) from TCGA_GBM: Agilent_4502A database. **(C)** Representative GlioVis plots of *RRM2* (left) and *RRM2B* (right) expression in different conditions (i.e. GBM subtype, IDH mutant status, etc.) from TCGA_GBM: RNAseq database. **(D)** Representative correlation plot of *RRM1_RRM2* (top) or *RRM1_RRM2B* (bottom). Correlations in Rembrandt (left), TCGA_GBM: RNAseq (middle), or TCGA_GBM: Agilent-4502A (right) databases on GlioVis. **(E)** The Human Protein Atlas RRM2 and RRM2B protein expression. **(F)** Baseline expression of genes of interest in cancer and neural stem cell lines.

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns, not significant.

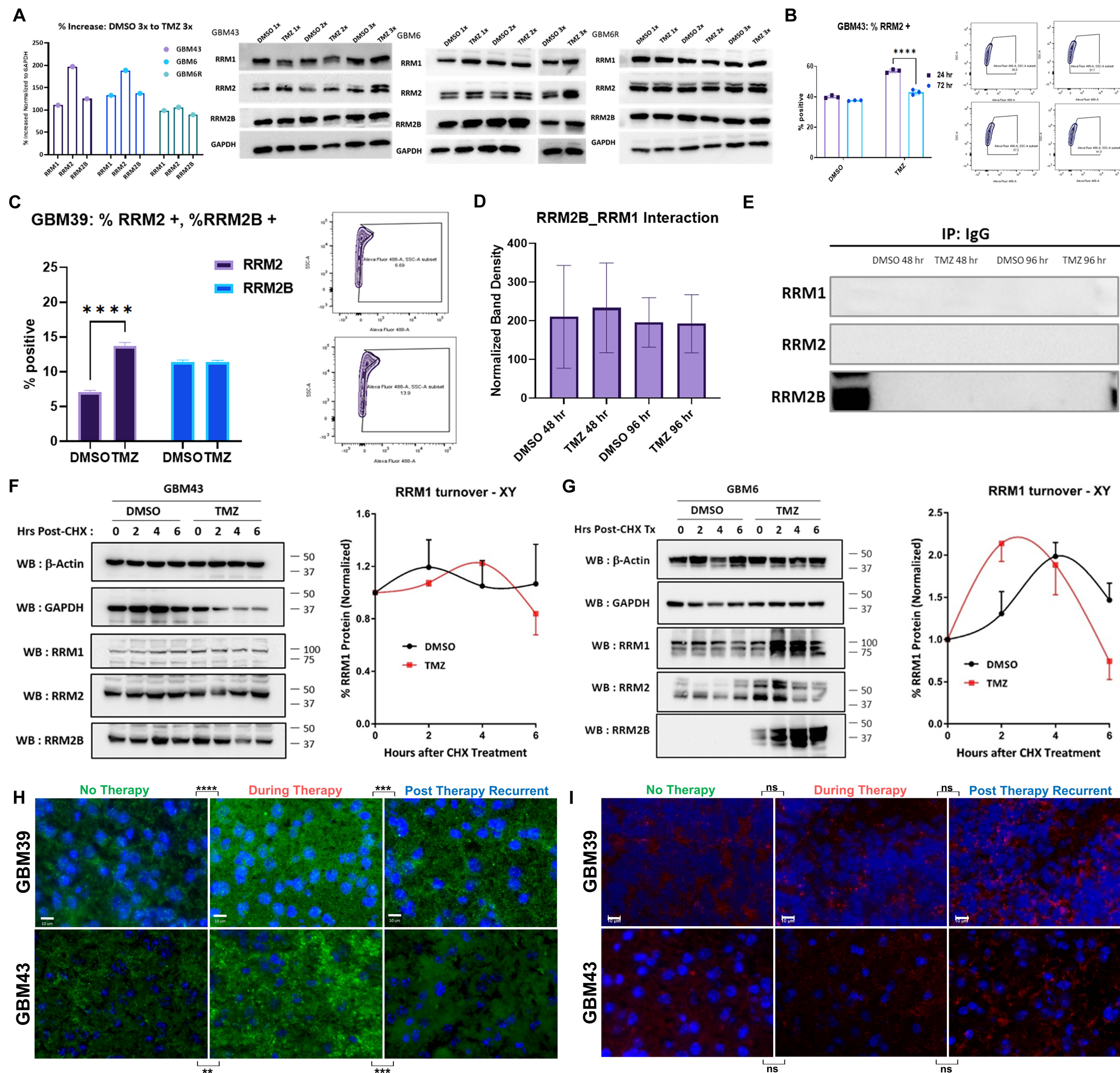


Figure S6: RNR gene expression during TMZ therapy

(A) Western-blot analysis and densitometry of GBM43, GBM6, and GBM6R cells treated 1x, 2x, or 3x exposure of DMSO (50uM) or TMZ (50uM). Stained for genes of interest. **(B)** Representative flow cytometry bar graphs for intracellular staining of RRM2 (Alexa Fluor 488) in DMSO or TMZ (50uM) for 24 or 72 hours. Representative flow cytometry gating corresponding to Figure S6B. **(C)** Figure 4A validated in GBM39. Representative flow cytometry gating. **(D)**

Using Fiji ImageJ software, RRM2B bands were normalized to RRM1. Bar graphs of IP interaction found in Figure 4B. **(C)** IP: IgG western blot corresponding to Figure 4B. **(F-G)** Cycloheximide Chase Assay performed in GBM43 and GBM6 with DMSO (50uM) or TMZ (50uM) for 0, 3, 4, or 6 hours. Corresponding western blot analysis of RRM2, RRM2B, and RRM1 and chase assay graphs of RRM1. RRM2 and RRM2B chase assay graphs shown in Figure 4 C-D. Validated in GBM43 (left) and GBM6 (right). **(H-I)** Immunohistochemistry of 'no therapy,' 'during therapy,' and 'post therapy recurrent' GBM tissue stained for RRM2 (Alexa Fluor 488) or RRM2B (Alexa Fluor 647). Validated in GBM39 (top) and GBM43 (below). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$; ns, not significant.

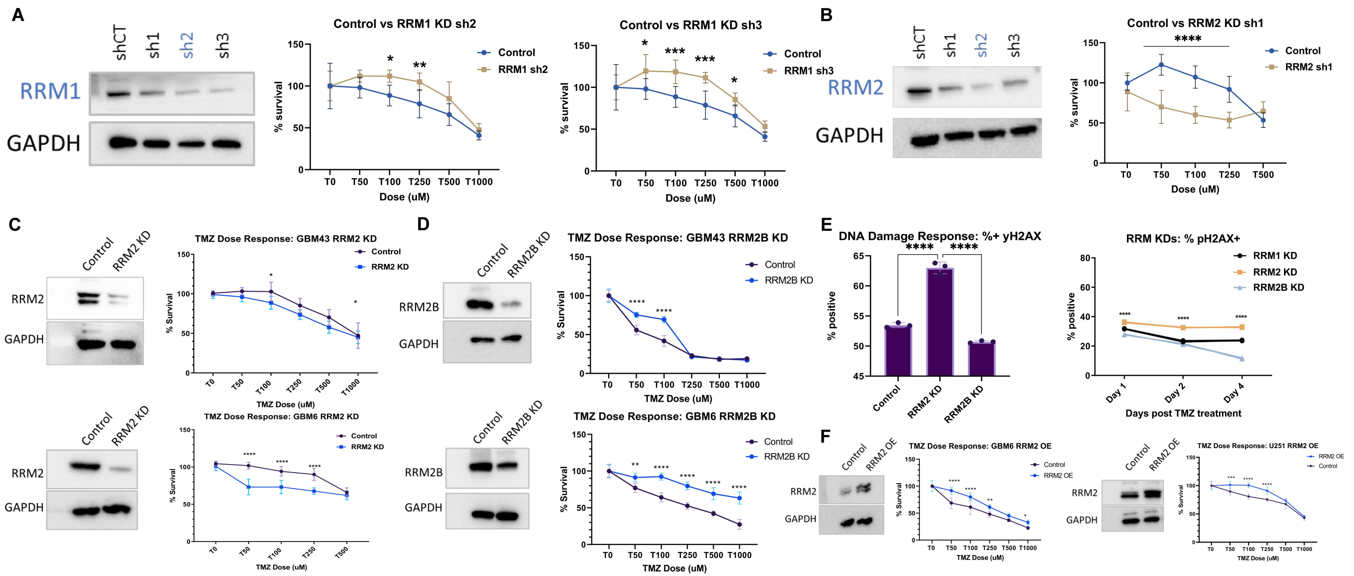


Figure S7: RNR-KDs response to TMZ

(A) Western blot of shRNA-mediated RRM1-KDs. MTT assay on RRM1-KDs in TMZ dose response. **(B)** Western blot of shRNA-mediated RRM2-KDs. MTT assay on RRM2-KDs in TMZ dose response. **(C)** Figure 5A validated in additional cell lines. **(D)** Figure 5C validated in additional cell lines. **(E)** Representative flow cytometry graphs of yH2AX (Alexa Fluor 647) expression in shControl and RNR-KD cells post TMZ treatment. **(F)** Figure 5E western blot and MTT assay validated in additional cell lines.

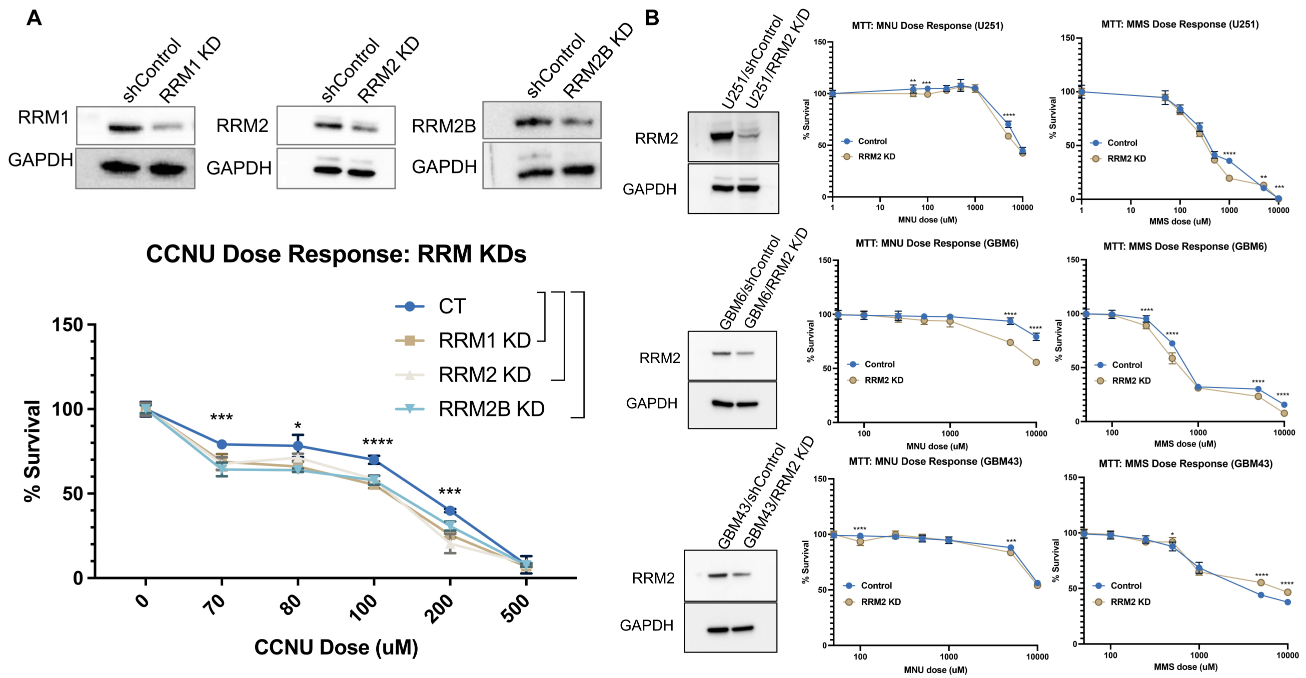


Figure S8: RRM2-KD drug-induced phenotype is TMZ specific

(**A**) Western blot of shRNA-mediated RNR-KDs. All control cell lines created by shRNA scramble vectors and are considered shControl. MTT assay on shControl and RNR-KD cells treated in CCNU dose response. Validated in U251. (**B**) Western blot of shRNA-mediated RNR-KDs. MTT assay on shControl and RRM2-KD cells treated in MNU (left) or MMS (right) dose response. Validated in U251, GBM6, GBM43.

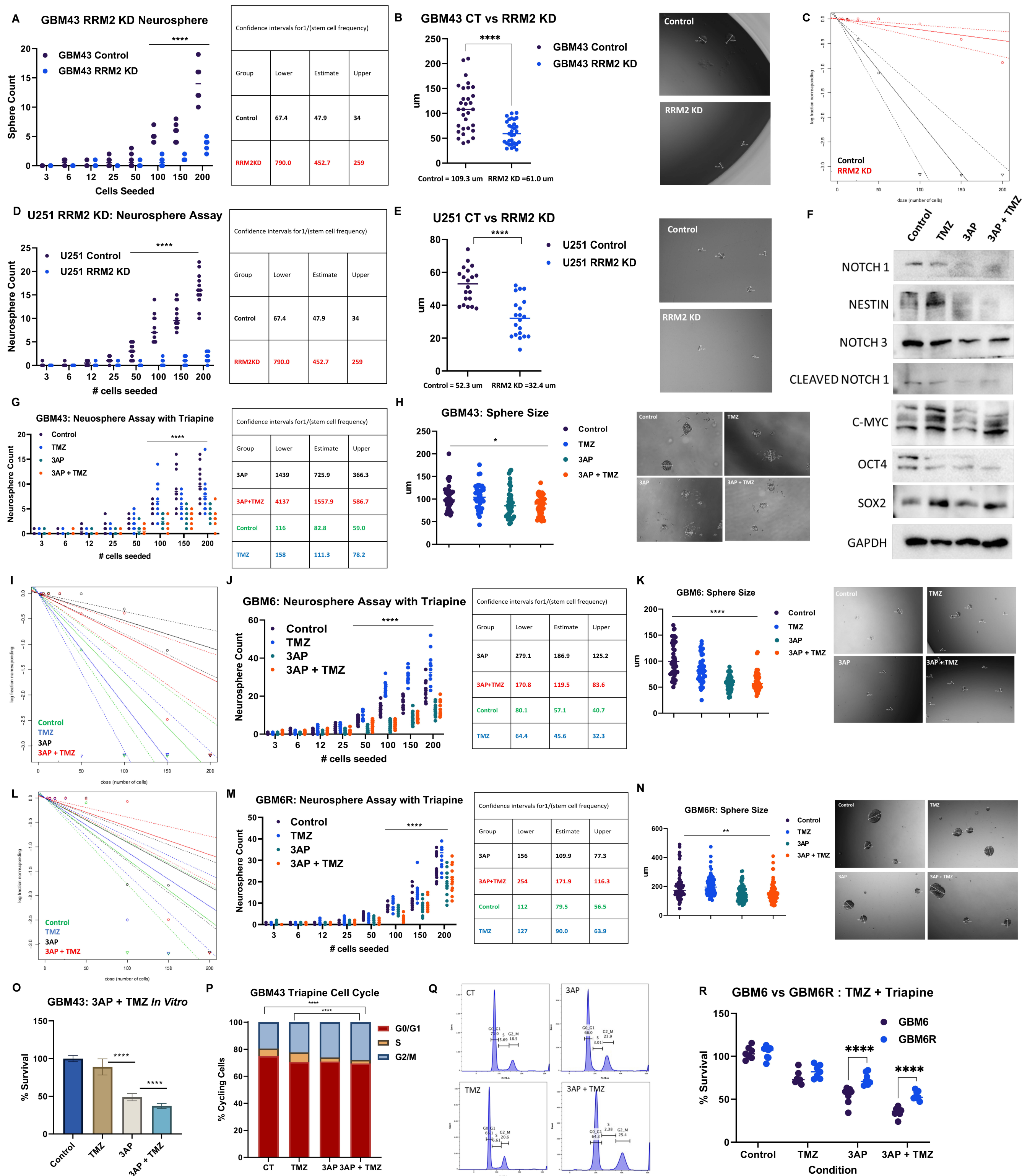


Figure S9: RRM2-KD and 3-AP Triapine on GBM stemness

(A-B) Neurosphere assay in GBM43 Control and RRM2-KD. **(C-E)** Neurosphere assay of U251 Control and RRM2-KD neurosphere assay. **(F)** Figure 5I validated in GBM6. **(G-H)** Neurosphere

assay of GBM43 cells treated with DMSO (50uM), TMZ (50uM), 3-AP (2uM), or TMZ + 3AP. **(I-K)** Figure S9G-H validated in GBM6. **(L-N)** Figure S9G-H validated in GBM6R. **(O)** MTT of GBM43 cells treated with DMSO, TMZ, 3-AP, or TMZ + 3AP. **(P-Q)** Cell-cycle flow cytometry of GBM43 cells treated with DMSO, TMZ, 3-AP, or TMZ + 3AP. **(R)** GBM6 and GBM6R cells treated in TMZ dose response. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns, not significant.

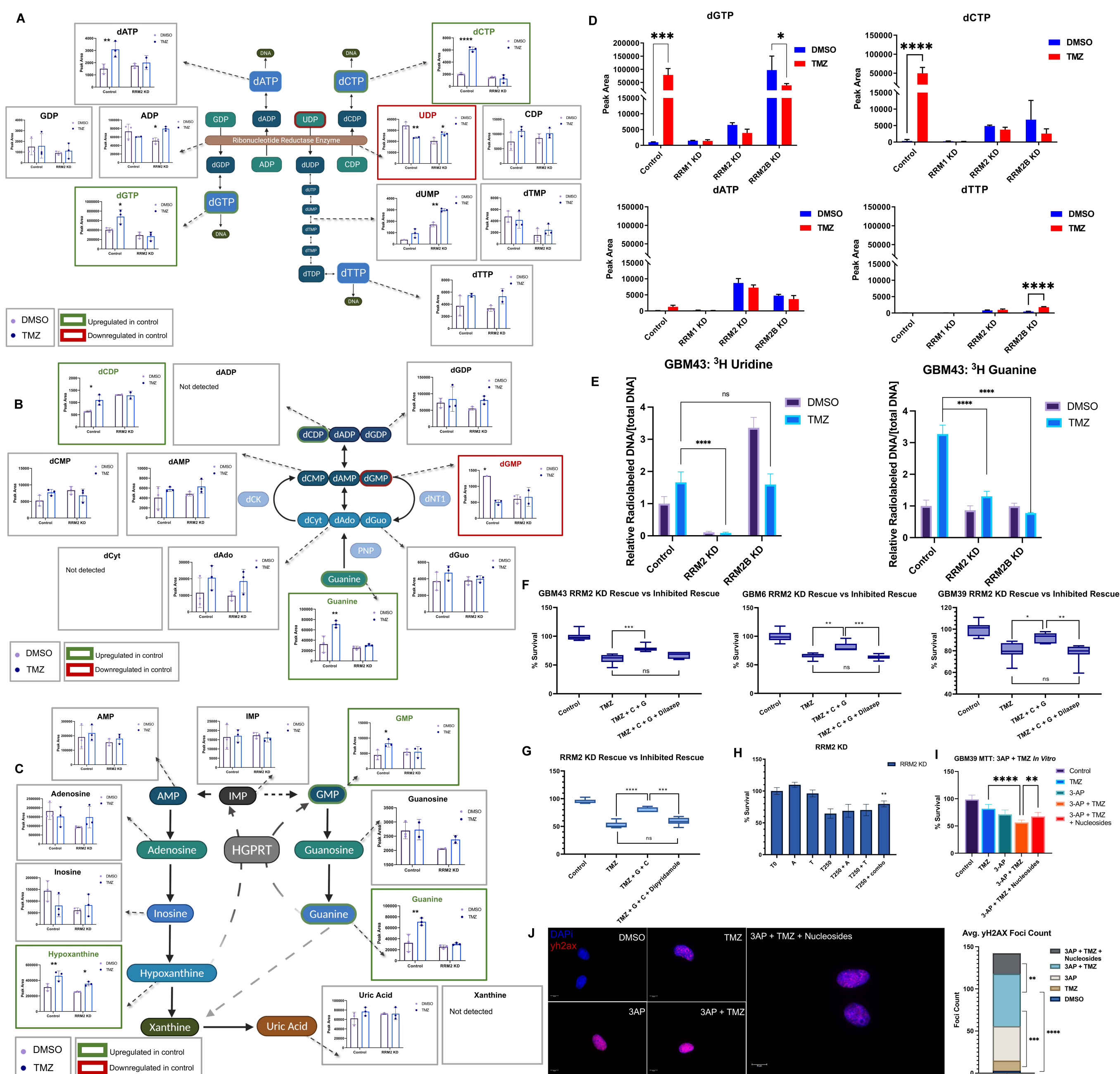


Figure S10: Targeted metabolomics reveals key metabolites necessary for RRM2-KD cells to combat TMZ

(A) Schematic of the RNR-mediated *de novo* dNTP synthesis and corresponding bulk metabolomics graphs of metabolites. **(B-C)** Schematic of the purine salvage pathways and corresponding bulk metabolomics analysis graphs of metabolites included in this pathway. **(D)** Targeted metabolomics analysis of dNTP production in control cells, shRNA-mediated RRM1-,

RRM2-, and RRM2B-KD cells. **(E)** Figure 6B validated in GBM43. Includes GBM43 RRM2B-KD relative radiolabeled DNA. **(F)** Figure 6F MTT validated in three more cell lines: GBM43 RRM2-KD, GBM6 RRM2-KD, and GBM39 RRM2-KD. **(G)** MTT assay of RRM2-KD cells treated with TMZ + deoxycytidine (25uM) and deoxyguanosine (25uM) nucleosides + Dipyridamole (5uM). Corresponding to Figure 6F. **(H)** MTT of shRNA-mediated RRM2-KD cells treated with DMSO, TMZ, TMZ + deoxyadenosine (25uM) and thymine (25uM) nucleosides. **(I)** Figure 6G validated in GBM39. **(J)** ICC of RRM2-KD cells when treated with DMSO, TMZ, 3-AP, 3-AP + TMZ, or 3-AP + TMZ + deoxycytidine and deoxyguanosine nucleosides. Representative bar graph of ICC quantification. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns, not significant.

Chemicals	**Source**	**Identifier**	**Concentrations**
DMSO	Sigma-Aldrich	D8418	*In vitro* 50uM; *In vivo* 2.5 mg/kg
TMZ	Merck	Temodal, 0085-1425-04	*In vitro* 0-1000uM; *In vivo* 2.5 mg/kg
1-(2-Chloroethyl)3-cyclohexyl-1-nitrosourea (CCNU)	Sigma-Aldrich	Lomustine, L0745000	*In vitro* 0-500uM
3-AP Triapine	MedChem Express	HY-10082	*In vitro* 2-5uM; *In vivo* 20-40 mg/kg
N-Nitroso-N-methylurea (MNU)	Selleck Chemicals, LLC	No. E0158 (100mg)	*In vitro* 0uM – 10mM
Methyl methanesulfonate (MMS)	Selleck Chemicals, LLC	No. E0609 (100mg)	*In vitro* 0uM – 10mM
Dilazep dihydrochloride	Tocris Bioscience	481	*In vitro* 5 uM
Diprydamole	Tocris Bioscience	691	*In vitro* 5 uM
2′-Deoxyguanosine monohydrate	Sigma-Aldrich	312693-72-4	*In vitro* 25 uM
2′-Deoxycytidine	Sigma-Aldrich	951-77-9	*In vitro* 25 uM
2′-Deoxyadenosine monohydrate	Sigma-Aldrich	16373-93-6	*In vitro* 25 uM

Thymidine	Sigma-Aldrich	50-89-5	*In vitro* 25 uM
shRNA KD/OE	**Source**	**Identifier**	
RRM1 KD	Horizon	RHS4430-200157988	
RRM2 KD	Horizon	RHS4430-20019181	
RRM2 OE	Genscript	N/a	
RRM2B KD	Horizon	RHS4430-177651	
Antibodies	**Source**	**Identifier**	**Concentrations**
RRM1 RB	Proteintech	10526-1-AP	WB 1:1000; IP 3ug; IF 1:400; FACS 1:200
RRM2 RB	Proteintech	11661-1-AP	WB 1:1000; IF 1:400
RRM2 MS	Abcam	ab57653	WB 1:800; IF 1:400; FACS 1:100
p53R2 RB	Abcam	ab8105	WB 1:1000; IF 1:500; FACS 1:400
GAPDH MS	Proteintech	60004-1-Ig	WB 1:4000
DLX6	Proteintech	23216-1-AP	IF 1:400
CLSP	Proteintech	23206-1-AP	IF 1:400
yH2AX Alexa Fluor 647 Conj. RB	Cell Signaling	9720S	IF 1:500; FACS 1:200
Notch 1 RB	Cell Signaling	3608S	WB 1:1000
Notch 3 RB	Proteintech	55114-1AP	WB 1:1000
Cleaved Notch 1 RB	Cell Signaling	4147S	WB 1:1000
Nestin RB	Proteintech	19483-1-AP	WB 1:1000

cMyc RB	Cell Signaling	18583S	WB 1:1000
Oct4 RB	Cell Signaling	2750S	WB 1:1000
Sox2 RB	Cell Signaling	23064S	WB 1:1000
Rabbit IgG RB	Proteintech	30000-0-AP	IP: 3ug
Anti-mouse IgG, HRP	Cell Signaling	7076S	WB 1:4000
Anti-rabbit IgG, HRP	Cell Signaling	7074S	WB 1:4000
Alexa Fluor 647 RB	Abcam	ab150079	IF 1:1000
Alexa Fluor 488 MS	Abcam	ab150113	IF 1:1000
Kits	**Source**	**Identifier**	
Human tumor cell isolation kit	Miltenyi Biotech	130-108-339	
Midi prep	Qiagen	12143	
Analysis Tools			
DropRunner			
Seurat 3.0			
Velocyto			
Prism, GraphPad v9.0			
Cytoscape			
gProfiler			
Enrichr			
String			

FlowJo, LLC			
Fiji, ImageJ Software			