Supplementary Material

Combination of ribociclib and gemcitabine for the treatment of medulloblastoma

Allison Pribnow[1,$,#], Barbara Jonchere[1,#], Jingjing Liu[2], Kyle S. Smith[3], Olivia Campagne[4] , Ke Xu[5], Sarah Robinson[1], Yogesh Patel[3,*] Arzu Onar-Thomas[6], Gang Wu[5], Clinton F. Stewart[4], Paul A. Northcott[3], Jiyang Yu[2], Giles W. Robinson[7], Martine F. Roussel[1,&]

Departments of Tumor Cell Biology[1], Computational Biology[2], Developmental Neurobiology[3], Pharmaceutical Sciences[4], Center for Applied Bioinformatics[5], Biostatistics[6], Neuro-Oncology[7], St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN, 38105
# co-authors contributed equally to the work

Material and Methods

RNA-sequencing and GSEA analysis

Paired-end RNA sequencing (RNA-seq) reads from human and mouse tumors were mapped to GRCh37-lite and MGSCv37, separately, as previously described (1). Mapped bam files of human tumors were cleansed by XenoCP (2) to remove the contaminating mouse reads. Read counts were quantified by HTSeq-Count (version 0.11.2) based on GENCODE release 19 for the human tumors and GENCODE release M1 for mouse tumors (3). Differentially expressed genes were identified with edgeR (version 3.28.0) and limma (version 3.42.0) (4,5). Gene set enrichment analyses (GSEA) were conducted by stand-alone GSEA software (version 2.2.3) against hallmark gene sets from MSigDB (version 5.2) on the normalized expression matrix composed of samples from two different treatment arms (6,7).

Reconstruction of medulloblastoma interactome and inference of gene activity

After removing 1,165 probe sets with low or invariable expression across samples, 20476 probe sets were used as SJARACNe input. Based on Gene Ontology classification (8), we compiled a list of driver (hub) genes, which consists of 2,002 transcription factor genes and 9,626 signaling factors. A transcription factor and a signaling molecule network were generated separately using SJARACNe, with drivers (hubs) linked to their targets through interactions (edges) based on gene-gene relationship derived from their expression pattern. The transcription factor network contained 18,314 nodes (genes) and 263,440 edges; the signaling network included 18,333 nodes (genes) and 424,897 edges. After combining these two networks, the final data-driven MBi consisted of 28,004 nodes (genes) and 688,337 edges, among which, there were 9,602 unique hub genes, including 1,733 transcriptional factors and 7,869 signaling molecules.

After cleansing and quantification of the reads, RNA-seq gene expression values were log2(FPKM+0.1) transformed. For each dataset, genes were selected by two cutoffs before calculating driver activity: (a) removing the low expression genes, whose values in 80% of the samples were below the 10^{th} percentiles of all expression values, and (b) keeping 70% of genes with the highest standard deviation. For the sequenced mouse samples, the mouse geneSymbol was converted to human geneSymbol by using the biomaRt package (version=2.42.1). Pathway enrichment of top differentially activated genes were performed by Fisher's exact test against gene sets in MSigDB (version 5.2).

Reference

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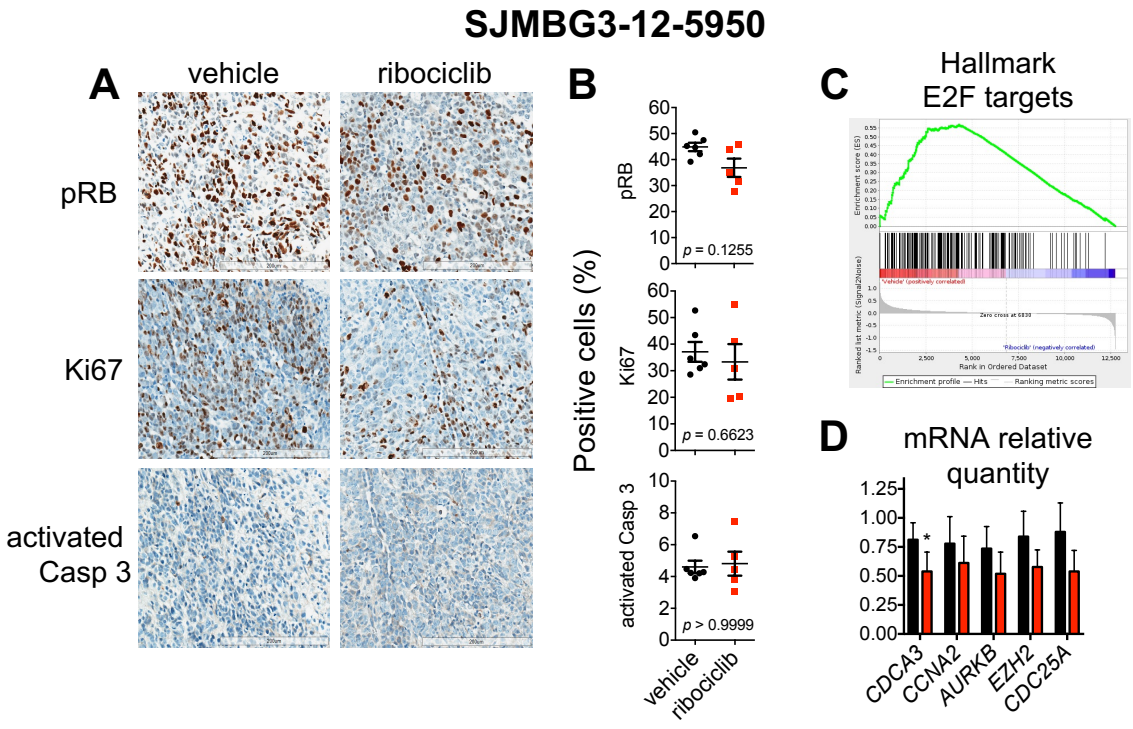
Supplementary Table

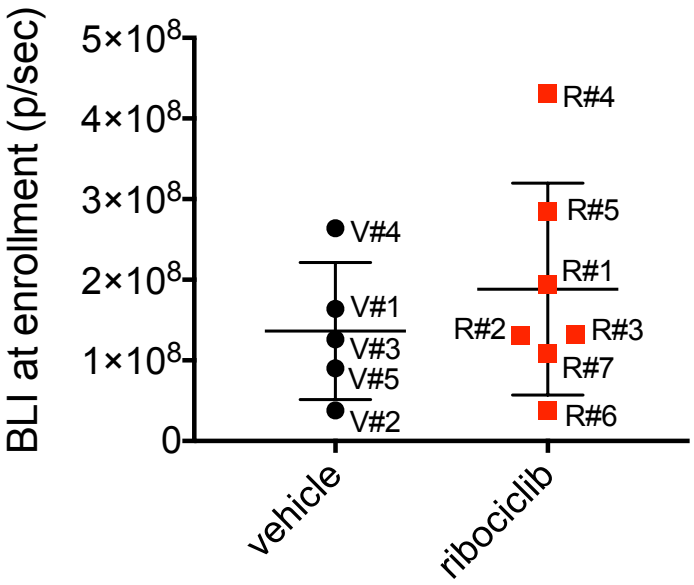
Once Supplementary Table provided as an excel file

Supplementary Figures

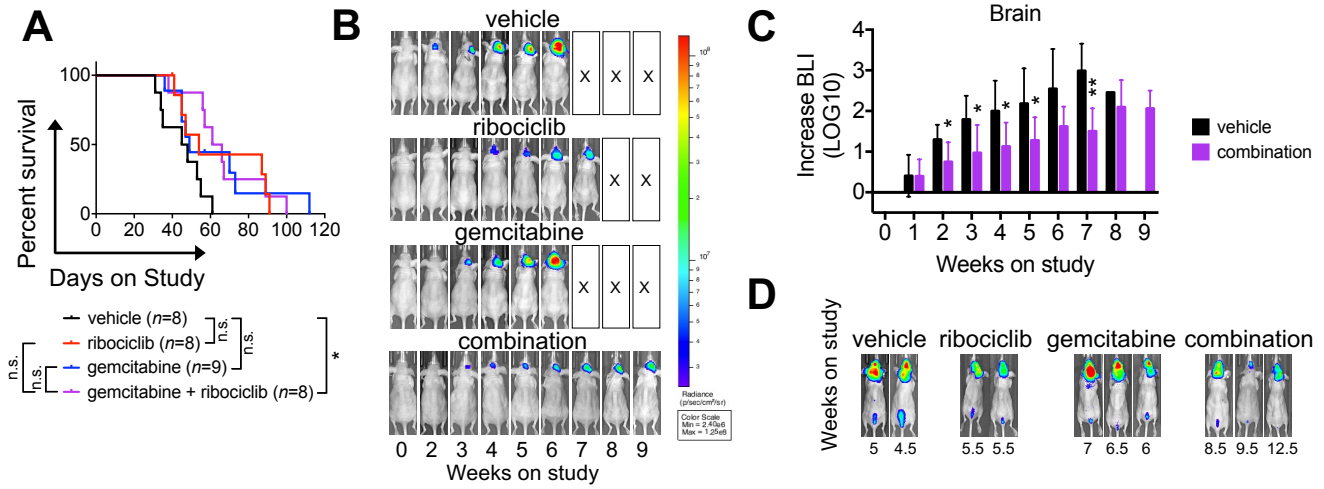
Ten Supplementary Figures



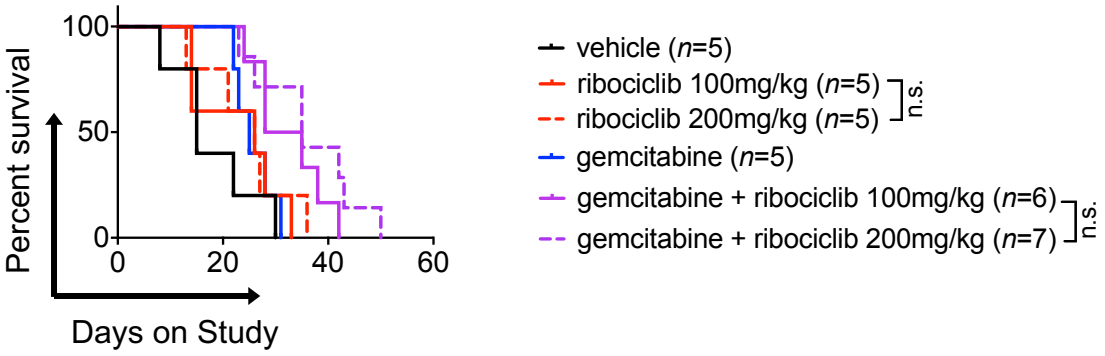
Supplementary Figure S1. Ribociclib suppresses the expression of E2F target genes in a human G3MB tumor. (**A-D**) Mice bearing human G3MB PDOX SJMBG3-12-5950 tumors treated with vehicle or ribociclib (100mg/kg), daily for 5 days and euthanized 4h post last dose. (**A**) Representative immunohistochemistry images of tumors stained with antibodies against pRb$^{Ser807/811}$ (pRB), Ki67 and activated Caspase-3. (**B**) Quantification of the percentage of positive cells corresponding to each staining (Mann-Whitney test, n=5 to 6). Note: one untreated tumor added to vehicle group. (**C**) Gene Set Enrichment Analysis (GSEA) showed significant depletion of HALLMARK_E2F_TARGETS in ribociclib versus vehicle treated tumors. (**D**) mRNA relative level of selected E2F target genes quantified by qRT-PCR (Mann-Whitney test, n=5 per group, $p \leq 0.05$ (*)).



Supplementary Figure S2. Brain bioluminescence signal at enrollment of the pharmacodynamic study presented in Fig 1G-J. Mice bearing mouse G3MB tumor #2416 submitted to bioluminescence imaging (BLI) seven days post-implant and enrolled in a pharmacodynamic study. Randomization in two treatment groups : vehicle (V#1-V#5) or ribociclib (R#1-R#7).

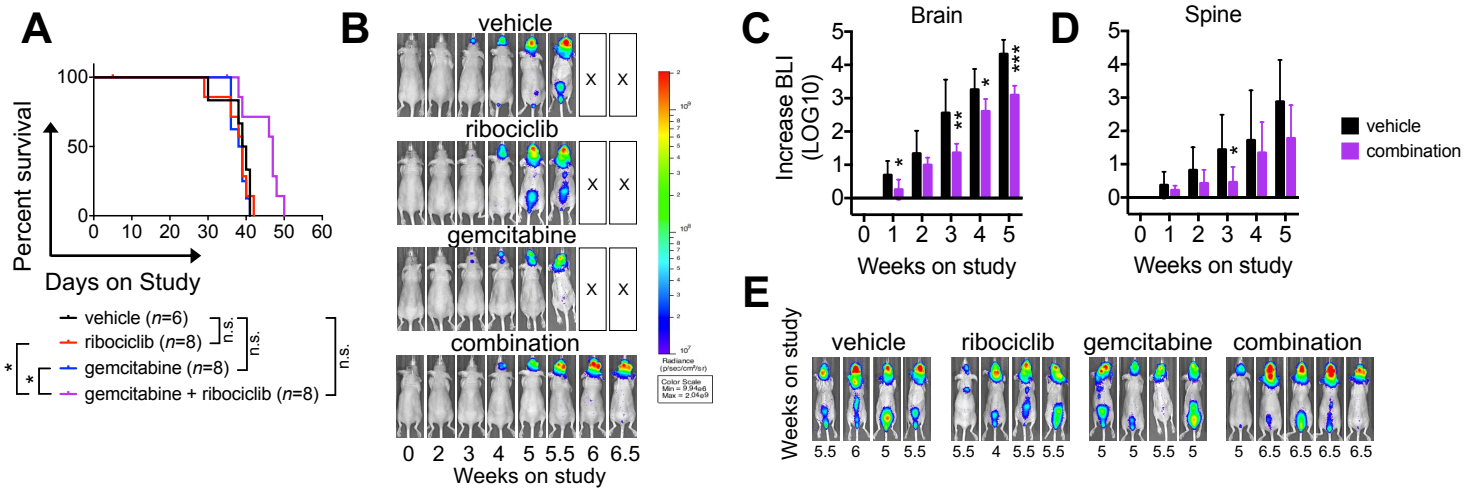


Supplementary Figure S3. Combination therapy of ribociclib and gemcitabine improves survival of mice bearing human G3MB PDOX SJMBG3-12-5950. (A-D) Mice bearing SJMBG3-12-5950 treated with vehicle (black lines), ribociclib (100mg/kg, continuous daily, by oral gavage (OG), red lines), gemcitabine (60mg/kg, intravenously (IV), day1 & day15 every 2 weeks, blue lines), or the combination (purple lines) until moribund. (**A**) Kaplan-Meier survival for each treatment groups. Comparison between treatment groups using log-rank test adjusted for multiple comparisons (not significant (n.s.) and adjusted $p \leq 0.05$ (*). Mice were censored if they died or required humane euthanasia not related to tumor and without neurologic symptoms (e.g., death during sedation for imaging). Mice underwent twice weekly bioluminescence imaging (BLI). **(B)** BLI pictures at different time points from enrollment through moribund stage for one representing mouse per treatment group. Mice were selected based on tumor and spinal growth median behavior. (**C**) BLI increase from enrollment (LOG10 transformation) for the brain (Mann-Whitney test, $p \leq 0.05$ (*), $p \leq 0.01$ (**)). (**D**) Endpoint BLI images of mice with visible spinal signal based on the scale.

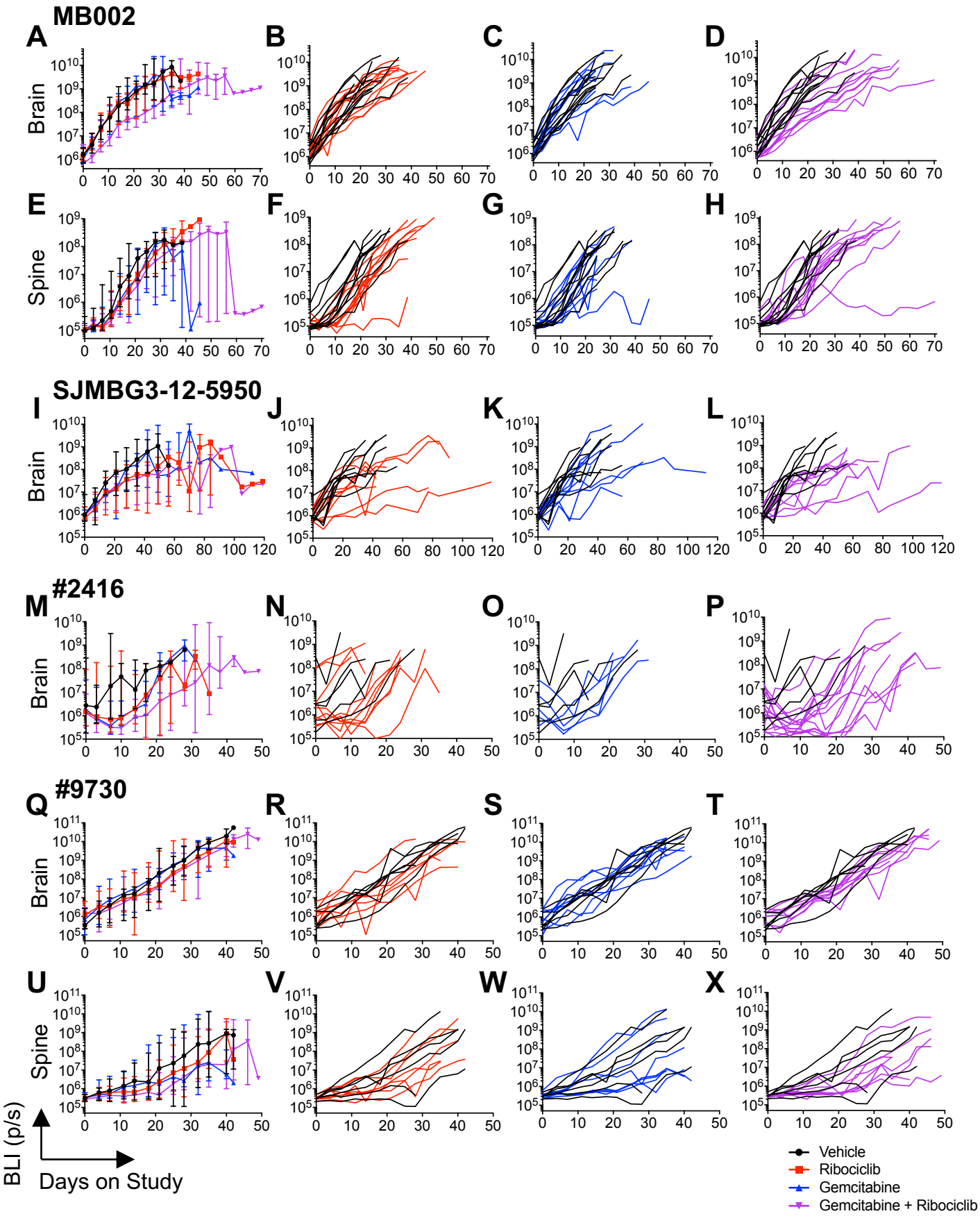


Supplementary Figure S4. Combination therapy of ribociclib and gemcitabine in mice bearing mouse G3MB tumor #2416. Mice bearing the mouse G3MB tumor #2416 treated with vehicle (black lines), ribociclib (100mg/kg, day1-21 (3 weeks), OG, red lines), ribociclib (200mg/kg, day1-21 (3 weeks), OG, red doted lines), gemcitabine (60mg/kg, IV, day1 & day15 every 2 weeks, blue lines), the combination of gemcitabine (60mg/kg, IV, day1 & day15) and ribociclib (100mg/kg, day1-21, OG, purple lines) or the combination of gemcitabine (60mg/kg, IV, day1 & day15) and ribociclib (200mg/kg, day1-21, OG, purple doted lines). Mice were treated with sequential cycles of therapy until moribund. Kaplan-Meier survival for each treatment groups. Comparison between treatment groups using log-rank test adjusted for multiple comparisons. Mice were censored if they died or required humane euthanasia not related to tumor and without neurologic symptoms (e.g., death during sedation for imaging).

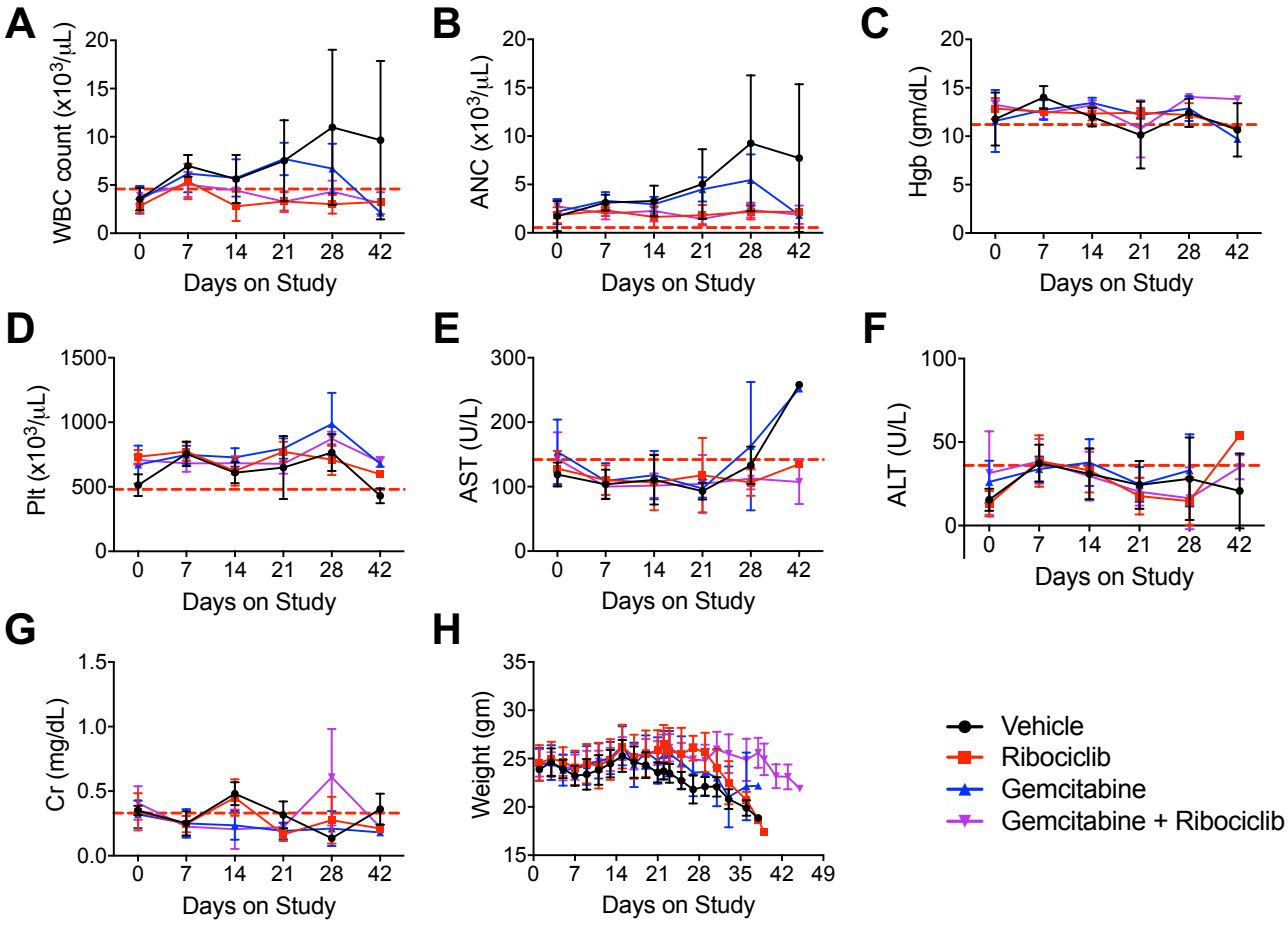
#9730



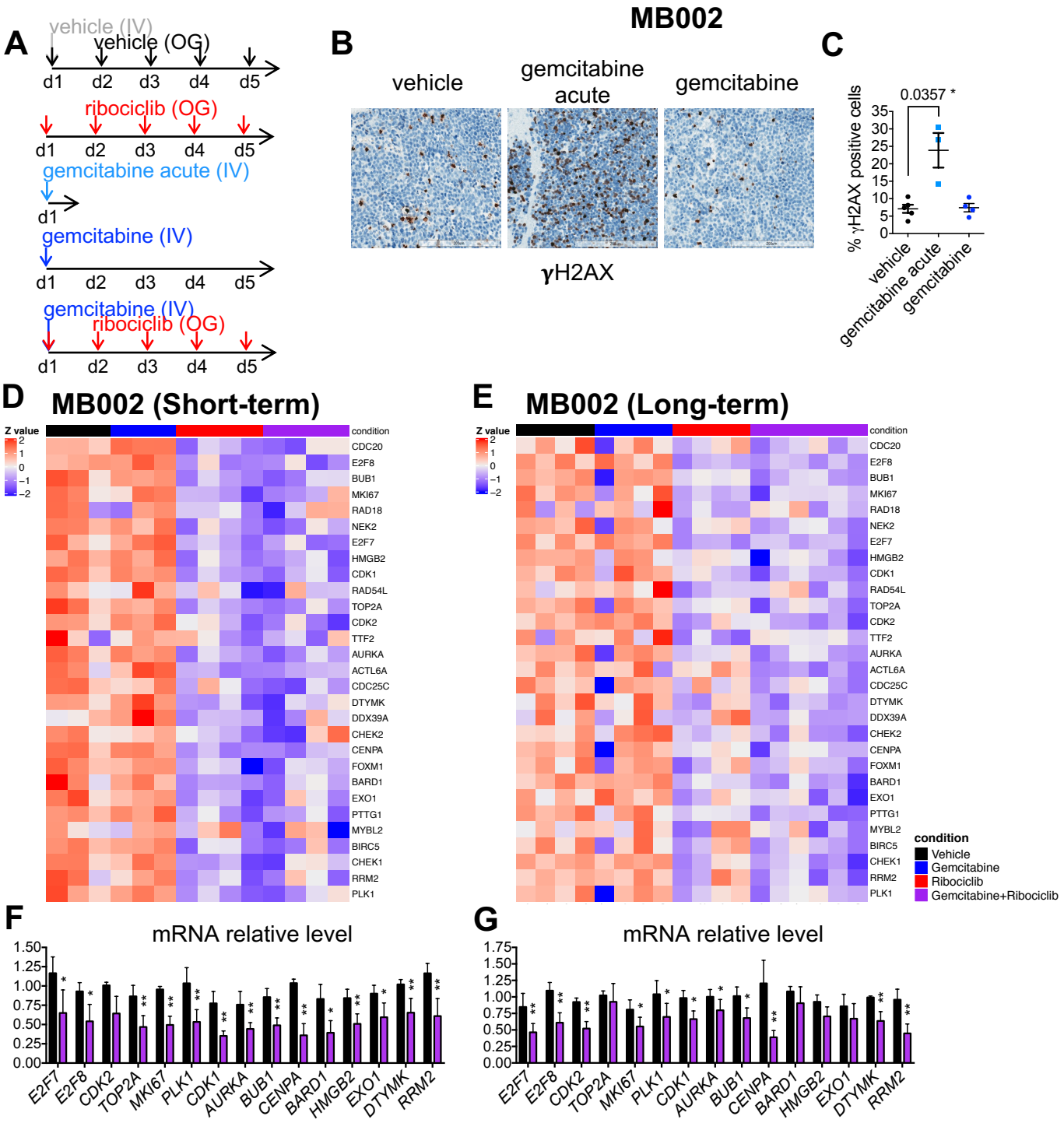
Supplementary Figure S5. Combination therapy of ribociclib and gemcitabine in mice bearing mouse G3MB tumor #9730. (A-D) Mice bearing the mouse G3MB tumor #9730 treated with vehicle (black lines), ribociclib (100mg/kg, day1-21 (3 weeks), OG, red lines), gemcitabine (60mg/kg, IV, day1 & day15 every 2 weeks, blue lines), or the combination of gemcitabine (60mg/kg, IV, day1 & day15) and ribociclib (100mg/kg, day1-21, OG, purple lines). Mice were treated with sequential cycles of therapy until moribund. (**A**) Kaplan-Meier survival for each treatment groups. Comparison between treatment groups using log-rank test adjusted for multiple comparisons (not significant (n.s.) and adjusted $p \leq 0.05$ (*). Mice were censored if they died or required humane euthanasia not related to tumor and without neurologic symptoms (e.g., death during sedation for imaging). Mice underwent twice weekly bioluminescence imaging (BLI). **(B)** BLI pictures at different time points from enrollment through moribund stage for one representing mouse per treatment group. Mice were selected based on tumor and spinal growth median behavior. (**C, D**) BLI increase from enrollment (LOG10 transformation) for brain (**C**) and spine (**D**) (Mann-Whitney test, $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***)). (**E**) Endpoint BLI images of mice with visible spinal signal based on the scale.


MB002
A
B
C
D
Brain
E
F
G
H
Spine
SJMBG3-12-5950
I
J
K
L
Brain
#2416
M
N
O
P
Brain
#9730
Q
R
S
T
Brain
U
V
W
X
Spine
BLI (p/s)
Days on Study
Vehicle
Ribociclib
Gemcitabine
Gemcitabine + Ribociclib

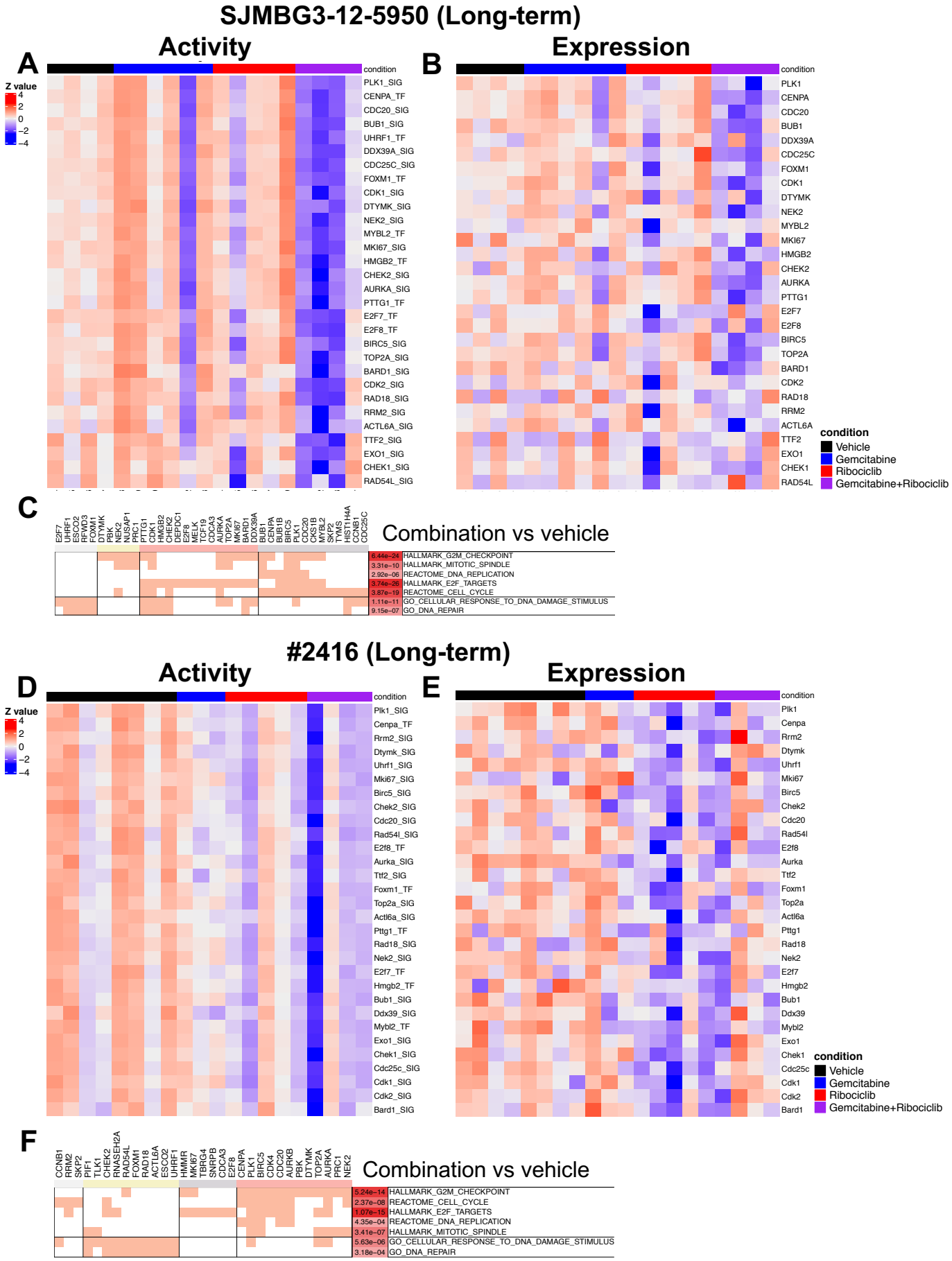
Supplementary Figure S6. Bioluminescence imaging of mouse and human G3MB treated with ribociclib and gemcitabine as single agent and in combination. Mice bearing human G3MB PDOXs MB002 (**A-H**) and SJMBG3-12-5950 (**I-L**) and mouse G3MB tumors #2416 (**M-P**) and #9730 (**Q-X**) treated with vehicle (black lines), ribociclib (red lines), gemcitabine (blue lines) or the combination of ribociclib and gemcitabine (purple lines) in **Fig. 2** and **Supplementary Fig S2** were imaged twice weekly by bioluminescence imaging (BLI) from enrollment through moribund stage. Median bioluminescence of the brain **(A, I, M, Q**) and spine (**E, U**). BLI of the brain (**B-D, J-L, N-P, R-T**) and spine (**F-H, V-X**) for each individual mice in all four treatment groups.



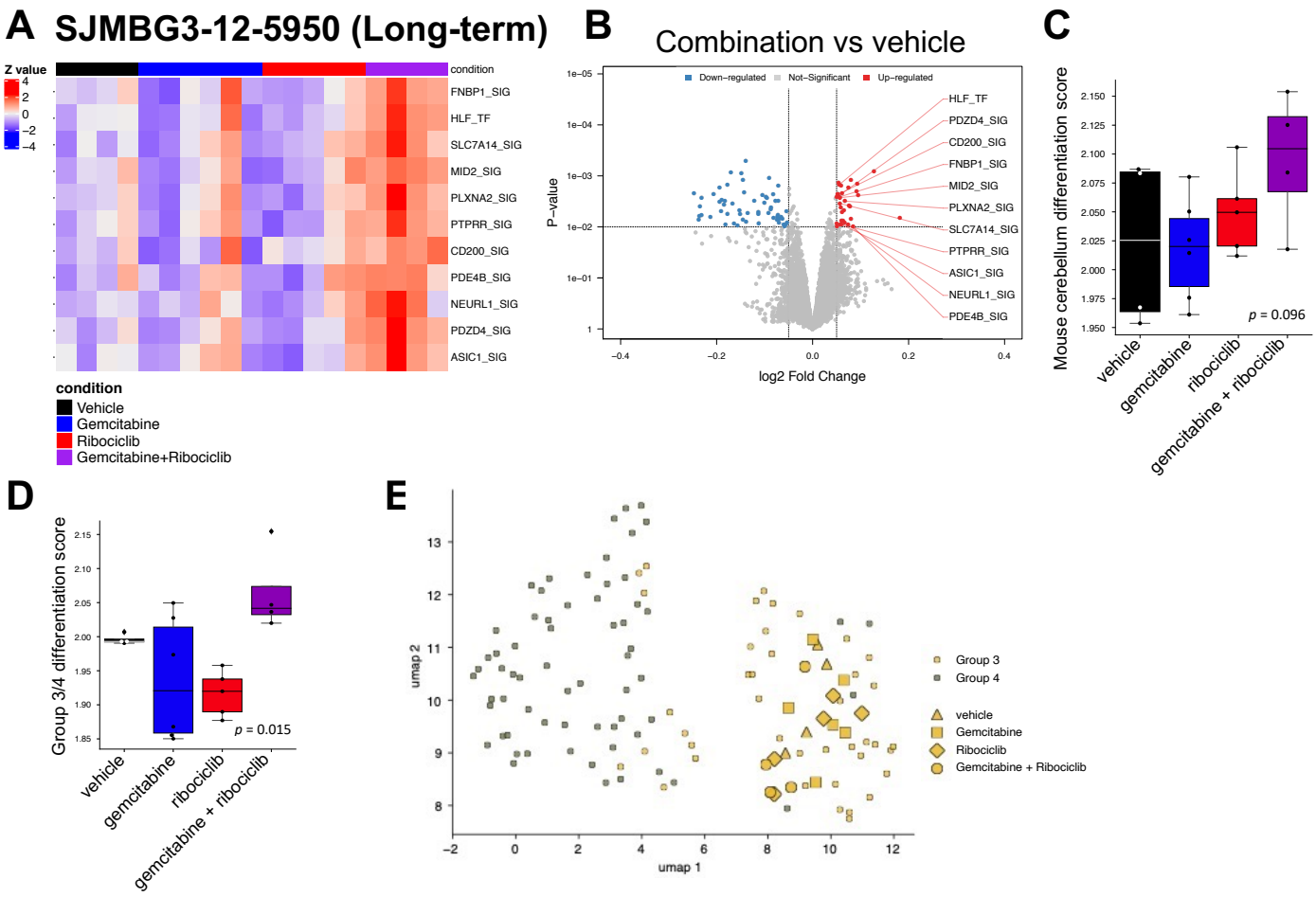
Supplementary Figure S7. Mouse toxicity profiles. Mice orthotopically implanted with the mouse G3MB tumor #9730 were treated with vehicle (black lines), ribociclib (red lines), gemcitabine (blue lines) or the combination of ribociblib and gemcitabine (purple lines). Complete blood counts of weekly blood collection include (**A**) white blood cell (WBC), (**B**) absolute neutrophil (ANC: absolute neutrophil count), **(C)** hemoglobin (Hgb), **(D)** platelet (Plt), and chemistry panel including **(E)** aspartate aminotransferase (AST), (**F**) alanine aminotransferase (ALT), and (**G**) creatine (Cr). **(H)** Body weight was measured before each dose. Blood counts and body weight were graphed as the mean values ± SD. Red dashed lines indicate the lower limit of normal for CD1 nu/nu mice. Similar data were found for mice bearing the human G3MB PDOX SJMBG3-12-5950 (data not shown).


A
vehicle (IV)
vehicle (OG)
d1 d2 d3 d4 d5
ribociclib (OG)
d1 d2 d3 d4 d5
gemcitabine acute (IV)
d1
gemcitabine (IV)
d1 d2 d3 d4 d5
gemcitabine (IV)
ribociclib (OG)
d1 d2 d3 d4 d5
B
MB002
vehicle
gemcitabine acute
gemcitabine
γH2AX
C
0.0357 *
% γH2AX positive cells
vehicle
gemcitabine acute
gemcitabine
D
MB002 (Short-term)
Z value
condition
CDC20
E2F8
BUB1
MKI67
RAD18
NEK2
E2F7
HMGB2
CDK1
RAD54L
TOP2A
CDK2
TTF2
AURKA
ACTL6A
CDC25C
DTYMK
DDX39A
CHEK2
CENPA
FOXM1
BARD1
EXO1
PTTG1
MYBL2
BIRC5
CHEK1
RRM2
PLK1
E
MB002 (Long-term)
condition
Vehicle
Gemcitabine
Ribociclib
Gemcitabine+Ribociclib
F
mRNA relative level
E2F7 E2F8 CDK2 TOP2A MKI67 PLK1 CDK1 AURKA BUB1 CENPA BARD1 HMGB2 EXO1 DTYMK RRM2
G
mRNA relative level
E2F7 E2F8 CDK2 TOP2A MKI67 PLK1 CDK1 AURKA BUB1 CENPA BARD1 HMGB2 EXO1 DTYMK RRM2

Supplementary Figure S8. DNA damage after gemcitabine treatment and expression of selected genes in a human G3MB tumor treated with gemcitabine and ribociclib. **(A)** Short-term treatment schedule with gemcitabine (60mg/kg) and ribociclib (100mg/kg). **(B)** Representative immunohistochemistry images of tumors treated with vehicle or gemcitabine and collected 4 hours (acute) or 4 days (on day 5) post-dose. Tumors were immuno-stained with an antibody against gamma-H2AX. (**C**) Quantification of the percentage of positive cells in each treatment (Mann-Whitney test, $p \leq 0.05$ (*)). **(D, E)** RNA-Seq derived expression heatmaps of selected genes in tumors from the four treatment groups after short-term and long-term treatment, respectively. **(F, G)** Relative mRNA level of a selection of genes was quantified by qRT-PCR in vehicle and combination treated tumors after short-term and long-term treatment, respectively (Mann-Whitney test, n=4 to 7 per group for short-term and n=4 to 6 per group for long-term, $p \leq 0.05$ (*), $p \leq 0.01$ (**)).


SJMBG3-12-5950 (Long-term)
Activity
Expression
A
B
Z value
4
2
0
−2
−4
condition
PLK1_SIG
CENPA_TF
CDC20_SIG
BUB1_SIG
UHRF1_TF
DDX39A_SIG
CDC25C_SIG
FOXM1_TF
CDK1_SIG
DTYMK_SIG
NEK2_SIG
MYBL2_TF
MKI67_SIG
HMGB2_TF
CHEK2_SIG
AURKA_SIG
PTTG1_TF
E2F7_TF
E2F8_TF
BIRC5_SIG
TOP2A_SIG
BARD1_SIG
CDK2_SIG
RAD18_SIG
RRM2_SIG
ACTL6A_SIG
TTF2_SIG
EXO1_SIG
CHEK1_SIG
RAD54L_SIG
PLK1
CENPA
CDC20
BUB1
DDX39A
CDC25C
FOXM1
CDK1
DTYMK
NEK2
MYBL2
MKI67
HMGB2
CHEK2
AURKA
PTTG1
E2F7
E2F8
BIRC5
TOP2A
BARD1
CDK2
RAD18
RRM2
ACTL6A
TTF2
EXO1
CHEK1
RAD54L
condition
Vehicle
Gemcitabine
Ribociclib
Gemcitabine+Ribociclib
C
Combination vs vehicle
E2F7
UHRF1
ESCO2
RFWD3
FOXM1
DTYMK
PBK
NEK2
NUSAP1
PTTG1
CDK1
HMGB2
CHEK2
DEPDC1
E2F8
MELK
TPX2
CDCA3
AURKA
TOP2A
MKI67
BARD1
DDX39A
BUB1
CENPA
BUB1B
BIRC5
PLK1
CDC20
CKS1B
MYBL2
SKP2
TYMS
HIST1H4A
CCNB1
CDC25C
6.44e−24 HALLMARK_G2M_CHECKPOINT
3.31e−10 HALLMARK_MITOTIC_SPINDLE
2.92e−06 REACTOME_DNA_REPLICATION
3.74e−26 HALLMARK_E2F_TARGETS
3.87e−19 REACTOME_CELL_CYCLE
1.11e−11 GO_CELLULAR_RESPONSE_TO_DNA_DAMAGE_STIMULUS
9.15e−07 GO_DNA_REPAIR
#2416 (Long-term)
Activity
Expression
D
E
Z value
4
2
0
−2
−4
condition
Plk1_SIG
Cenpa_TF
Rrm2_SIG
Dtymk_SIG
Uhrf1_SIG
Mki67_SIG
Birc5_SIG
Chek2_SIG
Cdc20_SIG
Rad54l_SIG
E2f8_TF
Aurka_SIG
Ttf2_SIG
Foxm1_TF
Top2a_SIG
Actl6a_SIG
Pttg1_TF
Rad18_SIG
Nek2_SIG
E2f7_TF
Hmgb2_TF
Bub1_SIG
Ddx39_SIG
Mybl2_TF
Exo1_SIG
Chek1_SIG
Cdc25c_SIG
Cdk1_SIG
Cdk2_SIG
Bard1_SIG
Plk1
Cenpa
Rrm2
Dtymk
Uhrf1
Mki67
Birc5
Chek2
Cdc20
Rad54l
E2f8
Aurka
Ttf2
Foxm1
Top2a
Actl6a
Pttg1
Rad18
Nek2
E2f7
Hmgb2
Bub1
Ddx39
Mybl2
Exo1
Chek1
Cdc25c
Cdk1
Cdk2
Bard1
condition
Vehicle
Gemcitabine
Ribociclib
Gemcitabine+Ribociclib
F
Combination vs vehicle
CCNB1
RRM2
SKP2
PIF1
TLK1
CHEK2
RNASEH2A
RAD54L
FOXM1
RAD18
ACTL6A
ESCO2
UHRF1
HMMR
MKI67
TBRG4
SNRPA3
E2F8
CENPA
PLK1
BIRC5
CDK4
CDC20
AURKB
PBK
DTYMK
TOP2A
AURKA
PRC1
NEK2
5.24e−14 HALLMARK_G2M_CHECKPOINT
2.37e−08 REACTOME_CELL_CYCLE
1.07e−15 HALLMARK_E2F_TARGETS
4.35e−04 REACTOME_DNA_REPLICATION
3.41e−07 HALLMARK_MITOTIC_SPINDLE
5.63e−06 GO_CELLULAR_RESPONSE_TO_DNA_DAMAGE_STIMULUS
3.18e−04 GO_DNA_REPAIR

Supplementary Figure S9. **Combination of ribociclib and gemcitabine decreases the activity of genes involved in cell cycle regulation and DNA damage response in human and mouse G3MB tumors**. Mice bearing human SJMBG3-12-5950 PDOX (**A, B , C**) or mouse tumor #2416 **(D, E, F**) tumors were treated with vehicle (black bar), gemcitabine (60mg/kg, blue bar), ribociclib (100mg/kg, red bar) or both (purple bar) following the treatment schedules presented in Fig. 2A and B, respectively. Tumors were harvested at moribund stage (long-term). NetBID was used to infer gene activity from RNA-seq data and a medulloblastoma-specific interactome. (**A, D**) Activity heatmaps of selected genes in tumors from the four treatment groups. (**B, E)** RNA-Seq derived expression heatmaps of selected genes in tumors from the four treatment groups. **(C, F)** Clustering of genes involved in down-regulated pathways.



Supplementary Figure S10. Long-term combination of ribociclib and gemcitabine increases the activity of genes conferring neuronal identity and the differentiation score in human G3MB. Mice bearing human PDOX SJMBG3-12-5950 were treated with vehicle (black bar), gemcitabine (blue bar), ribociclib (red bar) and combination of ribociclib and gemcitabine (purple bar) as for tumors in **Fig. 4**. Tumors harvested at moribund stage (long-term) were analyzed following the NetBID pipeline or for cell fate evaluation after RNA sequencing. **(A)** Activity heatmap of selected genes in tumors from the four treatment groups. **(B)** Volcano plot highlighting genes with differential activity between tumors treated with gemcitabine and ribociclib versus vehicle (network size > 25; $p < 0.01$ and logFC < -0.05 or logFC > 0.05). **(C)** Differentiation scores for each tumor computed from a single-cell RNA-seq dataset from the developing mouse cerebellum (Mann-Whitney test, gemcitabine + ribociclib versus vehicle). **(D)** G3/G4 differentiation score (Mann-Whitney test, gemcitabine + ribociclib versus vehicle). **(E)** Clustering of the tumors obtained from the preclinical studies with primary G3 and G4MB tumors.