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Supplementary material

Delineation of *MGMT* hypermethylation as biomarker for veliparib-mediated temozolomide-sensitizing therapy of Glioblastoma

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SUPPLEMENTARY METHODS

Western blotting: Protein lysates from fresh frozen tissue ~20-50 mg were obtained by pulverizing in kinase buffer. After 20 min incubation on ice, lysates were cleared by centrifugation and immunoprecipitation and immunoblotting performed as previously described (Gupta et al, Clin Can Res, 2014, 20: 3730-3741). To assess levels of γH2AX and Histone H3, acid extraction of nuclear proteins was performed. Briefly, the frozen tissue was triturated in ice-cold PBS/0.5% TritonX100, incubated on ice for 10 minutes, centrifuged and supernatant discarded. Remaining nuclei were extracted with 0.2 N hydrochloric acid and soluble nuclear proteins were recovered (Gupta et al, Clin Can Res, 2014, 20: 3730-3741). Protein (5-10 µg) were denatured and resolved on 5-20% acrylamide gradient gels, transferred on to PVDF membrane, blocked in 5% nonfat dry milk and then probed with appropriate primary antibodies diluted in 5%BSA (as listed below) overnight at 4ºC and followed by horseradish peroxidase-conjugated secondary antibodies. Signals were detected by using enhanced chemiluminescence method, and images captured in c300 imager (Azure Biosystems, Dublin, CA).

Primary Antibody	Source and cat #	dilution
Chk1 (rabbit polyclonal)	Millipore, cat# E04-207	1:1000
Chk2 (mouse monoclonal)	Millipore, cat# JBC1889929	1:1000
KAP1 (rabbit polyclonal)	Santa Cruz, cat# sc-136102	1:1000
PARP1 (rabbit polyclonal)	Cell signaling, cat# 9532	1:5000
PAR (rabbit polyclonal)	Trevigen, cat# 4336-BPC-100	1:5000
pChk1 (rabbit polyclonal)	Cell signaling, cat# 2341	1:1000
pChk2 (rabbit polyclonal)	Cell signaling, cat# 2661	1:1000
pKAP1 (rabbit polyclonal)	Abcam, cat# ab70369	1:1000
Histone-H3 (rabbit polyclonal)	Cell signaling, cat# 9717	1:1000
γH2AX (rabbit polyclonal)	Cell signaling, cat# 2577	1:1000
β-Actin (rabbit polyclonal)	Cell signaling, cat# 4967	1:5000

MGMT (goat polyclonal) R&D, cat# AF3794 1:1000

GBM subtype analyses: GBM subtype analysis was performed by Next Generation mRNA Sequencing. Briefly, single sample gene set enrichment was performed as previously described (Verhaak et al, Cancer Cell, 2010, 17: 98-110). To ensure comparability between the scores of the four subtype signatures, a resampling procedure was performed to generate null distributions for each of the four subtypes. After generating a large number of virtual samples, in which a gene expression level is assigned through random selection from the same gene in the remainder of the samples, the four ssGSEA scores for each signature were re-calculated. Following this procedure, 1 million random ssGSEA scores were generated for each subtype, resulting in a null distribution from which empirical p-values for the raw ssGSEA scores of each sample could be derived.

Analyses of p53, PTEN and EGFR alterations: Analysis of p53, PTEN or EGFR was performed as described previously (Brat et al, Am J Pathol 1999, 154: 1431-1438). Briefly, the purified genomic DNA (50-200ng) was PCR amplified in a 25-ul PCR reaction using specific primers and PCR conditions provided below- that were

***p53*:**

Pairs of primers: designed to flank exons 4-8 of TP53 gene were:

Exon-5: TTCAACTCTGTCTCCTTCCT / CAGCCCTGTCGTCTCTCCAG

Exon-6: TGGTTGCCCAGGGTCCCCA / CGGAGGGCCACTGACAACCA

Exon-7: AAGGCGCACTGGCCTCATCTT / GCACAGCAGGCCAGTGTGCAG

Exon-8: AGGACCTGATTTCCTTACTGC / TGCACCCTTGGTCTCCTCCAC

PCR conditions: 10 minute denaturation at 95°C, 35 cycles of 30 sec at 94°C, 30 sec at 55-60°C and 1 min at 72°C, and finally 10 min elongation at 72°C.

PTEN:

Paired primers listed below for each exon of PTEN amplifications were-

Exon-1: GCCACCAGCAGCTTCTGCC/ CTAAGAGAGTGACAGAAAGG

Exon-2: 1) ACCTTTTATTACTCCAGCTA/ TTCCTGTATACGCCTTCAAG

2) CAAACATTATTGCTATGGGA / ATCTTTTTCTGTGGCTTAGA

Exon-3: GCTCATTTTGTTAATGG / TAGAAGATATTTGCAAGC

Exon-4: ATAAAGATTCAGGCAATGTT / ATCGGGTTTAAGTTATACAA

Exon-5: 1) AGTTTGTATGCAGGCAATGTT / ATCATTACACCAGTTCGTCCC

2) GCAGCAATTCACTGTAAAGC / CAGATCCAGGAAGAGGAAAGG

Exon-6: 1) TTTTCAATTTGGCTTCTCTT / CATCTTGTGAAACAACAGTGCC

2) CCTGTTAAAGAATCATCTGG / CTGTTCCAATACATGGAAGG

Exon-7: 1) TTCCTGTGAAATAATACTGG / GAACTCTACTTTGATATCACC

2) TTCATGTACTTTGAGTTCCC / CCTTATTTTGGATATTTCTCCC

Exon-8: 1) TGCAAATGTTTAACATAGGTGA / TTCCTTGTCATTATCTGCACG

2) CAAGAAATCGATAGCATTTGC / ATACATACAAGTCAACAACCCC

Exon-9 1) TAAGATGAGTCATATTTGTGGG / CAGAGTCAGTGGTGTCAGA

2) TAGAGGAGCCGTCAAATCCA / CATGGTGNNTTATCCCTCTT

PCR conditions: 10 minute denaturation at 95°C, 50 cycles of 30 sec at 94°C, 30 sec at 48-55°C and 1 min at 72°C, and finally 10 min elongation at 72°C.

***EGFR* alterations**: Primers for differential PCR amplification of EGFR along with a reference gene β-globin were as follows-

1. EGFR: AGCCATGCCCGCATTAGCTC / AAAGGAATGCAACTTCCCAA)
2. β-globin: TGACTCCTGAGGAGAAGTCTGC / TCACCACCAACTTCATCACGT

PCR conditions: 10 minute denaturation at 95°C, 30 cycles of 2 min at 95°C, 1 min at 53°C and 1 min at 72°C, and finally 10 min elongation at 72°C.

After PCR, the entire reaction product was electrophoresed on a 2% agarose gel and stained with ethidium bromide and examined under Gel doc to document amplification of appropriately sized fragments. Purified PCR amplicon subjected to shrimp alkaline phosphatase (Exo-SAP) treatment as per instruction manual (New England BioLab Inc. Ipswich, MA) and submitted to Mayo Clinic Sequencing core facility for Sangers sequencing. The mutations were detected using Mutation Surveyor software V4.0.9 (Softgenetics, State College, PA).

Analyses of IDH1 and IDH2 mutations: IDH1 and IDH2 mutation analysis was performed as described previously (Jenkins RB et al, Nat Genet 2012, 44: 1122-1125). Briefly, the genomic DNA (200ng) was PCR amplified in a 25-ul PCR reaction using primers-

IDH1: AATATTCTGGGTGGCACGGTCTTC / CACATACAAGTTGGAAATTTCTG GGC

IDH2: CTGTCTTCCGGGAGCCCATCATCT / AGAGACAAGAGGATGGCTAGGCGAG

PCR conditions: 1 minute denaturation at 95°C, then 35 cycles of 1 min at 94°C, 1 min at 65°C and 1 min at 72°C, and finally 10 min elongation at 72°C, shrimp alkaline phosphatase (Exo-SAP) treated amplicons submitted for Sangers sequencing.

Mutations analyzed using Mutation Surveyor software V4.0.9 (Softgenetics, State College, PA).

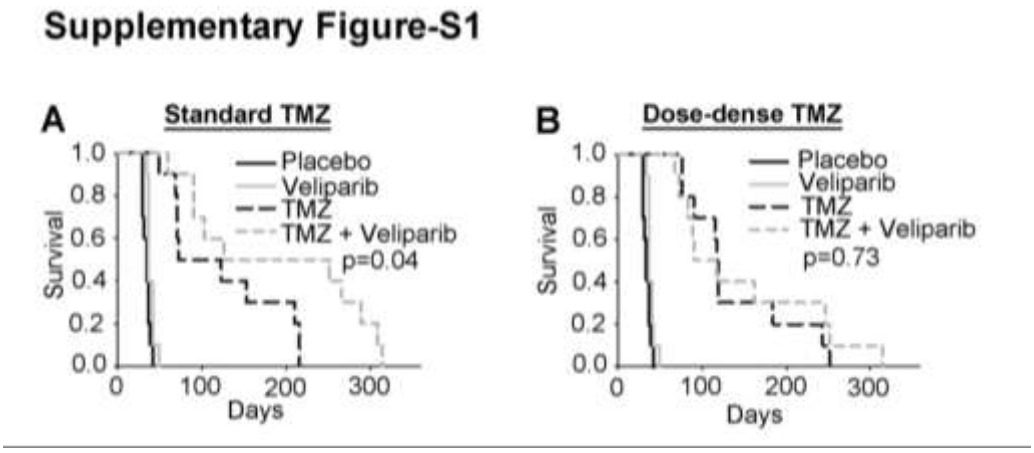
Analyses of TERT promoter mutations: Mutations in TERT promoter were analyzed as described previously (Eckel-Passow JE et al, New Engl J Med, 2015, 372(26):2499-508). Briefly, the TERT promoter (a 244 base pair (bp) segment spanning the C228 and the C250) was amplified from ~200ng genomic DNA using 2 pmol of the primers GCACAGACGCCCAGGACCGCGCT and TTCCCACGTGCGCAGCAGGACGCA using PCR conditions set at 94ºC for 1 minute and 35 cycles of 1 min at 94°C, 1 min at 65°C and 1 min at 72°C, and finally 10 min elongation at 72°C, 1 µl of the amplified DNA from this reaction was then used as template for a second PCR reaction with 2 pmol of the following primers-

CAGGAAACAGCTATGACCATGATTACGGCACAGACGCCCAGGACCGCGCT

CGTTGTAAAACGACGGCCAGTGAATTGTTCCCACGTGCGCAGCAGGACGCA

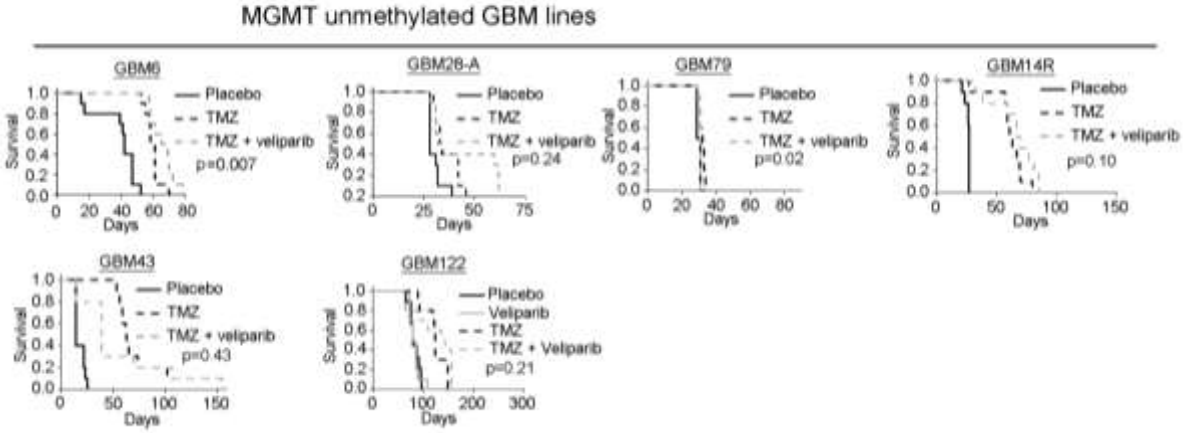
Using PCR conditions described above. Amplified DNA was then Sanger sequenced and C228T or C250T mutations were detected using Mutation Surveyor software V4.0.9 (Softgenetics, State College, PA).

SUPPLEMENTARY FIGURES S1-S5



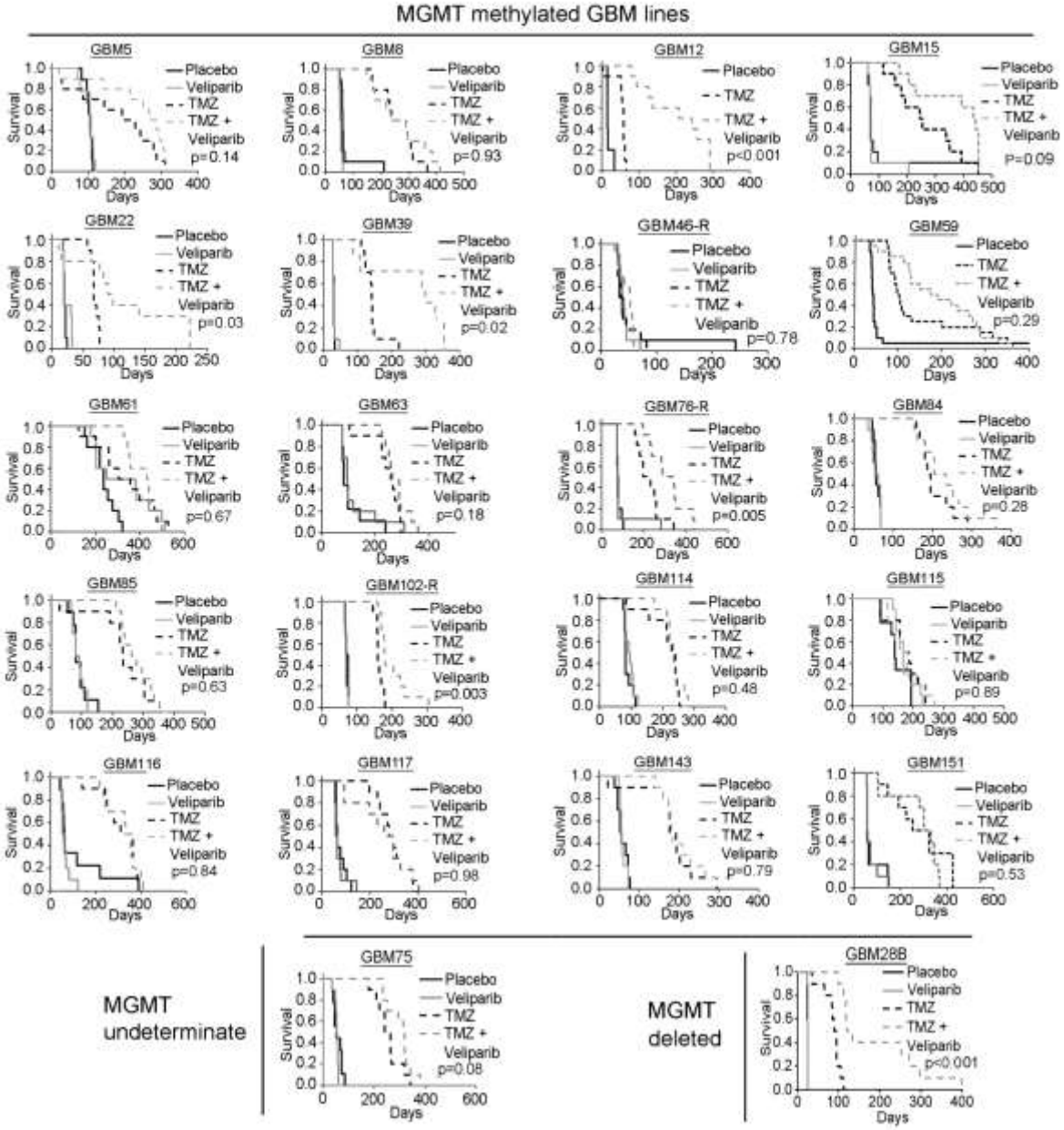
Supplementary Figure 1: Evaluation of different dosing schedules for placebo, TMZ alone or combined with veliparib in GBM 28B. Comparisons of standard versus dose dense TMZ schedules with or without concomitant veliparib with 10 mice randomized per group. Kaplan-Meier survival curves following- A) standard TMZ, 50 mg/kg ± veliparib, 12.5 mg/kg bid M-F of week 1 only, treatment was repeated every 28 days x 3 cycles; B) dose dense TMZ, 25 mg/kg ± veliparib, 12.5 mg/kg bid M-F of weeks 1-3 every 28 days x 3 cycles. The p-values denote a log rank test comparing survival in TMZ/veliparib versus TMZ alone. All statistical tests were two-sided.

Supplementary Figure S2



Supplementary Figure 2: Evaluation of veliparib, TMZ alone or TMZ/veliparib efficacy in MGMT unmethylated xenografts lines. Kaplan Meier graphs for indicated PDX models after treatment with 3 cycles of standard TMZ, 50 mg/kg ± veliparib, 12.5 mg/kg bid x 5 days every 28 days with 10 mice randomized per group for each xenograft line (except placebo group of GBM122 had 9 mice). P values provided are for the log rank comparison between TMZ/veliparib versus TMZ alone, and a p value **<**0.05 was considered statistically significant. All statistical tests were two-sided.

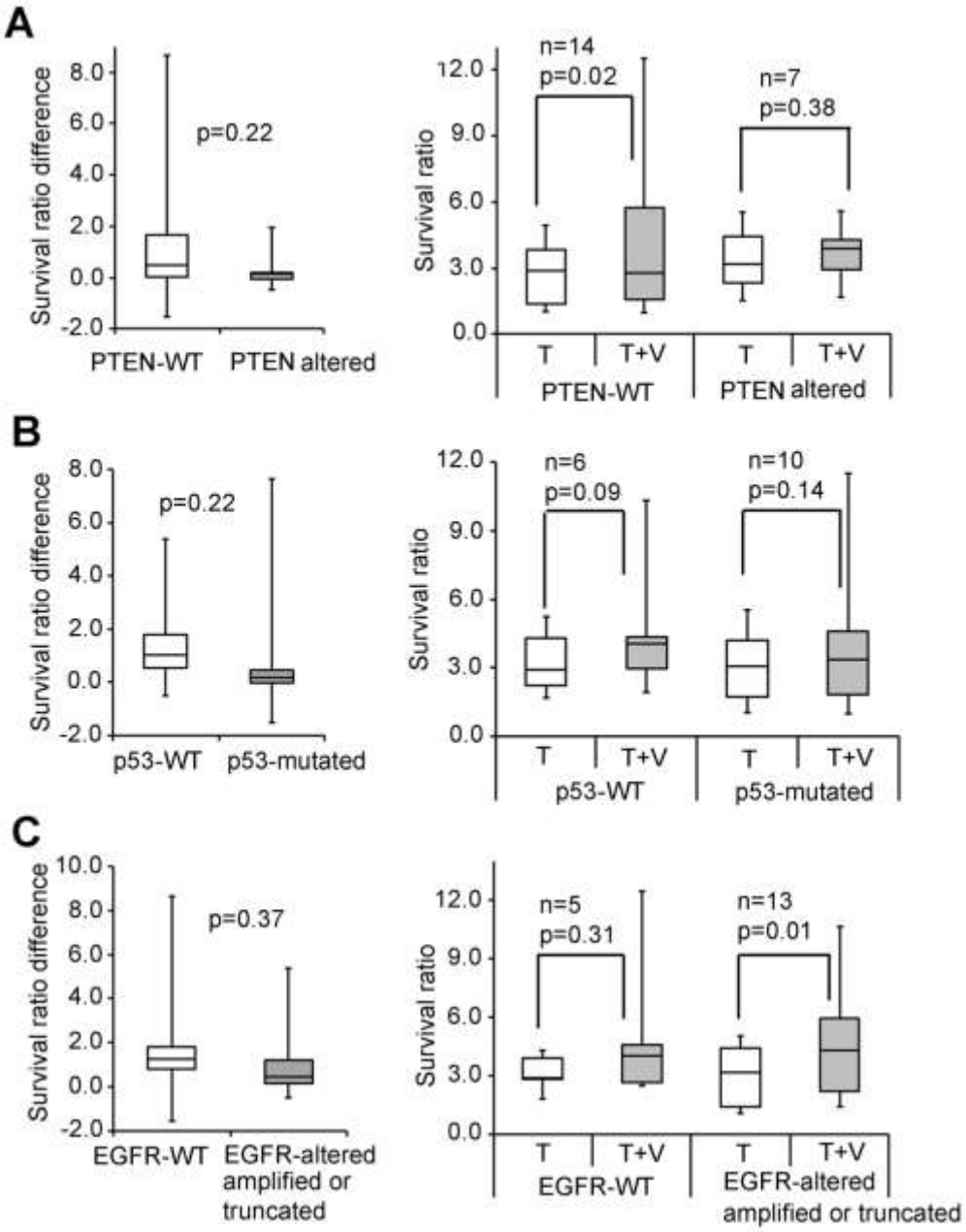
Supplementary Figure S3



Supplementary Figure 3: Evaluation of veliparib, TMZ or TMZ/veliparib efficacy in xenografts lines with MGMT hypermethylation, indeterminate MGMT status or deleted MGMT locus. Kaplan Meier graphs for indicated PDX models after treatment with 3 cycles of standard TMZ, 50 mg/kg ± veliparib, 12.5 mg/kg bid x 5 days every 28 days. 10 mice were randomized per group for each xenograft line (except that placebo, veliparib groups of GBM5, TMZ group of GBM8, placebo group for each of GBM63,

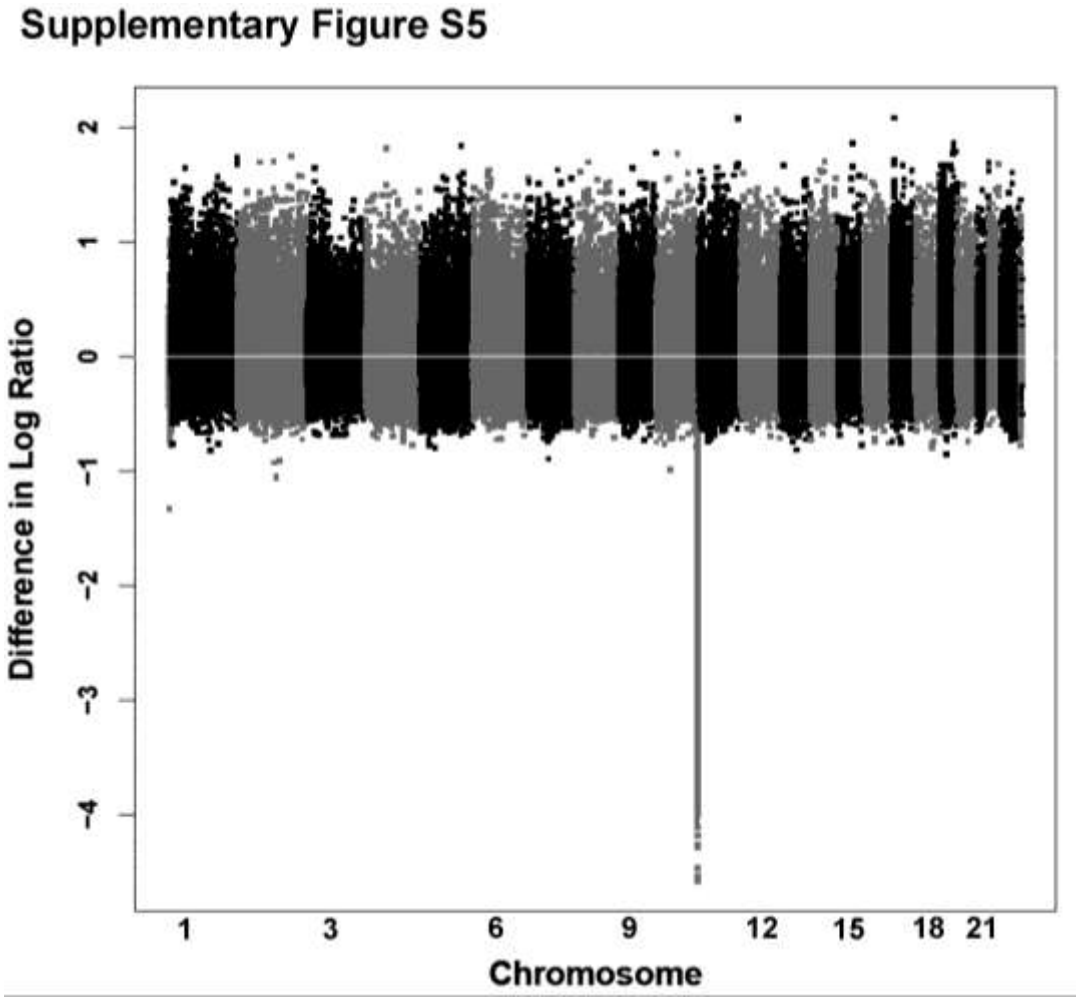
GBM85, GBM116 and GBM75 GBM lines had 9 mice; Placebo and TMZ+veliparib groups of GBM39 had 8 and 7 animals, respectively; GBM59 had 20 animal in each treatment group). P values provided are for the log rank comparison between TMZ/veliparib versus TMZ alone and a p value <0.05 considered statistically significant. All statistical tests were two-sided.

Supplementary Figure S4



Supplementary Figure 4: Comparison of TMZ/veliparib versus TMZ response in Mayo GBM PDX models based on PTEN, p53 or EGFR status. Ratio of median survival for treatments, TMZ/veliparib (T+V) or TMZ alone (T) relative to placebo (Survival ratio), and the difference of survival ratio for TMZ/veliparib minus survival ratio for TMZ alone (Survival ratio difference) are presented as box plots. Shown in the box plots are survival ratio difference (left) or survival ratios for the T vs. T+V (right) based on **A**) PTEN status, **B**) P53 status, **C**) EGFR status. PTEN-altered includes gene deletion

and/or mutation, and EGFR-altered includes amplification, truncation and/or mutation, p values denote Wilcoxon rank sum test (left) or paired signed rank test (right). All statistical tests were two-sided. [Abbreviations: PTEN= phosphatase and tensin homolog, WT= wild type, p53 = Tumor protein p53, EGFR = epidermal growth factor receptor, n = number of GBM lines, T = temozolomide, V = veliparib]



Supplementary Figure 5: Array comparative genomic hybridization (aCGH) analysis comparing GBM28A and GBM28B, shown in the image is the genome wide copy number variations on chromosome 1-23. Each color band represents separate chromosomes, and dots above zero represent increased copy number and dots below zero represent decreased copy number in GBM28B as compared to GBM28A.

Supplementary Table S1: Characteristics of various xenograft lines.

GBM	Patient characteristics				Molecular		EGFR†					IDH1
line	Age	sex	Diagnosis	Prior Treatment	subtype	MGMT*	Amplified	Mutation	PTEN ‡	TP53 §	TERT#	/ IDH2
GBM5	57	M	Newly		Proneural	Me	N	WT	WT	WT	C228T	WT/ WT
GBM6	65	M	Newly		Classical	U	N	VIII	WT	R273C	C228T	WT/ WT
GBM8	74	F	Newly		Proneural	Me	Amp	WT	HD	WT	C228T	WT/ WT
GBM12	68	M	Newly		Neural	Me	N	G719A	WT	Splice	C250T	WT/ WT
GBM14	57	M	Recurrent	RT, Gefitinib, BCNU	Classical	U	N	WT	INDEL	WT	C228T	WT/ WT
GBM15	NA	NA	Newly		Classical	Me	Amp	WT	W	NA	C228T	WT/ WT
GBM22	80	M	Newly		Classical	Me	N	WT	W	R273C	C250T	WT/ WT
GBM28	67	M	Newly		Mesenchymal	U		E602Q	G132D	M246T	C228T	WT/ WT
GBM39	51	M	Newly		Mesenchymal	Me	Amp	VIII	WT	WT	C250T	WT/ WT
GBM43	69	M	Newly		Mesenchymal	U	N		WT	F270C	NA	WT/ WT
GBM46	55	M	Recurrent	RT-Erlotinib, BCNU, TMZ	Mesenchymal	Me	Amp	VIII	WT	Q353K	C228T	WT/ WT
GBM59	82	F	Newly		Mesenchymal	Me	Amp	VIII	HD	WT	C228T	WT/ WT
GBM61	44	F	Newly		Neural	Me	Amp	WT	WT	WT	C228T	WT/ WT
GBM63	50	F	Newly		Proneural	Me	Amp	WT	INDEL	T253N, R175H	C228T	WT/ WT
GBM75	62	F	Newly		NA	indeterminate	Amp	WT	WT	NA	C228T	WT/ WT
GBM76	38	M	Recurrent	Erlotinib, RT-TMZ, Ad.-TMZ+ Erlotinib, SAHA	Classical	Me	N	A289T	I253N N69D	WT	C250T	WT/ WT
GBM79	71	F	Newly		Mesenchymal	U	Amp	INDEL	WT	C725Y, C242Y	C228T	NA/ WT
GBM84	49	F	Newly		Neural	Me	Amp	WT	WT	NA	C228T	WT/ WT
GBM85	77	M	Newly		Proneural	Me	NA	NA	V217G	C135Y	C228T	WT/ WT
GBM102	68	M	Recurrent	RT-TMZ, ad TMZ > Surg.	Classical	Me	NA	NA	HD	NA	C228T	WT/ WT
GBM114	68	M	Newly		NA	Me	NA	NA	WT	NA	C228T	NA/ WT
GBM115	75	F	Newly		Proneural	U	NA	NA	WT	NA	C228T	WT/ WT
GBM116	56	F	Newly		Mesenchymal	Me	NA	NA	HD	G265E	C228T	WT/ WT
GBM117	63	M	Newly		Classical	Me	NA	NA	HD	R175H	C228T	WT/ WT
GBM122	58	F	Newly		NA	U	NA	NA	HD	NA	C228T	WT/ WT
GBM143	67	M	Recurrent	RT-TMZ, ad TMZ > Surg.	NA	Me	NA	NA	ND	NA	C228T	NA/ WT
GBM151	51	F	Newly		Classical	Me	N	VIII	ND	NA	C228T	WT/ WT

*: MGMT- Me= MGMT hypermethylated, U= MGMT unmethylated; intermediate= MGMT status right at the cutoff;
†: EGFR- N= EGFR normal, Amp= EGFR amplified, INDEL= internal deletion, NA= information not available;
‡: PTEN- WT= wild type, HD= PTEN homozygous deletion, ND= PTEN analysis not done;
§: P53- WT= wild type, or R273C, M246T, F270C, Q353K, T253N, R175H, C725Y, C242Y, C135Y, G265E: specific amino acid changes in protein;
#: TERT - C228T, C250T= cytosine nucleotide at specified location in *TERT* promoter is changed to thymidine, NA= information not available.