Supplemental Figure Legends.

Supplemental Figure 1.
A. Flow cytometry analysis of A172 cells in SubG1 phase of cell cycle (DNA content <2n) when treated with 100uM temozolomide**.**
B. Left panel: western blot of transient expression of APNG compared to empty vector (EV). Densitometry analysis was undertaken to show elevated levels of APNG compared to EV transfected controls. Right Panel: Comparing IC 50 values as measured by cell viability of C6 and SKMG cells compared to C6 and SKMG cells transiently transfected with APNG.
C. Cleaved Caspase Assay of C6 cells and SKMG cells transiently transfected with APNG compared to empty vector controls.
D. Flow cytometry analysis of GBM6 cells in SubG1 phase of cell cycle (DNA content <2n) when treated with 100uM temozolomide. *P<0.01, **P<0.05.

Supplemental Figure 2.
A. Western blot demonstrating effectiveness of siRNA knockdown on APNG and MGMT in T98G cells**.**
B. Cell viability of T98G Cells were treated with varying amounts of temozolomide.
C. Cell viability of GBM6 cells treated with varying amounts of methyl methane sulfonate (MMS).
D&E. Measurement of activated Caspases 3&7 in A172 and T98G cells when treated with 100uM MMS.
*P<0.05 **P=0.001.

Supplemental Figure 3.
A. Anchorage independent growth assay of GBM6 cells grown in soft agar with 100uM temozolomide.
B. Summary of anchorage independent growth assay with colony numbers and colony size. *P<0.05.

Supplemental Figure 4.
A. Table summarizing number of mice in each treatment group. GBM explant number was assigned from a previous study(31).
B. Xenograft GBM explants were subjected TMZ treatment and compared within each treatment with respect to median overall survival based on combined APNG IHC expression and MGMT promoter methylation.
C. Xenograft GBM explants were subjected radiation treatment (RT) or placebo and compared within each treatment with respect to median overall survival based on combined APNG IHC expression and MGMT promoter methylation. *p<0.05.

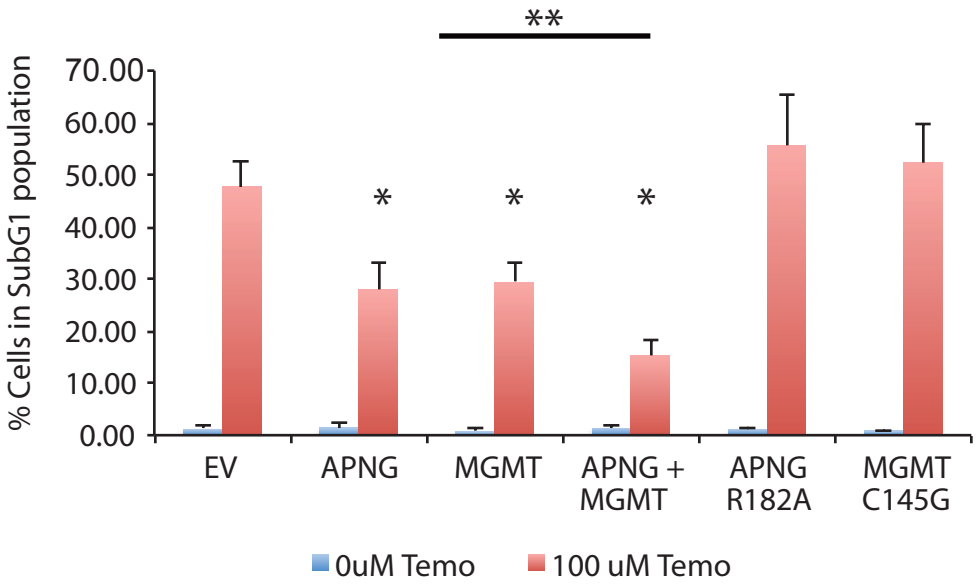
Supplemental Figure 5.
A. REMBRANDT data set: APNG (MPG) expression (RNA) and effect on survival. Unified gene was selected as a gene marker. Website access (January 18, 2011).
B. From Figure 5C, age is not statistically different among good and poor survivor groups.

Supplemental Figure 6.
A. Overall survival in patients with respect to APNG expression in the EORTC-NCIC TMZ clinical trial with respect to MGMT unmethylated promoter and given combinatorial therapy of TMZ and radiation. APNG positive GBMs had a poorer prognosis approaching significance (P=0.068).
B. Overall survival in patients with respect to APNG expression in the EORTC-NCIC TMZ clinical trial with respect to MGMT unmethylated promoter and given only radiation. APNG expression seems not prognostic for overall survival (P=0.312), however, too few patients were in this subgroup for appropriate statistical analysis.

Supplemental Figure 7.
A. MSP-PCR gel assessing methylation status of APNG for 24 GBM samples that were bisulfite treated.
B. Survival Curve based on IHC expression of GBM patients used in **A**.
C. Survival Curve based on MSP-PCR expression of GBM patients from **A**.

Supplemental Figure 1

A



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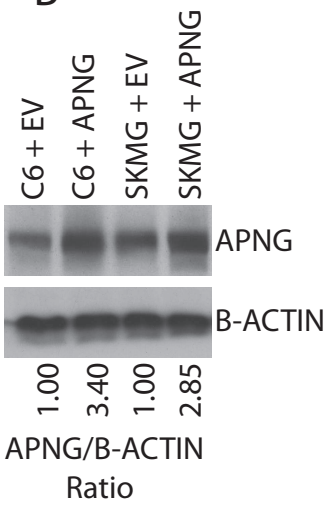

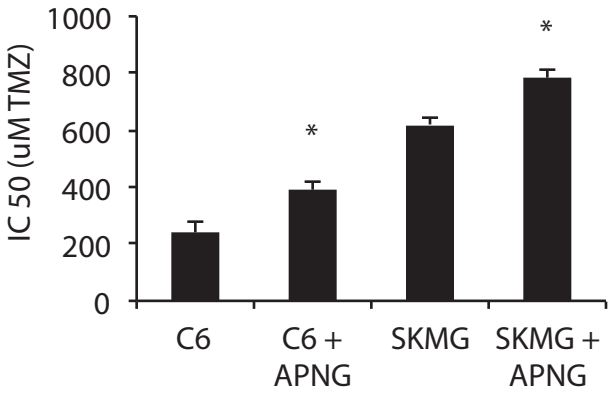

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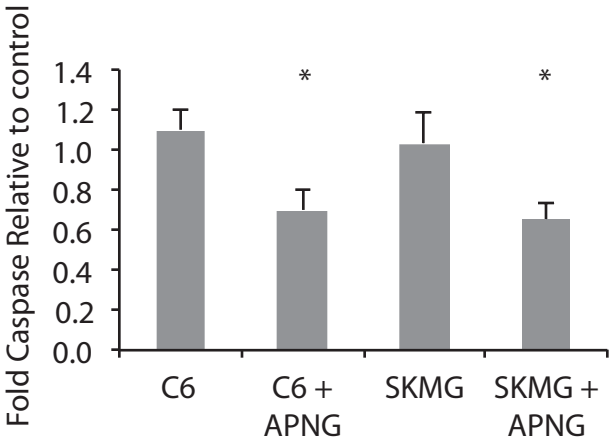

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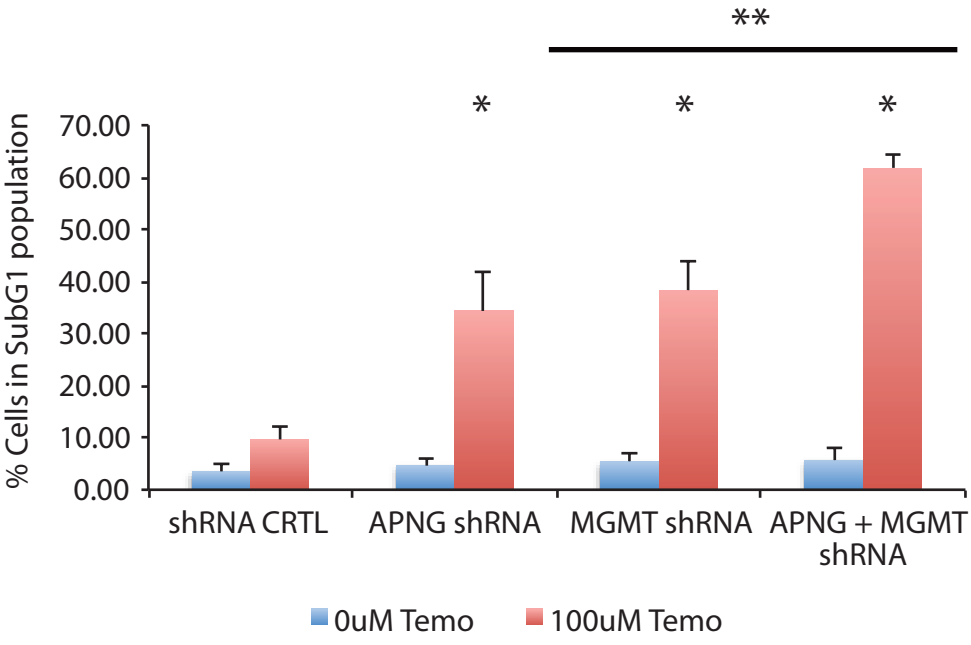
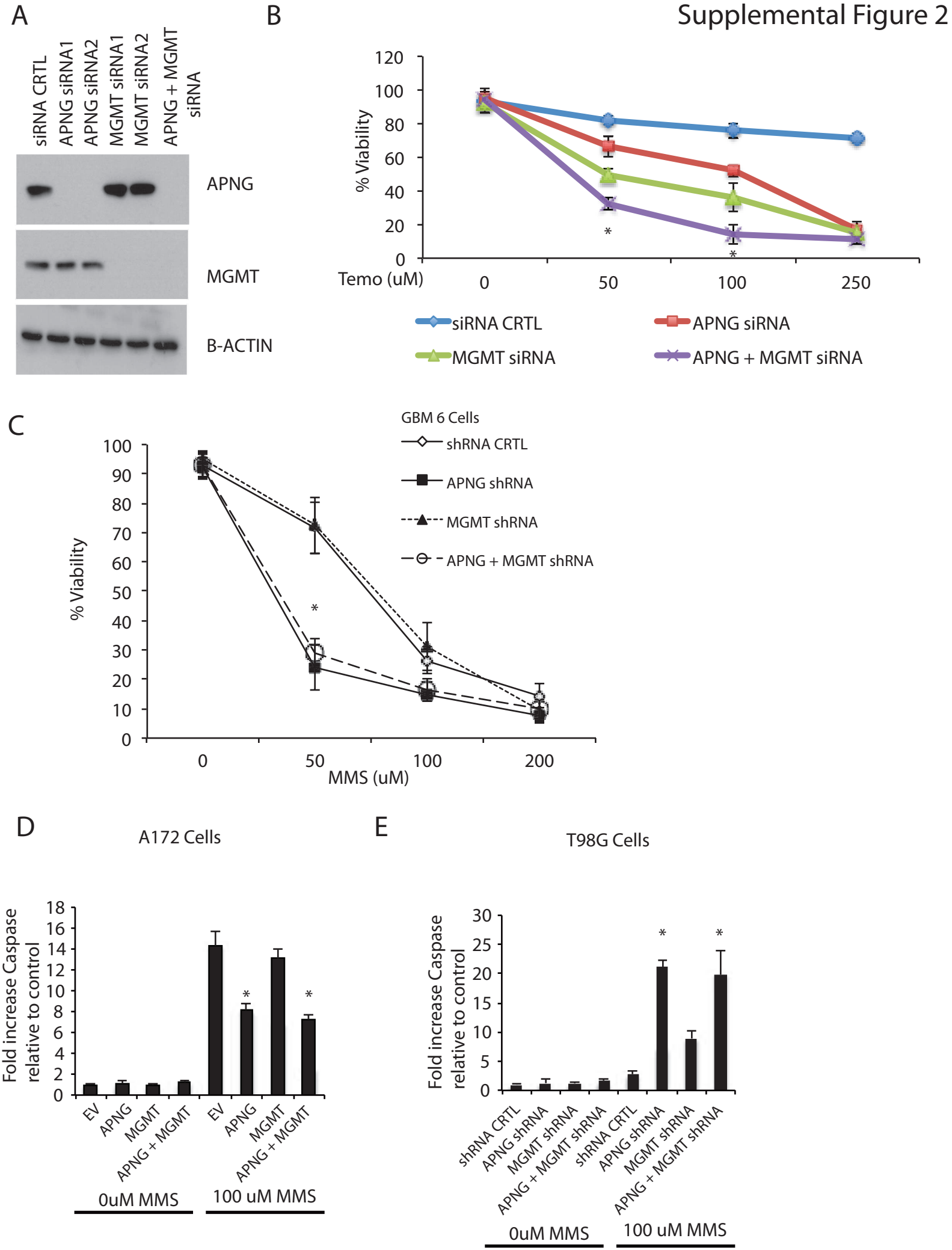
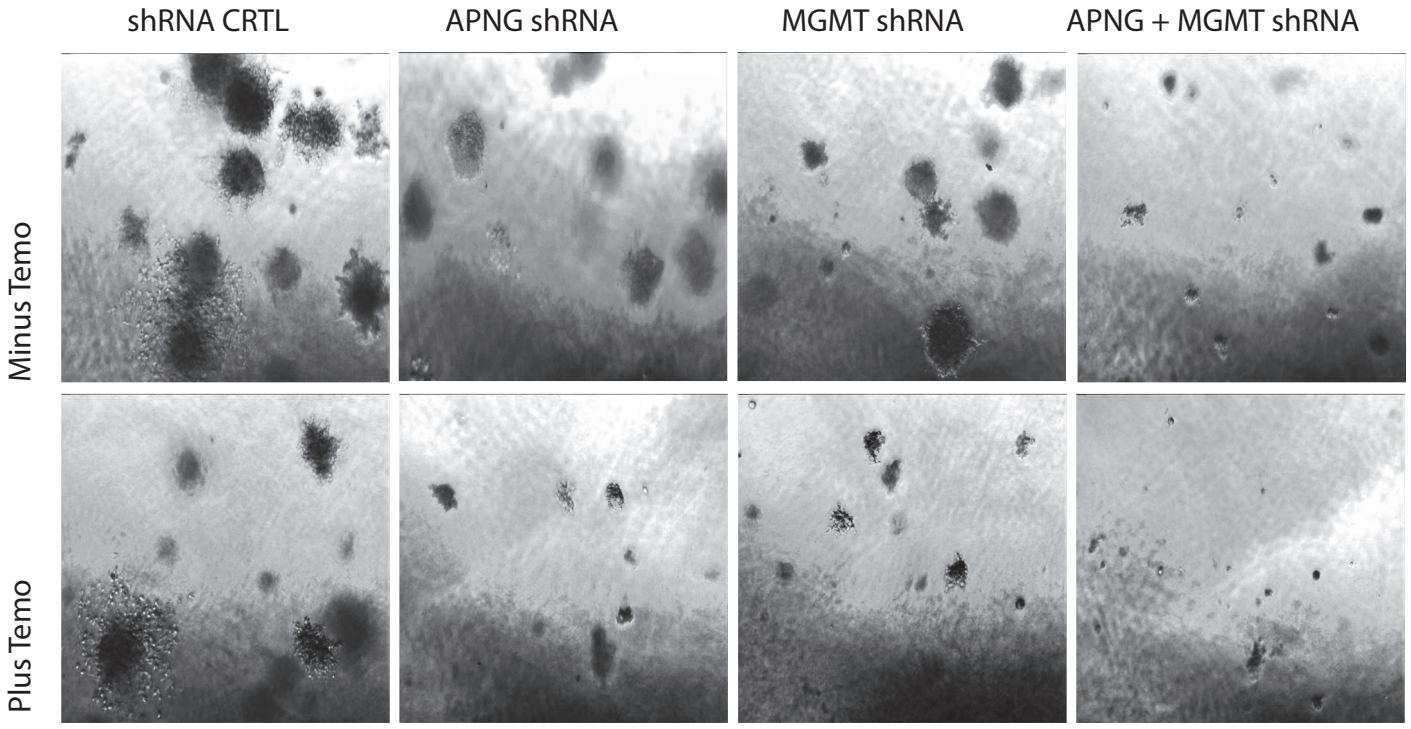


Supplemental Figure 2



A

Supplemental Figure 3



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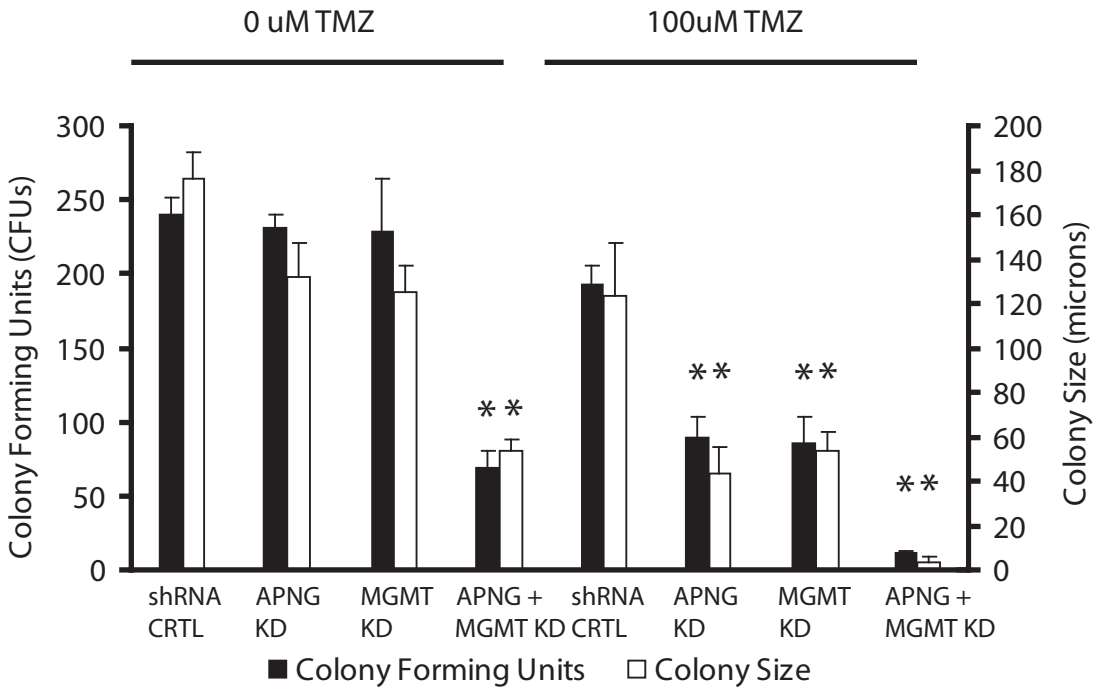
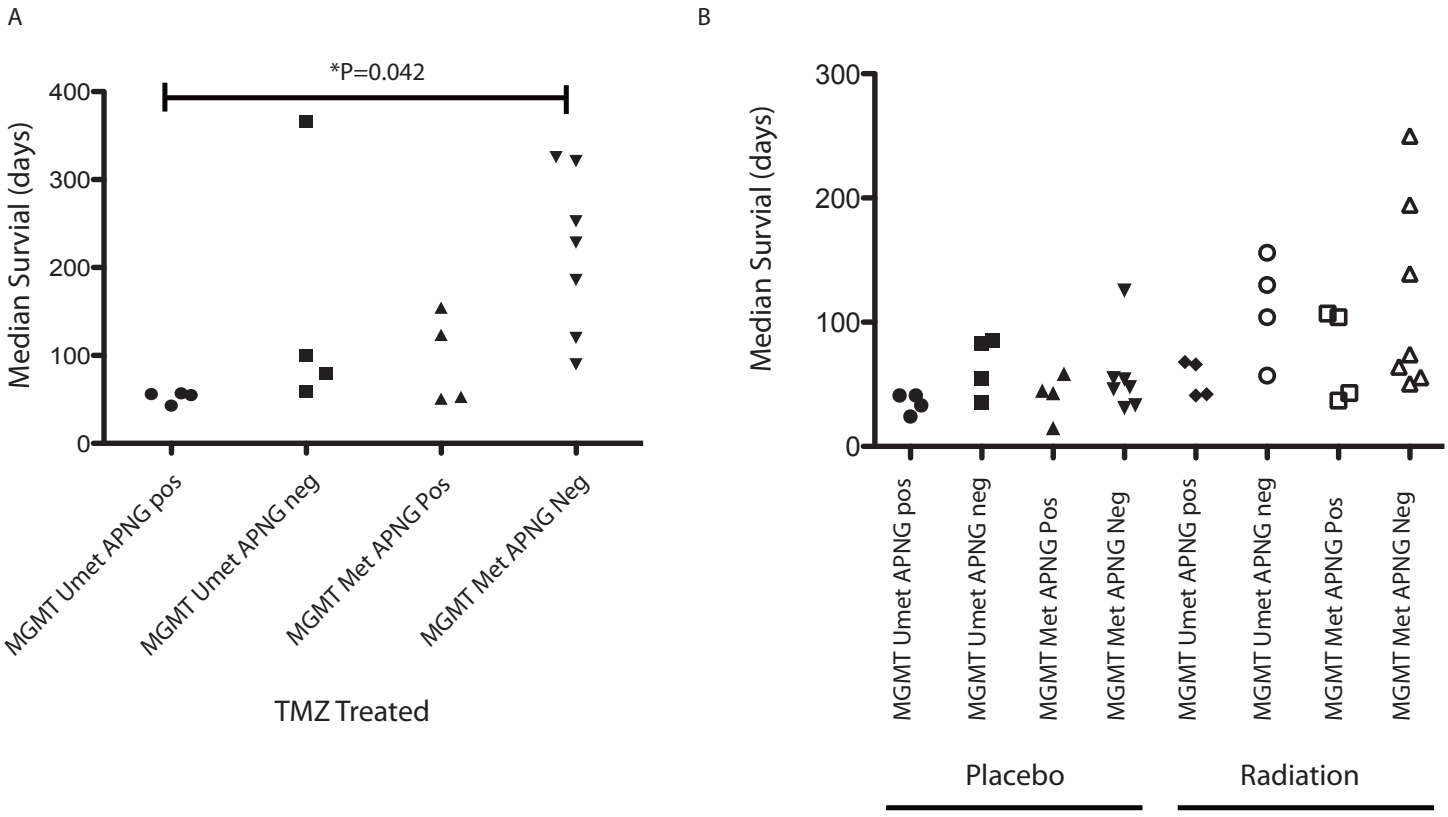
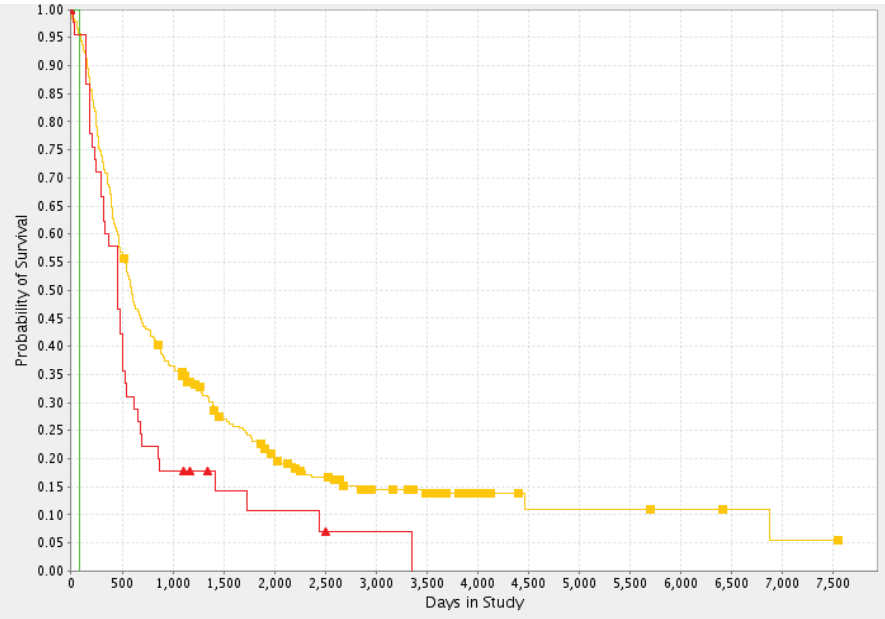



A
*P=0.042
Median Survial (days)
400
300
200
100
0
MGMT Umet APNG pos
MGMT Umet APNG neg
MGMT Met APNG Pos
MGMT Met APNG Neg
TMZ Treated
B
Median Survial (days)
300
200
100
0
MGMT Umet APNG pos
MGMT Umet APNG neg
MGMT Met APNG Pos
MGMT Met APNG Neg
MGMT Umet APNG pos
MGMT Umet APNG neg
MGMT Met APNG Pos
MGMT Met APNG Neg
Placebo
Radiation

A

Kaplan-Meier Survival Plot for All Glioma Samples with Differential APNG (MPG) Gene Expression



APNG Up-Reg. >= 2.0X (n=45)

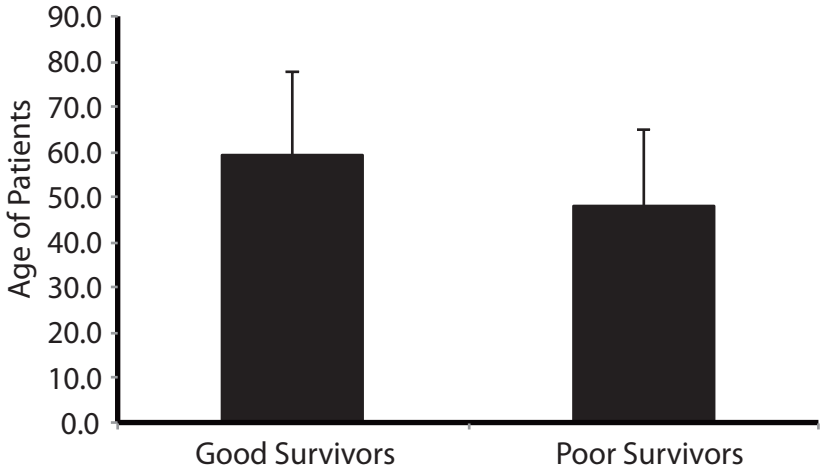
APNG Down-Reg. >= 2.0X (n=1)

APNG Intermediate (n=298)

MPG

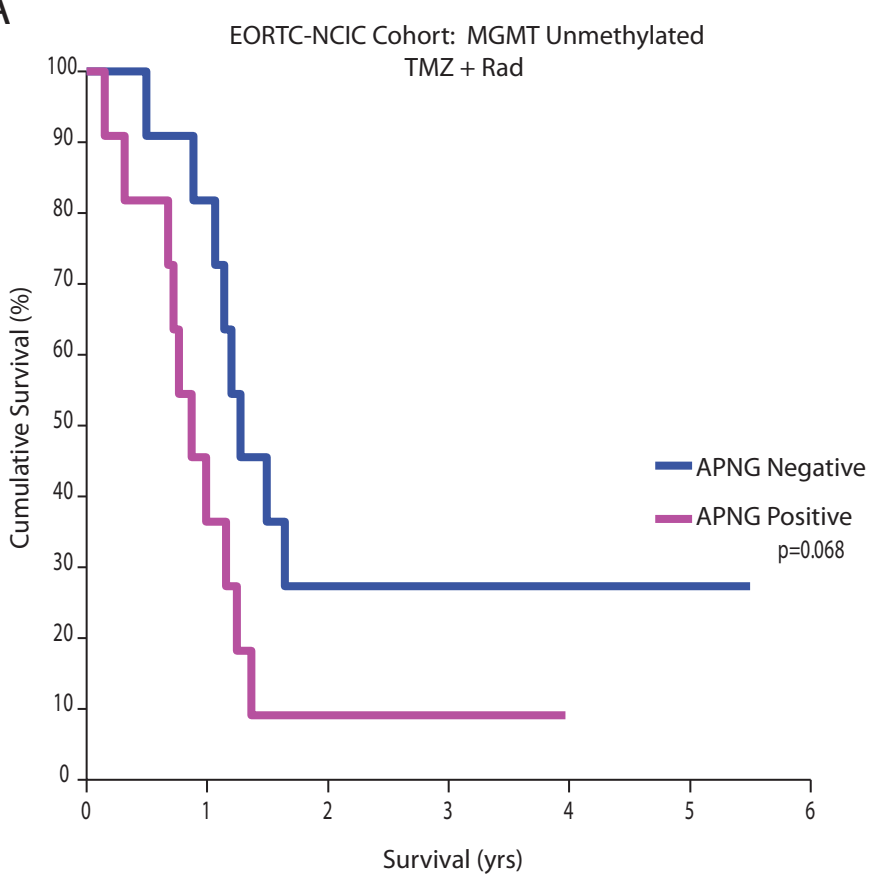
Up-Regulated vs. Intermediate: P= 0.0098262652

B



Supplemental Figure 6

A



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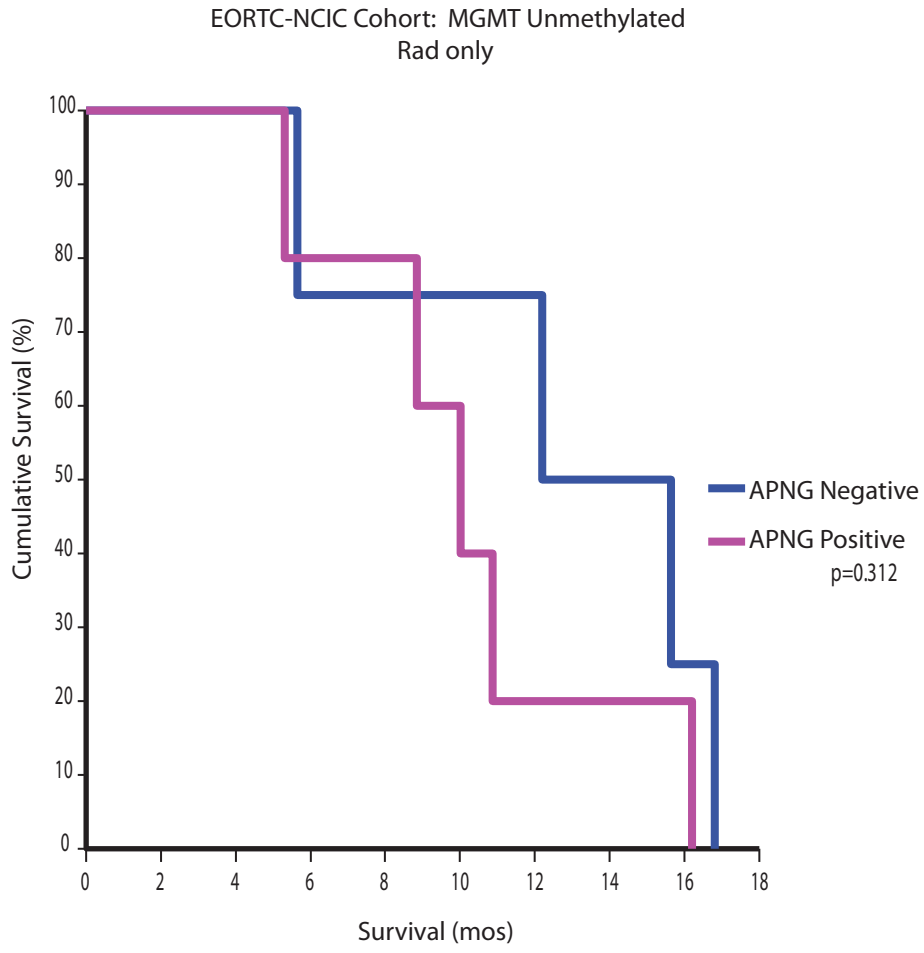

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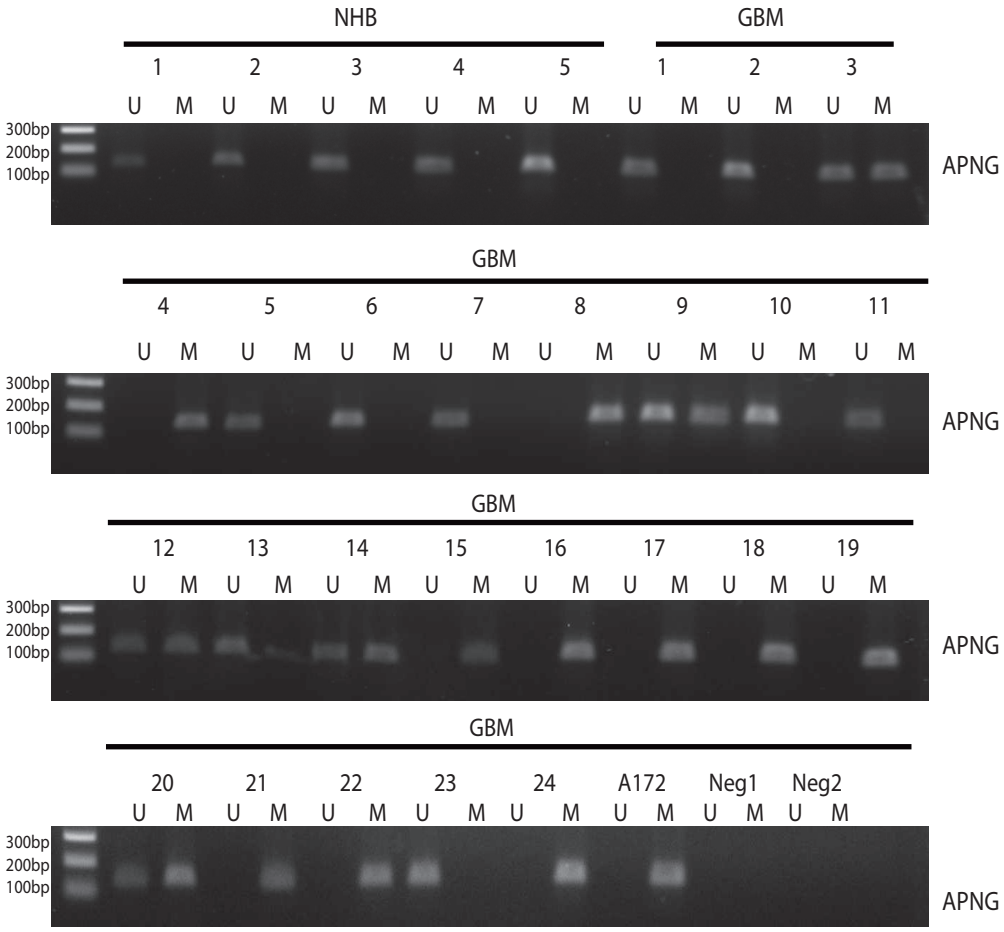
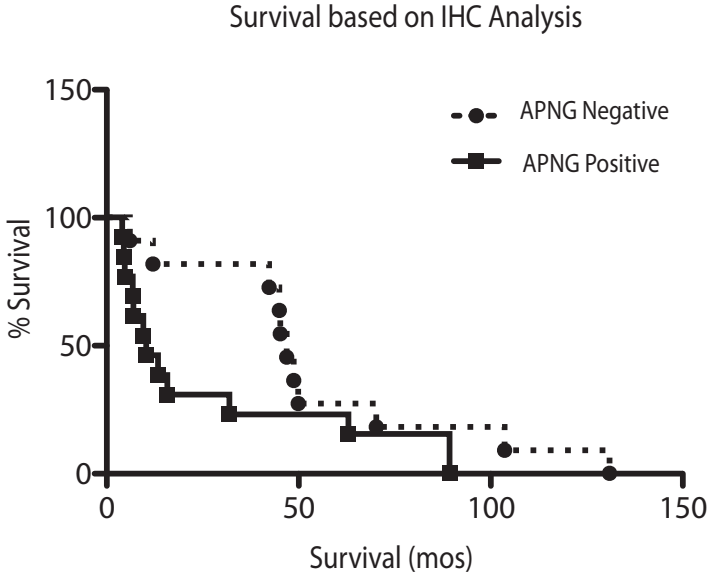


Summary

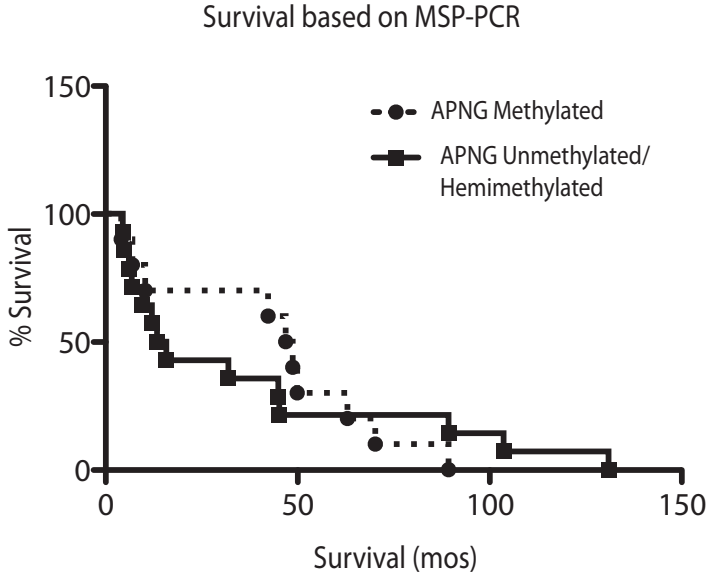
PCR Band	Total Number of Patients
Unmethylated	8
Hemimethylated	6
Methylated	10

B



p=0.038

C



p=0.415

Supplemental Table 1: Surivial study summary following placebo, TMZ, TMZ/RT treatment for 19 GBM xenograft lines

GBM	Rx (day)	MGMT	APNG	No of Mice	Survival (days) Placebo	No of Mice	Survival (days) TMZ	No of Mice	Survival (days) Radiation	No of Mice	Survival (days) TMZ/RT
5	22	M	Neg	8	55	10	321	10	139	9	349
6	37	U	Pos	8	41	6	57	7	42	4	98
8	44	M	Pos	8	58.5	8	123.5	8	107	8	189
10	36	U	Pos	7	41	7	55	9	66	6	76
12	11	M	Pos	10	15	10	53	10	37	10	85.5
14	24	M	Neg	10	33	10	186	10	55.5	9	188
15	29	M	Neg	10	54	10	252.5	9	250	10	252
16	23	M	Neg	8	47.5	8	325.5	8	64	8	343
22	38	M	Pos	8	43	8	154.5	8	43	8	325.5
26	65	U	Neg	8	82.5	8	99.5	7	130	7	130
28	30	U	Pos	8	33	7	56	7	68	8	82.5
34	46	U	Neg	8	85	8	366.5	8	156	7	391
36	44	M	Neg	8	125.5	8	228.5	8	194.5	8	307
38	45	U	Neg	9	55	10	80	9	57	9	75
39	28	M	Neg	10	31	10	120	10	50.5	10	202
43	22	U	Pos	5	24	8	43	7	41	5	55
44	31	U	Neg	9	35	7	59	9	104	10	113
46	30	M	Pos	10	45	10	51	9	104	10	114.5
59	39	M	Neg	9	46	9	90	9	74	10	254

Abbreviations: M = methylated promoter, U = unmethylated promoter, TMZ = temozolomide, RT = radiation therpay, Survival represents median surival, RX = day post tumour implantation when treatment was given. APNG expression was determined by IHC and scored as positive (pos) or negative (neg).

Supplemental Materials and Methods:

Primer sequences used in this study:

Gene	Use	Forward Primer	Reverse Primer
APNG	QRT	CATTTACGGCATGTACTTCTGC	ATGGTCTCCAGACCTTCCAG
HPRT1	QRT control	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
APNG	R182A mut primer	CTTGCGTCTTGCTGGCAGCACTGGAGCCCC	GGGGCTCCAGTGCTGCCAGCAAGACGCAAG
MGMT	C145G mut primer	CCATCCTCATCCCGGGCCACAGAGTGGTC	GACCACTCTGTGGCCCGGGATGAGGATGG
APNG	MSP umet primer	TTGTTGGTATTTTATTGGAAAATATGT	ATCATAAACCTAAATAAATCCTCAAA
APNG	MSP met primer	TCGTTGGTATTTTATCGGAAAATAC	GTCGTAAACCTAAATAAATCCTCGA
APNG	To Clone Promoter	GCGTCCACTTCCTGGATAAG	GCAGCACCTAAGTCCTCCTG
APNG	shRNA hairpin	CCGGCATCTATTTCTCAAGCCCAAACTCGAGTTTGGGCTTGAGAAATAGATGTTTTTG	
APNG	shRNA hairpin	CCGGGTACATCATTTACGGCATGTACTCGAGTACATGCCGTAAATGATGTACTTTTTG	
APNG	shRNA hairpin	CCGGCGACTTCCTAATGGCACAGAACTCGAGTTCTGTGCCATTAGGAAGTCGTTTTTG	
MGMT	shRNA hairpin	CCGGTCTTACCAGCAATTAGCAGCCCTCGAGGGCTGCTAATTGCTGGTAAGATTTTT	
MGMT	shRNA hairpin	CCGGCTTACCAGCAATTAGCAGCCCCTCGAGGGGCTGCTAATTGCTGGTAAGTTTTT	
MGMT	shRNA hairpin	CCGGTTACCAGCAATTAGCAGCCCTCTCGAGAGGGCTGCTAATTGCTGGTAATTTTT	

Quantitative Real Time Reverse Transcriptase PCR (qPCR)

Total RNA was isolated using an RNA extraction kit (RNeasy: Qiagen). cDNA was synthesized from 100 ng of total RNA using the Quantitect RT kit (Qiagen). Quantitative real-time PCR was performed on 40 ng of cDNA template in a final volume of 25 µl using the Chromo4 Real Time PCR detector (MJ Research) using SYBR green fluorescence. Real-time PCR data was analyzed using Opticon Monitor 3.1.3 analysis software and the delta delta CT method with HPRT1 as a reference gene. Primer sequences are shown in supplemental materials and methods.

Immunofluorescence

For immunofluorescence, 5 x 10^4 cells were grown on coverslips in 6-well media plates in D-MEM (Wisent) containing 10%FBS (Wisent). The following day, cells were fixed for 10 minutes in 4% Paraformaldhyde (PFA, Pierce Chemical Co., Rockford, IL) and permeabilized with 0.5% Triton X for 10 minutes. After removal of Triton X, cells were incubated with APNG monoclonal antibody (Santa Cruz Inc., 1:50) and detected using Alexa Fluor 488 labeled secondary antibodies (Invitrogen Inc). Cells were also stained with DAPI in the mounting medium). Images were captured on a Nikon E-600 microscope and analysed using Nikon ACT-1 software.

Site directed mutant constructs

Mutants were generated using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA Cat# #200521). Primer sequences are shown in supplemental materials and methods.

Methylation Assays (5-AZA treatment and MSP-PCR)

A172 cells were incubated in 5-aza-2'-deoxycytidine (AZA; Sigma, St. Louis, MO, USA Cat#A3656), or 5um AZA with 100nM Trichostatin A (Sigma Cat# T8552) for 96 hours. DNA extracted from cell lines or biopsies was bisulfite treated and purified using the EZ DNA methylation kit (Zymogen, BaseClear, Leiden, the Netherlands): sequencing was carried out as previously described (13). GBM samples were reviewed by our neuropathology co-author, Kenneth Aldape, MD., to ensure accuracy of diagnosis, as well as quality control to minimize normal tissue contamination. Tumour sections were cut, deparaffinized and DNA was isolated using a commercially available kit (Epicentre, Madison, WI). Genomic DNA (1ug) was then bisulfite converted using the Zymo EZ96 DNA methylation kit (Zymo Research, Orange, CA; cat # D5004), and then amplified by the fluorescence-based, MethyLight real-time PCR strategy as described previously (43). Primers and probes used for the real-time PCR have previously been described (33).

REMBRANDT Data

Data was obtained from the Repository of Molecular Brain Neoplasia data (REMBRANDT) site accessed on Jan 18 2011.

Soft Agar Assays

Control and variously modified A172 and GBM6 cells were counted and $1x10^4$ cells were mixed with an equal volume of 0.75% soft agar and plated onto 0.5% agar in 6 well culture plates,

followed by 4ml of DMEM containing 10% FBS. Colonies were counted 14 days later. Soft agar assays were repeated in triplicate.

Activated Caspase assays

Caspase 3/7 activity levels were measured using the Apo-One® Homogeneous Caspase 3/7 assay (Promega Corp., USA) that provides a profluorescent substrate and a cell lysis/activity buffer for Caspase 3/7 (DEVDase) activity. After induction of apoptosis, 100uL of Apo-One was added to each well, incubated for 3 hours and then fluorescence levels measured (485Ex/527Em).

Promoter Assay

The APNG promoter was cloned from a BAC clone (CTD-3077J14 obtained from The Centre of Applied Genomics at the Hospital for Sick Children) containing part of human chromosome 16p. Amplification of the 1kb fragment by PCR used the Phusion high fidelity polymerase (Thermo Scientific) and 50ng of BAC DNA in a 20ul reaction containing DMSO as per the manufacturer's protocol. An initial cycle of 98^{o}C (30sec) was followed by 35 cycles of 98^{o}C (15sec) and 72^{o}C (45sec), a final extension of 72^{o}C (10min) and 4^{o}C hold. The PCR product was gel purified, phosphorylated using polynucleotide kinase (New England Biolabs, Toronto Canada) and cloned into EcoRV-digested and shrimp alkaline phosphatase-treated pGL4.10 (New England Biolabs).

In vitro methylation was as follows, 6ug of APNG promoter was cut with Kpn1 (Fermentas, Toronto Canada) and HindIII (Fermentas). Vector and cut insert were gel extracted. The insert was treated with Sss1 (New England Biolabs) to methylate CpG sites in vitro or the insert was mock treated (non-methylated). Reactions were carried out as per manufactures instructions and products were purified using PCR cleanup columns (Qiagen). Sss1 treated insert or non-treated

insert was re-ligated back to vector isolated from initial digest with T4 Ligase (10 Weiss Units, Fermentas). 2ug of treated or mock treated APNG-luciferase constructed was transfected into 6 well plates containing $2x10^5$ U87 or T98G cells with 100ng of Renilla Luciferase co-transfected and used as a normalizing control. Cells were harvested 48h post transfection and luminesce was measured as previously described (44). Transfections were performed in triplicate with standard error of the mean reported.

N7-methylguanine Assay

An immuno-slot-blot method was used to quantify N7-meG in DNA (45). Genomic DNA was extracted from GBM cell lines using the Qiagen genomic DNA extraction kit (Qiagen), except that the proteinase K and ribonuclease A digests were carried out overnight at 4°C, followed by 1 h at 37°C. Sonicated DNA from samples and MNU-methylated calf thymus-DNA standards were alkali treated (70 mM NaOH, at 37°C for 30 min) to open the imidazole ring of N7-MeG, neutralized, and then heat-denatured (5 min, 100°C) and cooled on ice for 10 min to generate single stranded DNA. Samples were then immobilized on nitrocellulose (NC) filters in a slot-blot apparatus. The filter was baked at 80°C for 90 min and then blocked for 1 h with a 5% fat-free milk solution. Primary rabbit polyclonal antibody diluted 1:10 000 in PBS-Tween (0.1% v/v) containing 0.5% milk was applied overnight at 4°C. The NC filters were washed and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1:10 000 in PBS-Tween (0.1% v/v) containing 0.5% milk for 1 h at room temperature. Binding was detected by chemiluminescence and X-ray film and the band intensities were quantified by optical scanner (Storm 860; Amersham Biosciences, Piscataway, USA). The mean coefficient of variation of the triplicate ISB assays was 9.8% for both cases and controls. The limit of quantitation was ~0.31 fmoles/ug DNA.