SUPPLEMENTAL MATERIALS AND METHODS

Cell lines and cell culture.

Primary tissue cultures were derived with consent from patient tumor samples obtained by the Duke Brain Tumor Center. These patient-derived cultures were maintained in human neural stem cell (NSC) media (STEMCELL, cat# 05751), supplemented with EGF, FGF, and Heparin and plated onto laminin coated plates. All experiments were performed within the first 20 passages. The human U251MG cell line (Sigma, cat #09063001) and the transformed astrocyte model (see below; Lonza, cat #CC-2565) were cultured using the same medium conditions. The U-138 MG (ATCC HTB-16) cell line was maintained in Minimum Essential Medium Eagle (Sigma cat #M4655), supplemented with 10% fetal bovine serum (FBS; Corning cat #35-010-CV), Sodium Pyruvate (Thermo cat #11360), and non-essential amino acids (Thermo cat #11140). All cell lines were maintained in a humidified atmosphere at 37°C and with 5% CO_2. Cells were tested for Micoplasma at the Duke Cell Culture Facility, and cell line authentication was performed on each cell line using short tandem repeat (STR) profiling to match derivative cell lines to parental primary tissue culture and to confirm the identity of U251MG parental and *MTAP* knockout clones.

Drug Sensitivity Assays

Cell viability was analyzed using CCK-8 (DojinDo, cat# ck04). Cells were plated out in 96-well laminin-coated plates (2,000 cells/well in 100 µl medium), and were allowed to adhere for at least 12 hours prior to adding drugs. At each experimental time point, 10 µl of CCK-8 solution was added to each well and incubated for 4-12 hours. Plates were read at 450nm by a multimode microplate reader (Infinite M200 PRO; Beckman). L-Alanosine was purchased from Santa Cruz Biotechnology (Cat# 207791) or Medkoo Biosciences (Cat#200130) in powder form and was suspended either in 1N NaOH (*in vitro* Experiments) or in normal saline (for *in vivo* treatment). Temozolomide was purchased from Medkoo Biosciences (Cat# 100810) and was suspended in DMSO.

Plasmid construction and generation of derivative cell populations.

The CRISPR system was used for knockout of *MTAP* in U251MG. The plasmid pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid #48138). Double-stranded oligonucleotides were inserted into restricted enzyme BbsI-linearized PX458 to construct plasmids for CRISPR targeting of human *MTAP*. For transient plasmid transfection, plasmids (2 plasmids at 1:1 ratio for achieving the desired gene

deletion/mutations) and Transfex (ATCC, cat# ACS-4005) were mixed and used for cell transfection according to manufacturer's instructions. Three to four days after the transfection, green fluorescent protein–positive (GFP+) cells were sorted via fluorescence-activated cell sorting (BD FACSVantage SE cell sorter, Duke Cancer Institute) to obtain the GFP+ population. Sorted cells were plated at single-cell densities and allowed to expand for 21 days, at which point DNA was prepped from each colony to screen for a deletion in *MTAP* (exon 1-exon 3) using PCR amplification across the deleted region as shown in **Supplementary Fig. S4**. MTAP overexpression in *MTAP*-null cell lines was achieved using the retroviral vector, MigR1 (1).

Lentiviral CRISPR/CAS9 was used to knockout *MTAP* in the transformed astrocyte model. LentiCRISPR v2 plasmid was purchased from Addgene (cat #52961). The same guides as with pX458-sgRNA-MTAP construct were used and the cloning protocol was based on Zhang Feng Lab protocol. Lentivirus was generated by the Duke RNAi facility. The transformed astrocytes were infected using 3 multiplicity of infection (MOI) in a 6-well plate with 8 ug/mL of polybrene. Fresh media was added the following day. No selection methods were used.

Preparation of RNA and RT- qPCR.

Total RNA was extracted using quick-RNA mini prep kit (Zymo Research, cat# 11-328) or Allprep DNA/RNA/Protein mini kit (Qiagen, cat #8004) following the manufacturer's protocols. Concentration of RNA was determined by Nanodrop Lite Spectrophotometer (Thermo Scientific). For gene expression analysis, reverse transcription was performed to convert total RNA into single-strand complementary DNA (cDNA) using the RNA to cDNA EcoDry Premix (Clontech, cat #639547). Subsequently, qPCR was performed following the aforementioned qPCR procedure. Each reaction included a cDNA template equivalent of 10 ng of total RNA, using the following program: 95°C, 3 minutes; 95°C, 15 seconds and 63°C 25 seconds; 95°C 15 seconds and 61°C 25 seconds; 40 cycles of 95°C 15 seconds and 60°C 25 seconds, then a standard dissociation curve from 65°C to 95°C of 5 seconds/ 5 degree increment. The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was used as the internal control for RT-qPCR as it was shown to be a reliable internal control gene for gliomas (2).

Oligos and primers.

All oligos and primers used for the study were synthesized by IDT or Eton Bio and are listed in **Supplementary Table 4**, below. Quantitative PCR was performed using KAPA SYBR Fast 2x Universal master mix (KK4602) according to the manufacturer's protocols on a BIO-RAD CFX96 Real-Time System.

Supplementary Table 4. Oligos and Primers

Assay	Gene	Primer Name	Sequence (5'-3')
RT-PCR	GAPDH	hGAPDH_RT_ F	TTGCCATCAATGACCCCTTCA
		hGAPDH_RT_ R	CGCCCCACTTGATTTTGGA
	EGFR	EGFR_RT_2F	CCCACTCATGCTCTACAACCC
		EGFR_RT_2R	TCGCACTTCTTACACTTGCGG
	MTAP	MTAP_RT_F	TGATGGAAGTCATTCTTGTGCC
		MTAP_RT_R	GGAGGGTTTCTGACCATTCTGTG
	PROM1	PROM1_RT_1F	CAGAGTACAACGCCAAACCA
		PROM1_RT_1R	AAATCACGATGAGGGTCAGC
	GFAP	hGFAP_RT_2F	GCACGCAGTATGAGGCAATG
		hGFAP_RT_2R	TAGTCGTTGGCTTCGTGCTT
	TUBB3	TUJ1_RT_1F	GGGCATCTCTTGAGAACAAA
		TUJ1_RT_1R	GCACGTACTTGTGAGAAGAG
	NES	Nestin_RT_1F	CAGCGTTGGAACAGAGGTTGG
		Nestin_RT_1R	TGGCACAGGTGTCTCAAGGGTAG
	GALC	hGALC_RT_1F	CCAGAGCCCTATACGTTCTCA
		hGALC_RT_1R	CCATGGCAACCCAATGAGTG
Copy Number qPCR	Line1	hLine1_89bp_F	TCACTCAAAGCCGCTCAACTAC
		hLine1_89bp_R	TCTGCCTTCATTTCGTTATGTACC
	EGFR	hEGFR_CNexon6_F	AGCGACTGCCTGGTAAGATG
		hEGFR_CNexon6_R	AATTGACAGCTCCCCCACAG
	MTAP	hMTAP_CNexon8_F	ATGTTTCCTGCGTCCTCACT
		hMTAP_CNexon8_R	GATAGGCAAGGGCATGAAAG
Methylation Specific qPCR	PROM1	Forward	CTACAGGAAATGGATGCTGT
		Reverse	GTGGGGATCTGCCTCAGTC
		Probe	/56-FAM/GCTCAGCCGGTCCAATCAGA-/3IABkFQ/
CRISPR sgRNA	MTAP	hMTAP_sgRNAex1_sense	CACCGGCTCATCTCACCTTCACGG
		hMTAP_sgRNAex1_antisense	AAACCCGTGAAGGTGAGATGAGCC
		hMTAP_sgRNAex6_sense	CACCGTCTGCCCGGGAGCTAAAACG
		hMTAP_sgRNAex6_antisense	AAACCGTTTTAGCTCCCGGGCAGAC
Cloning	MTAP	hMTAP_ORF_F	ttaataCTCGAgcagacatggcctctggca
		hMTAP_ORF_R	tatgccGAATTCttaatgtcttggtaataaaacagaa
MTAP KO screening primers	MTAP	hMTAPscreen#1	GGGAGGAAGAGGAGGAGTCA
		hMTAPscreen#2	GGGATCATTTGCAAGCGTA
		hMTAPscreen#3	AAGAAATCAACTTGGTAAACATTGG
		hMTAPscreen#4	CACCCAGAAACCCATGCTAT

Reprogramming and transformation of normal human astrocytes.

Normal human astrocytes (Lonza, cat #CC-2565) were obtained from Duke Cell Culture Facility. Reprogramming and transformation were performed as previously described (3). Briefly lentivirus cocktails of the 4 core factors (OMRP) were prepared via packaging in 293FT cells in NSC media supplied with 3% FBS. Normal human astrocytes within passage 6 were incubated overnight in the lentivirus cocktail in the presence of 6 μg/ml of polybrene and were subsequently cultured in the same NSC media with 3% FBS. Cells were transduced with CRISPR lentivirus for *MTAP* knockout 7-10 days after initial transduction. Otherwise, DMSO (1:6000) or the MTAP inhibitor, MTDIA (MedKoo Biosciences, Inc, cat #407244) was added to the media (1 μg/ml or 3 μg/ml of MTDIA) for 2 more weeks, at which point cultures were switched to FBS-free NSC media, in the presence of vehicle control (DMSO) or MTDIA. Cells were used for RT-PCR analysis of gene expression or FACS analyses starting from week 4 post transduction (and 3 weeks after starting MTDIA incubation). All antibodies used for FACS analyses were from Miltenyi, including anti-CD133-PE (cat #130-080-801), anti CD133-APC (cat #130-098-829), Annexin V-PE (cat #130-108-112), IgG1-PE (cat #130-092-212), and IgG1-APC (cat #130-098-846).

Cell Differentiation.

In order to test the effect of *MTAP* status on cell differentiation, tumor cells were plated in polyornithine and laminin coated plates in differentiation medium, which consisted of neurobasal medium (Neurobasal®, Invitrogen cat #21104-049) supplemented with serum-free 2% B-27 (Invitrogen, cat #17504-044) and 2mM GlutaMAX-I (Invitrogren cat #35050-061). Cells were incubated for 7 to 10 days then harvested for gene expression analysis.

Drug sensitivity.

Temozolomide (Medkoo Biosciences, cat #100810) was suspended in DMSO. Cells were plated out in 96-well laminin-coated plates (2,000 cells/well in 100 μl medium), and adhered for at least 12 hours before adding drugs. Cell viability was analyzed using CCK-8 (DojinDo, cat #ck04). At each experimental time point, 10 μl of CCK-8 solution was added to each well and incubated for 4 to 12 hours. Plates were read at 450nm by a multimode microplate reader (Infinite M200 PRO; Beckman).

***In vivo* tumorigenesis.**

Animal use and care protocol was approved by the Institutional Animal Care and Use Committee (IACUC). Orthotopic intracranial tumors were generated using patient-derived cell line 13-0302 in 6 week old NOD-SCID-gamma (NSG) male mice. Briefly, cells were suspended in serum-free media and 25% methylcellulose and 300,000 cells were injected using a stereotaxic frame into the right caudate nucleus of anesthetized NSG mice in accordance with an IACUC-approved protocol. Mice were monitored for weight loss and neurological symptoms and were sacrificed after either losing 20% body weight or becoming moribund. Kaplan Meier curves and the log-rank test *P* value were generated using Graphpad Prism.

Tumorigenesis of transformed astrocytes was tested in athymic nu/nu (Nude) mice (6 weeks old; Jax strain 002019) obtained from the Duke Division of Laboratory Animal Resources Breeding Core. Cells to be transplanted were cultured for 4 weeks after transformation in NSC media as described above in the presence of MTDIA (dissolved in DMSO) or DMSO control. Cells were then transduced with Lentiviral PLX304-Luciferase, were selected for 3 days with blasticidin, and cultured for 2 more weeks in NSC media with or without MTDIA. At the time of transplantation, cells were counted and suspended in serum free medium with 25% methylcellulose and were injected at concentrations of either 10,000 cells per animal or 200,000 cells per animal. Each cell line was injected into equal numbers of males and females. Tumor growth was estimated using bioluminescent imaging as described below. Mice were monitored for weight loss and neurological symptoms and were sacrificed after either losing 20% body weight or becoming moribund. Kaplan Meier survival curves and the log-rank test *P* value were generated using Graphpad Prism.

Bioluminescent imaging.

Mice implanted with luciferase-expressing xenografts were imaged on an IVIS XR imaging system using the Living Image software (PerkinElmer). Prior to imaging, mice were weighed and injected with 15 mg/kg luciferin (GoldBio Cat# LUCNA-1) suspended in saline. At 12 minutes after luciferin injection, total flux (photons/second) was measured within a defined region surrounding the intracranial tumor site. Exposure was set to consistently defined parameters for all images and time points.

***In vivo* L-Alanosine Treatment**

For subcutaneous xenografts, tumors were generated using patient derived cell lines in athymic nude mice. Xenograft tissues were harvested, minced, suspended, and injected subcutaneously into the flank of

additional athymic nude mice age 6-12 weeks (10 mice per treatment arm). Treatment began when all tumors in the cohort were above the minimum threshold of 200mm^3. L-Alanosine (Medkoo Biosciences, Cat#200130) was suspended in normal saline and 225mg/kg were injected intraperitoneally following a 5 day on, 2 day off schedule for the duration of 21 days. Tumor volume was measured by handheld calipers. Animals were sacrificed once the tumor size had reached both 1000mm^3 and 5 x its original size at the beginning of the study.

To evaluate efficacy of L-alanosine for the treatment of intracranial tumors, luciferase-expressing, patient-derived cell lines were injected into the right caudate nucleus of nude mice using a stereotaxic frame as described above. Treatment was initiated 5 weeks after tumor implantation. Mice received daily I.P. injections of L-Alanosine 225 mg/kg for the specified duration. Tumor response to treatment was monitored by bioluminescence on an IVIS Lumina XR imager and analyzed using Living Image software. Animals were sacrificed when moribund.

Genomic DNA preparation and q-PCR experiments.

Using QIAamp DNA Mini Kit (QIAGEN, cat #51306) or Allprep DNA/RNA/Protein mini kit (Qiagen, cat #8004), gDNA (genomic) was extracted following the manufacturer's protocol. Concentration and purity of DNA was assessed by Nanodrop Lite and Qubit 2.0 Fluorometer (Life Technologies, CA). Gene copy number assays were performed using genomic primers within the target gene (Supplementary Table 1) and compared to Line1 amplification. All qPCR was SYBR green label-based and was performed on CFX96 Real-Time System (BIO-RAD). The qPCR data were analyzed using Bio-Rad CFX Manager 3.1. For each reaction, the melting curve was checked to confirm the specificity of the qPCR reaction.

Methylation assays.

Bisulfite conversion of DNA was done using the Zymo EZ DNA Methylation kit (cat# D5001, Zymo Research, Irvine, CA, USA) prior to running the Illumina Methylation EPIC protocol. For each sample, 500 ng of high molecular weight DNA were used and bisulfite conversion followed the manufacturer's protocol with the following exceptions: constant vortexing for 10 minutes was done during step 2 instead of frequent vortexing, and the alternative thermocycler conditions were used for step 4 and 5, with the thermocycler program as follows: 95ºC for 30 seconds, 50ºC for 60minutes x16 cycles, then 4ºC hold.

The Illumina MethylationEPIC array(cat# WG317-1001, Illumina, San Diego, CA, USA) was processed at the Duke Molecular Physiology institute following the manufacturer's Infinium HD Methylation protocol. Briefly, the samples were denatured and amplified overnight for 20 to 24 hours, and then fragmented, precipitated and resuspended. Samples were then hybridized to the Illumina Infinium Methylation EPIC BeadChip for 16 to 24 hours. Finally, the BeadChips were washed to remove any unhybridized DNA and then labeled with nucleotides to extend the primers to the DNA sample. The BeadChips were then imaged using the Illumina iScan system (Illumina, San Diego, CA, USA). Data was processed using GenomeStudio software. Methylation array data is uploaded to the GEO Repository. Accession number GSE130093, Title: MTAP loss promotes stemness and epigenetic reprogramming in glioblastoma.

Methylation-specific PCR was determined using a quantitative DNA methylation assay. Briefly 20 ng of total DNA was incubated in the presence or absence of methylation-sensitive restriction enzymes at 37°C for 1 hour, followed by real-time PCR. Non-methylated DNA, which is sensitive to restriction enzyme digestion, shows a lower cycle threshold (Ct) value after enzyme digestion than the same sample incubated without enzymes. Levels of methylation (%) were determined using the formula $100x2^{-\Delta CT}$, where ΔCT is the difference in Ct value from the cut reaction minus the non-cut reaction. Methylation standards were run for comparison. Restriction enzymes were purchased from NEB labs. Primers and probes were synthesized by Integrated DNA Technologies.

Global DNA methylation was measured using the MethylFlash Global DNA Methylation (5-mC) enzyme-linked immunosorbant assay (ELISA) Easy Kit (Colorimetric) (EpiGentek, cat #p-1030) in a 96-well microplate format following the manufacturer's protocols. The DNA was quantified using the Qubit 2.0 Fluorometer (Life Technologies, CA). Plates were read at 450nm by a multimode microplate reader (Infinite M200 PRO; Beckman).

Immunoblotting.

Total proteins were extracted from cells using RIPA Buffer (Thermo Scientific, cat #89901) or with the Allprep DNA/RNA/Protein mini kit (Qiagen, cat# 8004). Protein concentrations were determined by Pre-Diluted Protein Assay standards (Thermo Scientific, cat #NA165380) using Pierce BCA Protein Assay Kit (Thermo Scientific, cat #RD231228). Along with NuPAGE Sample Reducing Agent (10X; Life Technologies, cat #NP0004) and NuPAGE LDS Sample Buffer (4X; Thermo Scientific, cat #NP0007) 20 ug of total protein was loaded onto Novex NuPAGE 4% to 12% Bis-Tris Protein Gels (Thermo Scientific, cat #NP0322BOX) and transferred to

nitrocellulose membranes (BIO-RAD, cat #1620090). Membranes were blocked with 5% milk in washing buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.05% Tween 20) at room temperature for 2 hours. Membranes were then incubated at 4°C overnight with anti-MTAP (Cell Signaling technology, cat #4158; 1:1,000 dilution), anti-PRMT5 (Cell Signaling Technology, cat #2252; 1:1000 dilution), anti-Beta Actin (clone D6A8; Cell Signaling Technology, cat #8457; 1:2,000 dilution) or anti-H4R3me2s (Sigma-Aldrich, cat #SAB4300870; 1:1000 dilution). The secondary antibody used in all cases was anti-rabbit IgG, HRP-linked Antibody (Cell Signaling Technology, cat #7074s; 1:2500 dilution). Immunoblot bands were detected using SuperSignal West-pico Chemiluminescent Substrate (Thermo Scientific cat #34080), and scanned/quantified via the ChemiDoc MP Imaging System (Bio-Rad).

Immunohistochemistry.

Brain tumor sections were stained using antibodies against CD133 (Cell Signaling, cat# 64326) or IgG isotype control (Cell Signaling, cat# 3900) on the Leica Bond RX^{M} automated system. Stained slides were scanned using the Vectra automated pathology imaging system (PerkinElmer) and positive cells were quantified using inForm software (PerkinElmer).

Gene expression microarray.

The RNA was extracted as described above. Samples were analyzed using the Affymetrix Human Genome U133 Plus 2.0 array according to the manufacturer's protocols by the Duke Sequencing and Genomic Technologies Shared Resource. Data was analyzed using the Affymetrix Expression Console and Affymetrix Transcriptome Analysis Console v3.0 software. Data is uploaded to the GEO Repository. Accession number GSE114867, Title: Expression data from patient-derived primary glioblastoma cell lines.

Analysis of TCGA data.

All TCGA data was downloaded from the online portal https://tcga-data.nci.nih.gov/docs/publications/tcga/ and through cbioportal.org (4,5). The most recently published 2013 GBM data set was used for all analyses. For each analysis, the maximum number of complete cases available (confirmed *IDH1/2* wildtype) were used unless otherwise stated, as IDH mutations are known to independently influence epigenetics and cellular differentiation. Analyses performed include gene expression (385 samples), methylation (72 samples), and patient survival (242 samples). We utilized *MTAP* expression levels to categorize patients rather than gene copy number because

MTAP is known to be silenced epigenetically in a variety of cancer types (6-9), and our analysis of DNA methylation and gene expression data in patients suggests it can also be epigenetically silenced in GBM (Supplementary Fig. S5). For most of the analyses, samples were divided into 3 groups based on MTAP expression (low, medium, high) and a *t* Test was used to compare the "low" and "high" groups to find differences in gene expression and methylation between these two groups.

Pathway analysis.

Pathway analysis was done using the DAVID 6.8 platform at david.ncifcrf.gov (10,11). For pathway analysis of gene expression differences between *in vitro* samples and between patient cohorts, all genes found to be "significant" by the parameters defined in the manuscript were included in the analysis. Unless otherwise stated, for pathway analysis of differentially methylated CpG islands and shores all probes with a methylation difference greater than 10% were included, with a maximum of 3000 genes allowed by DAVID (if more than 3,000 probes showed greater than 10% methylation difference, those with the highest difference were included).

Statistical analysis.

Statistical tests were performed using Statgraphics Centurion (ANOVA, Kruskal Wallis, Mann Whitney U) and Graphpad Prism (*t* tests and log rank test for Kaplan-Meier curves). All experiments were repeated to ensure reproducibility of results. Unless otherwise indicated, pooled data from multiple experiments was used for each figure. A *P* value cutoff of 0.05 was used to determine significance in all cases except where corrections were applied for larger data sets (ie. Bonferonni).

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