Nuclear Receptor 4A1 (NR4A1) and NR4A2 Regulate Expression of O^6-Methylguanine-DNA-Methyltransferase (MGMT) in Glioblastoma and Can Be Targeted by NR4A1/2 Ligands

Evan Farkas[1], Srijana Upadhyay[1], Arafat Rahman Oany[1], Wai Ning Tiffany Tsui[1], Gargi Sivaram[2], Sofia Melgoza[2], Karol Bomsztyk[3,4,5], Daniel Mar[3,4], Ana Gutierrez[6], Svetlana A. Mikheeva[6], Andrei Mikheev[6], Robert Rostomily[6] and Stephen Safe[1*]

[1] Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, Texas 77843

[2] Department of Biochemistry and Biophysics, College of Agriculture and Life Sciences, Texas A&M University, College Station, Texas 77843

[3] UW Medicine South Lake Union, University of Washington, Seattle, WA 98109, USA

[4] Institute for Stem Cell and Regenerative Medicine, University of Washington, Seattle, WA 98109, USA

[5] Matchstick Technologies, Inc, Kirkland, WA 98033, USA

[6] Department of Neurosurgery, Houston Methodist Hospital and Research Institute, Houston, Texas 77030

*Correspondence should be addressed: Stephen Safe, Email: ssafe@cvm.tamu.edu; Tel. (979) 845-5988; Fax. (979) 862-4929

Supplementary Table 1 (Antibodies):

Antibody	**Source**	**Catalog No./Identifier**	**WB Dilution**
β-Actin	Sigma	A5316	1:1000
NR4A1 (Western)	Abcam	Ab283264	1:500
NR4A2 (Western)	Santa Cruz Biotechnology	sc-376984	1:1000
Sp1 (Western)	Proteintech	21962-1-AP	1:1000
MGMT (Human)	Cell Signaling	#2739	1:1000
MGMT (Mouse)	R&D Systems	#MAB3299	1:1000
Sp1 (E3) (ChIP)	Santa Cruz Biotechnology	Sc-17824	1:500
NR4A1 (C-5) (for ChIP)	Santa Cruz	Sc-365113 X	N/A
NR4A2 (F-5) (for ChIP)	Santa Cruz	Sc-376984 X	N/A
5-Methylcytosine (5-mC) (For MeDIP)	Epigentek	#A-1014	N/A

Supplemental Table 2:

(qRT-PCR Primers)

Primer	Sequence
Human MGMT	Forward: 5' ACGAAATAAAGCTCCTGGGC 3' Reverse: 5' CTTGCTGGAAAACGGGATGG 3'
Mouse MGMT	Forward: 5’ GGAATGATGAGTGGACGTGC 3’ Reverse: 5’ CGTGCTGGTGTTTCATGTCA 3’
Human NR4A1	Forward: 5' GGACAACGCTTCATGCCAGCAT 3' Reverse: 5' CCTTGTTAGCCAGGCAGATGTAC 3'
Mouse NR4A1	Forward: 5’ GTGCAGTCTGTGGTGACAATGC 3’ Reverse: 5’ CAGGCAGATGTACTTGGCGCTT 3’
Human NR4A2	Forward: 5' CGACATTTCTGCCTTCTCC 3' Reverse: 5' GGTAAAGTGTCCAGGAAAAG 3'
Mouse NR4A2	Forward: 5’ GTGCAGTCTGTGGTGACAATGC 3’ Reverse: 5’ CAGGCAGATGTACTTGGCGCTT 3’
Human Sp1	Forward: 5' ACGCTTCACACGTTCGGATGAG 3' Reverse: 5' TGACAGGTGGTCACTCCTCATG 3'
Mouse Sp1	Forward: 5’ CTCCAGACCATTAACCTCAGTGC 3’ Reverse: 5’ CACCACCAGATCCATGAAGACC 3’
Human β-Actin	Forward: 5' CACCATTGGCAATGAGCGGTTC 3' Reverse: 5' AGGTCTTTGCGGATGTCCACGT 3'
Mouse β-Actin	Forward: 5’ AGTGTGACGTTGACATCCGTA 3’ Reverse: 5’ GCCAGAGCAGTAATCTCCTTC 3’

Supplementary Table 3:

(ChIP Primers)

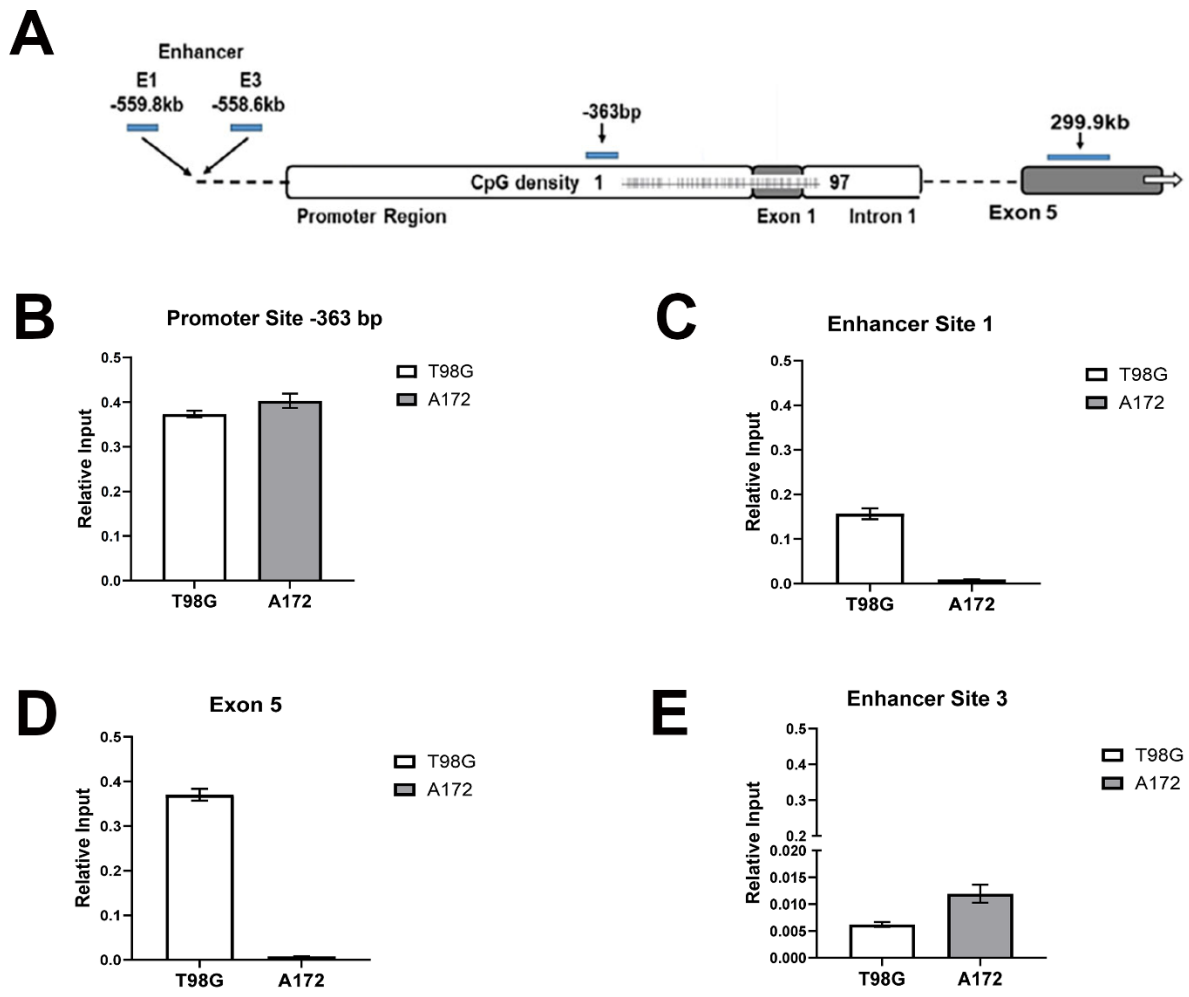
Primer	**Sequence**
Human MGMT (Binding Site 1)	Forward: 5' GCAAAGCGTTCTAGGGGCG 3' Reverse: 5' GGATTCTCACTAAGCGGGCG 3'
Human MGMT (Binding Site 2)	Forward: 5’ CCAGACCAGCAGAGACCCT 3’ Reverse: 5’ CCAGACCAGCAGAGACCCT 3’
Mouse MGMT (Binding Site 1)	Forward: 5' TCTGGCACTGAGAAATACG 3' Reverse: 5' GCCATTATGAGGTACAGAGG 3'
Mouse MGMT (Binding Site 2)	Forward: 5’ CAGGGCTTTCTTTCAGGATC 3’ Reverse: 5’ GAGCTGCACACTGGGAAG 3’

Supplementary Table 4:

(MeDIP Primers; Buffer Prep Info)

Primer	**Sequence**
Human MGMT Enhancer Site 1	Forward: 5' TACCAGGAATGATGTGGGGTTA 3' Reverse: 5' GGCATCTTCCCCTTCACAT 3'
Human MGMT Enhancer Site 3	Forward: 5’ CCATGCAGGCATTTCTACCT 3’ Reverse: 5’ GAGCTCGCAGGAGCAACTAC 3’
Human MGMT Promoter -363	Forward: 5' CCATTTGGCAAACTAAGGCA 3' Reverse: 5' CCCTTCGGCCGGTACAA 3'
Human MGMT Exon 5	Forward: 5’ GGCACCGCTGTATTAAAGGA 3’ Reverse: 5’ ATAGAGCAAGGGCAGCGTTA 3’
PBS (made with nuclease free reagents and ultrapure distilled RNAse/DNAse free H_2O)	137 mM NaCl, 10 mM Sodium phosphate, 2.7 mM KCl, pH 7.4; TE: 10 mM Tris-HCl, 1 mM EDTA, pH 7.5
Immunoprecipitation (IP) buffer (made with nuclease free reagents and ultrapure distilled RNAse/DNAse free H_2O)	150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 5 mM EDTA, NP-40 (0.5% vol/vol), Triton X-100 (1.0% vol/vol)
Elution buffer-Proteinase K (made with nuclease free reagents and ultrapure distilled RNAse/DNAse free H_2O)	25mM Tris Base, 1% IP Buffer, 1mM EDTA, 80 µg/ml Proteinase K

SUPPLEMENTARY FIGURES/FIGURE LEGENDS



Supplementary Figure 1: Evaluation of Human GBM Cell's MGMT Promoter Methylation Status

Locations of designed primers used for Matrix-MeDIP Assay, performed as described in "Methods", along the human MGMT promoter relative to the Transcription Initiation Site (TSS) (**a**). Relative methylation of MGMT promoter sites in untreated T98G and A172 human GBM cells (**b-e**).