Characterization and validation of MGMT-binding partners using a proteomic-based approach in glioblastoma

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June 2016

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

Table of Contents

Abstract

Glioblastoma (GBM) is the most common primary malignant brain tumor in adults and is one of the most lethal human cancers. This is due to its highly vascularized and invasive nature, which makes complete surgical resection and treatment difficult. O^6-methylguanine-DNA methyltransferase (MGMT), a DNA repair protein ubiquitously expressed in normal human tissues, plays a vital role in protection against alkylating agents used in GBM treatment. While MGMT is known to exert its DNA repair function (alkyl transfer) independently of other proteins, increasing evidence suggests a role of MGMT as an ancillary protein mediating additional functions through interactions with binding partners in other cancers.

Our previous studies revealed an inverse relationship between MGMT expression and GBM angiogenesis and invasion, challenging the paradigm that MGMT exerts its function in GBM solely as a DNA repair protein. However, the mechanism by which MGMT affects angiogenesis and invasion at the molecular level remains unknown. We hypothesized that interaction of MGMT with binding partners confers novel functions to MGMT beyond its known role as a DNA repair protein in GBM.

Using T98G, a human GBM cell line with constitutive expression of MGMT, we performed immunoprecipitation of endogenous MGMT followed by a mass spectrometry-based proteomic approach, which enabled the identification of 186 MGMT-binding partners in GBM. We identified proteins involved in mitochondrial metabolism, DNA repair and replication, the ubiquitin pathway, transcription regulation, RNA post-transcriptional processing, protein biosynthesis and trafficking, cellular metabolism, cell cycle and division, response to stress and cell death, and cell migration and invasion. We further analyzed our results using MetaCore software (GeneGO, Inc., MetaCore database) to determine related functional pathways of greatest significance in the context of GBM. Annexin A2 and heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) were among the binding partners identified with high confidence in our proteomic and bioinformatics analysis for their roles in angiogenesis and invasion. Using human GBM cell lines isogenic for MGMT, we validated the interaction of MGMT with hnRNPA1 and annexin A2. Confocal fluorescence colocalization studies and quantitative colocalization image analysis showed the predominance of nuclear colocalization of MGMT with hnRNPA1 and cytoplasmic colocalization of MGMT with annexin A2.

To ascertain the biological significance of our findings, we examined the role of ionizing radiation in the expression of MGMT, hnRNPA1, and annexin A2 protein levels, as well as the activation of key signaling pathways involved in response to genotoxic stress. Notably, activation of extracellular signal-regulated kinases (Erk1/2) was suppressed while induction of wild-type p53 and its downstream target gene p21 was more pronounced in MGMT-positive cells exposed to radiation compared to MGMT-negative cells in a time-dependent manner.

Our results provide the first evidence supporting the interaction of MGMT with binding partners in the context of GBM. Elucidating how MGMT is involved in novel functional activities will reveal how MGMT affects GBM angiogenesis and invasion at the molecular level. Additional studies are warranted to investigate radiosensitivity of GBM cell lines in relation to MGMT expression and determine the effects of radiation on the interaction of MGMT with relevant binding partners to ultimately overcome radioresistance in GBM treatment.

Résumé

Le glioblastome (GBM), la tumeur maligne primaire du cerveau la plus fréquente chez les adultes est l'un des cancers les plus mortels. Ceci est dû à sa nature hautement vascularisée et invasive, ce qui rend difficile la résection chirurgicale complète et le traitement. L'O6-méthylguanine-ADN méthyltransférase (MGMT), une protéine de réparation de l'ADN exprimée ubiquitairement dans les tissus humains normaux est essentielle pour la protection contre les agents alkylants utilisés pour traiter le GBM. Alors que MGMT est connu pour sa fonction de réparation d'ADN (alkyl-transférase) indépendamment d'autres protéines, son rôle a été suggéré en tant que protéine auxiliaire qui interagit avec des partenaires de liaison dans d'autres types de cancer.

Nos études antérieures ont révélé une relation inverse entre l'expression de MGMT et l'angiogenèse et l'invasion de GBM, contestant le paradigme que MGMT exerce sa fonction exclusivement comme protéine de réparation d'ADN dans le GBM. Cependant, le mécanisme par lequel MGMT affecte l'angiogenèse et l'invasion au niveau moléculaire reste inconnu. Nous avons émis l'hypothèse que l'interaction de MGMT avec des partenaires de liaison confère de nouvelles fonctions à MGMT au-delà de son rôle connu comme protéine de réparation d'ADN en GBM.

Utilisant T98G, une lignée de cellules de GBM humaines, avec l'expression constitutive de MGMT, nous avons effectué une immunoprécipitation de MGMT endogène, suivie par une approche protéomique basée sur la spectrométrie de masse, et permettant l'identification de 186 partenaires de liaison de MGMT en GBM. Nous avons identifié des protéines impliquées dans le métabolisme mitochondrial, la réparation et la réplication de l'ADN, la voie d'ubiquitination, la régulation de la transcription, post-transcription de l'ARN, la biosynthèse et le trafic de protéines, le métabolisme cellulaire, le cycle et la division cellulaires, la réponse au stress et la mort cellulaire, ainsi que la migration et invasion. Nous avons ensuite analysé nos résultats en utilisant le logiciel MetaCore (GeneGo, Inc., base de données MetaCore) pour déterminer les voies ayant la plus grande importance dans le contexte du GBM. L'annexine A2 et la ribonucléoprotéine hétérogène nucléaire A1 (hnRNPA1) ont été parmi les partenaires de liaison identifiées de manière confidente dans notre analyse protéomique et bioinformatique pour leur rôle dans l'angiogenèse et l'invasion. En utilisant des lignées de cellules de GBM humaines isogéniques pour MGMT, nous avons pu valider l'interaction de MGMT avec hnRNPA1 et annexine A2. Des

études de colocalisation par fluorescence confocale et des analyses quantitatives de colocalisation d'images ont démontré la prédominance de la colocalisation nucléaire de MGMT avec hnRNPA1 et la colocalisation cytoplasmique de MGMT avec l'annexine A2.

Pour confirmer la signification biologique de nos résultats, nous avons examiné le rôle de l'irradiation ionisante dans l'expression des protéines MGMT, hnRNPA1, annexine A2, et dans l'activation de voies de signalisation clés dans la réponse au stress génotoxique. L'activation des kinases régulées par des signaux extracellulaires (Erk1/2) a été réduite, alors que l'induction de p53 non-muté et de son gène-cible p21 était plus prononcée dans les cellules MGMT-positives irradiées, par rapport aux cellules MGMT-négatives, d'une manière dépendante du temps.

Nos résultats montrent pour la première fois l'interaction de MGMT avec des partenaires de liaison dans le GBM. Élucider le rôle de MGMT dans de nouvelles activités fonctionnelles permettra de révéler les effets moléculaires de MGMT au niveau de l'angiogenèse et l'invasion du GBM. Des études additionnelles sont nécessaires pour examiner la radiosensibilité de lignées cellulaires de GBM en relation avec l'expression de MGMT et déterminer les effets de l'irradiation sur l'interaction de MGMT avec des partenaires de liaison relevant pour contourner la radiorésistance du GBM.

Acknowledgments

I would like to thank my supervisor, Dr. Siham Sabri, for her commitment and support in the completion of this thesis. Her guidance and expertise were instrumental in the development and achievement of this project.

I would like to express my appreciation for the support and assistance of my colleagues in the lab, whose knowledge and camaraderie were essential in my development and growth as a scientist. I would like to thank our Administrative Research Assistant, Ms. Elena Popova, for her hard work in keeping the lab running smoothly and for all of her help over the last two years. I would also like to express my gratitude to Dr. Min Fu and the staff of the Molecular Imaging Platform at the Research Institute of the McGill University Health Centre for their unremitting assistance in image acquisition and analysis.

Finally, I would like to thank my family, particularly my mother, for their continuous support and motivation in the completion of this degree.

Preface

Alexandra Candib was responsible for performing pathway analysis for MGMT-binding partners, immunofluorescence staining and confocal image analysis, co-immunoprecipitation studies (validating the interaction of MGMT-binding partners), experiments analyzing the effects of ionizing radiation, and manuscript preparation. Mr. Yaoxian Xu provided technical assistance preparing samples used for mass spectrometry analysis performed at the Proteomic Platform of the Institut de recherches cliniques de Montréal. Dr. Jad Ashami at the laboratory of Dr. Rolando Del Maestro (McGill University) conducted stable transfection of U87MG cells with a plasmid carrying exogenous MGMT (U87/MGMT) or an empty vector (U87/EV).

Dr. Siham Sabri was responsible for the conception, design, development of methodology, data analysis, manuscript revision, and provided grant-funded financial support to conduct this work.

List of Abbreviations

293T-FLAG/EV	293T cells expressing flag-tagged empty vector control
293T-FLAG/MGMT	293T cells expressing flag-tagged MGMT
Akt	protein kinase B
AP-2	activator protein 2
ApoE	apolipoprotein E
ATCC	American Type Culture Collection
BCA	bicinchoninic acid
BER	Base Excision Repair
BRCA2	breast cancer type 2 susceptibility protein
BT549	breast cancer cell line
c-FOS	FBJ murine osteosarcoma viral oncogene cellular homolog
c-Myc	Avian myelocytomatosis virus oncogene cellular homolog
C-terminal	carboxyl-terminus
Ca^{2+}	calcium ion
cAMP	cyclic adenosine monophosphate
CBP/p300	cAMP response element-binding protein (CREB) /p300
CBTRUS	Central Brain Tumor Registry of the United States
CDKN1A	Cyclin-Dependent Kinase Inhibitor 1A
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
CEM	leukemia cell line
Cl^-	chloride ion
CRISPR	clustered regularly interspaced short palindromic repeats
DAPI	4',6-diamidino-2-phenylindole
DDR	DNA damage response
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EGFR	epidermal growth factor receptor
EGR1	early growth response protein 1
Erk1/2	extracellular signal-regulated kinases 1 and 2

F-actin	filamentous actin
FAK	focal adhesion kinase
FDA	Food and Drug Administration
FGF-2	fibroblast growth factor 2
FLAG	fusion tag (AspTyrLysAspAspAspAspLys)
GBM	Glioblastoma
GI	GenInfo Identifier
GO	gene ontology
GTPase	guanosinetriphosphatase
Gy	Gray, unit of ionizing radiation dose
H3F3A	H3 Histone, Family 3A
HC	antibody heavy chain
HeLa	Human cervical adenocarcinoma cell line
hnRNA	heterogeneous nuclear RNA
hnRNP	heterogeneous nuclear ribonucleoprotein
hnRNPA1	heterogeneous nuclear ribonucleoprotein A1
hnRNPA/B	heterogeneous nuclear ribonucleoprotein A/B family
HPRD	Human Protein Reference Database
HT29	human colorectal adenocarcinoma cell line with epithelial morphology
IκBa	inhibitory subunit of NF-κB alpha
IDH	isocitrate dehydrogenase
IDH1	isocitrate dehydrogenase 1
INK4A	p16
IP	immunoprecipitation
K27	Lysine, positive 27
KRas	Kirsten rat sarcoma viral oncogene homolog
kVp	kilovoltage peak
LC	antibody light chain
LC	liquid chromatography
Let-7a	lethal 7a microRNA
mA	milliampere

MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MCF7	human breast cancer cell line
MDA-231	breast cancer cell line
MEBP	MGMT enhancer binding protein
MGMT	O^6-methylguanine-DNA methyltransferase
MLH1	MutL Homolog 1
MMR	Mismatch Repair
MNK	Mitogen-Activated Protein Kinase-Interacting Kinases
MNNG	*N*-methyl-*N*'-nitro-*N*-nitrosoguanidine
MRC5.SV40	fetal lung fibroblast strain MRC, infection with SV40 strain VA45-54-2
MRI	magnetic resonance imaging
mRNA	messenger RNA
MS/MS	tandem mass spectrometry
N-terminal	amino-terminus
N3-MeA	*N3*-methyladenine
N7-MeG	*N7*-methylguanine
NER	Nucleotide Excision Repair
NF1	neurofibromin 1
NF-κB	Nuclear Factor Kappa Beta
NOS	not otherwise specified
O4-MeT	*O4*-methylthymine
O6-MeG	*O6*-methylguanine
O^6BG	O^6-benzyl guanine
OCT1	Organic Cation Transporter 1
P11	S100 calcium-binding protein A10
p16	cyclin-dependent kinase inhibitor 2A
p21	cyclin-dependent kinase inhibitor 1A
p53	tumor protein p53
p63	tumor protein p63
pAkt	phospho-protein kinase B

PBS	phosphate-buffere saline
PC12	cell line derived from a pheochromocytoma of the rat adrenal medulla
PDGF	platelet-derived growth factor
PDGFRA	platelet-derived growth factor receptor, alpha
pErk	phospho-extracellular signal-regulated kinases
PINA	Protein Interaction Network Analysis
PKC	protein kinase C
PKM1	pyruvate kinase isoform 1
PKM2	pyruvate kinase isoform 2
PPAR-g	Peroxisome proliferator-activated receptor gamma
PtdIns(4,5)P_2	phosphatidylinositol-4,5-bishosphate
R/min	roentgens per minute
Rab11	Ras-related protein 11
RBD	RNA binding domain
RBP	RNA binding protein
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
RTK I	Receptor tyrosine kinases I
RTK II	Receptor tyrosine kinases II
S100A10	S100 calcium-binding protein A10
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	small hairpin RNA
SP3	Sp3 transcription factor
SR	serine-arginine-rich
SRF	serum response factor
STAT1	signal transducer and activator of transcription 1
SUMOylation	small ubiquitin-like modifiers post-translational modification
T47D	human breast cancer cell line
TBST	Tris-Buffered Saline with 1% Tween-20 solution
TCGA	The Cancer Genome Atlas
TMZ	temozolomide

tPA	tissue plasminogen activator
TP53	tumor protein 53
U87/EV	U87MG cell line transfected with empty vector control
U87/MGMT	U87MG cell line transfected with MGMT
VEGF	vascular endothelial growth factor receptor
WAF1	wild-type activating fragment-1
WCL	whole cell lysate
WHO	World Health Organization
ZEB1	zinc finger E-box binding homeobox 1

List of Figures

List of Tables

1. Introduction

1.1 Glioblastoma

1.1.1 Glioblastoma Pathology

Glioblastoma (GBM) is the most common primary malignant glioma in adults and is one of the most devastating human cancers. According to the Central Brain Tumor Registry of the United States (CBTRUS), GBMs make up 15.1% of all primary brain tumors and 46.1% of primary malignant brain tumors (Figure 1.1) [1]. Although GBM has a relatively low incidence of 3.2 per 100,000 individuals, the median survival is 12-15 months resulting in a disproportionate number of cancer-related deaths [1, 2].

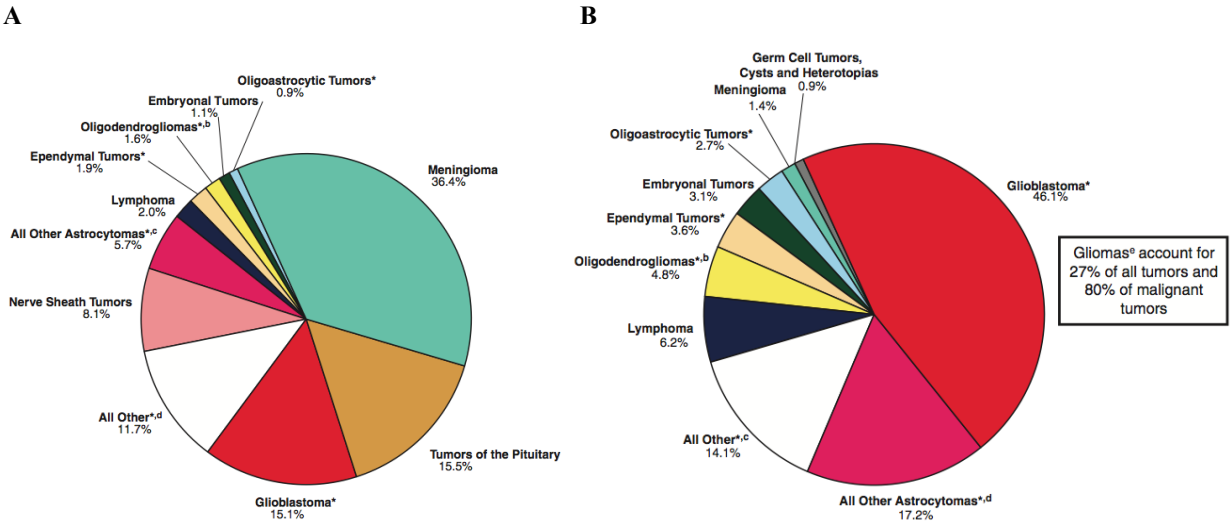

Figure 1.1 (A) Distribution of primary brain and central nervous system tumors (n=356,858). **(B)** Distribution of malignant primary brain and central nervous system tumors (n=117,023). Figure adapted from Ostrom *et al.* (2015) [1].

In terms of incidence, GBM is more prevalent in older adults and 1.6 times more common in males than females [1]. GBM is less common in children, making up 2.9% of all brain and central nervous system tumors in those 0 to 19 years of age. However, prevalence increases with age, with the highest incidence rates in patients 75 to 84 years of age [1].

GBMs arise from astrocytes but contain a variety of cell types and are characteristically heterogeneous within tumors and between patients. GBMs are typically located in the cerebral hemispheres of the brain, more often in the frontal and temporal lobes, but may be found anywhere in the brain or spinal cord. Additionally, GBMs are characteristically diffuse and invasive, forming finger-like projections into other regions of the brain and extending beyond macroscopic borders. These features make treatment extremely difficult, as illustrated in Figure 1.2, but while GBMs are highly infiltrative, they are not metastatic and rarely form tumors in other areas of the body.

Invasion and angiogenesis are integral to GBM tumor growth; however, the mechanisms that control these processes are not fully understood. Of all solid tumors, malignant brain tumors show the highest degree of vascular proliferation and the World Health Organization (WHO) uses the presence of microvascular proliferation as a diagnostic criterion for high grade astrocytomas and as an independent prognostic parameter [3].

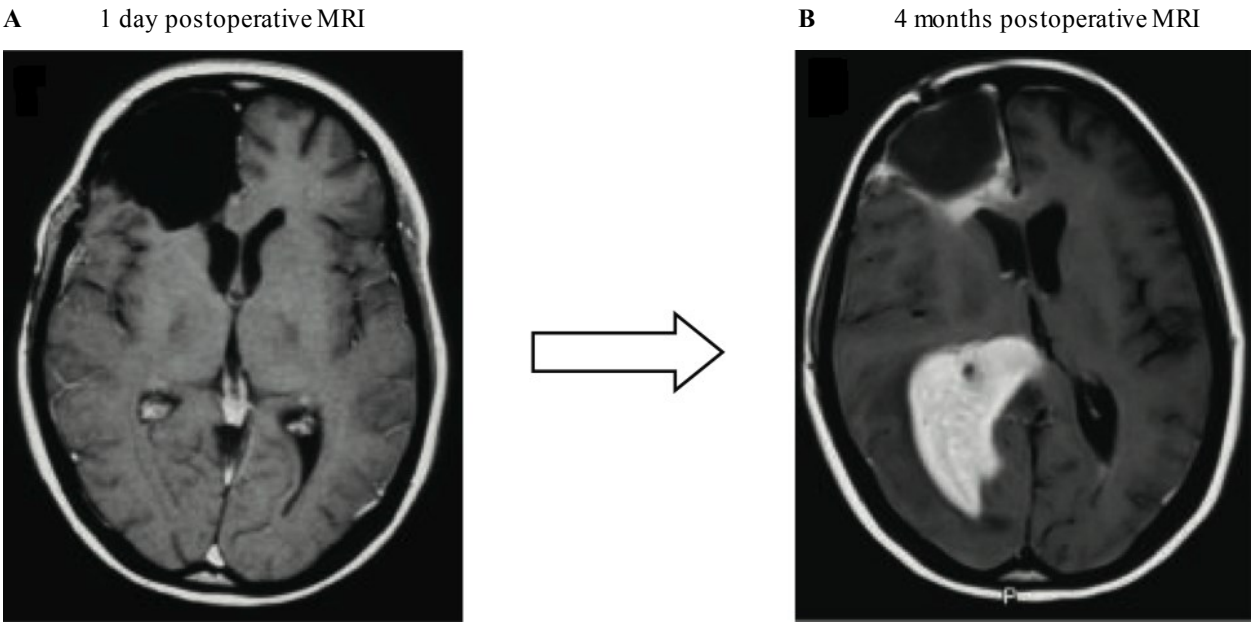

Figure 1.2 MRIs showing aggressive invasion of GBM. (**A**) MRI showing the resection hole one day after surgical resection of a right frontal GBM. (**B**) MRI of the same patient collected four months after surgery, fractionated radiotherapy, and chemotherapy with temozolomide (TMZ). A new tumor has formed and there is evidence of tumor reformation at the site of resection, indicating local relapse. Figure adapted from Martínez, R., 2012 [2] with modification.

Overall, the poor prognosis and high mortality of GBM can be attributed to its highly vascularized and invasive nature, which makes complete surgical resection near impossible and treatment problematic [4]. Though research has focused on elucidating the pathways involved in the increased invasiveness characteristic of GBM, the mechanisms by which these pathways are activated are not fully understood; there exist a multiplicity of intra- and intercellular interactions, communications, and architectures that we do not understand.

1.1.2 Glioblastoma Classification

GBMs display great inter- and intra-tumor heterogeneity and categorizing this heterogeneity has become an increasingly popular pursuit among researchers and clinicians. There is an emphasis on defining the extensive genetic and epigenetic variations that exist in GBM in order to generate distinct tumor profiles that may be used to predict patient response to treatment.

Initially, GBMs were classified as either primary or secondary. Primary GBMs make up approximately 90% of GBMs; they develop rapidly and manifest high-grade lesions from their outset [5]. In contrast, secondary GBMs develop more slowly through progression from lower grade diffuse astrocytoma or anaplastic astrocytoma [5]. While both are histologically indistinguishable, their genetic and epigenetic profiles differ [5]. The critical factor differentiating primary and secondary GBMs is isocitrate dehydrogenase 1 coding gene (*IDH1*) mutations, which are present in secondary GBMs and confer a hypermethylation phenotype [5]. *IDH1* mutations are considered initiator and lineage markers of gliomagenesis and are now reflected in the WHO diagnostic categories of GBMs [6].

More recent emphasis on the genetic makeup of GBM has led to the classification of GBM subtypes that correlate with clinical outcome and response to therapy [7]. Historically, gliomas have been grouped into four grades on the basis of histological presentation, with grade IV astrocytoma, or GBM, being the most aggressive [8]. In 2016, the WHO updated the classification system for tumors of the central nervous system with a stronger focus on molecular parameters in combination with histology [6]. The shift toward the incorporation of genetic alterations to define diagnostic categories, as opposed to being used for purely prognostic or predictive purposes, has lead to the creation of three GBM tumor types: Glioblastoma, *IDH*-

wild-type; Glioblastoma, *IDH*-mutant; Glioblastoma, NOS [6]. (Central nervous system tumor diagnoses consist of a histopathological name followed by genetic feature(s). Tumors lacking genetic mutation use the term *wildtype*. Tumors lacking a diagnostic mutation or sufficient information to assign a more specific code are given a *not otherwise specified*, or NOS, designation [6].) This change in the classification system is reflective of the current research emphasis on defining genetic and epigenetic characteristics of GBM.

In 2010, Verhaak *et al*. used integrated genomic analysis data from 200 GBM and two normal brain samples to generate four GBM subtypes [7]. These four gene cluster subtypes were then validated in an independent data set of 260 GBM expression profiles and three of the four gene clusters were successfully observed in a xenograft model (Figure 1.3) [7]. Four subtypes were defined and named on the basis of prior naming conventions and expression of defining genes: Proneural, Neural, Classical, and Mesenchymal [7]. The Proneural subtype is characterized by mutations in *IDH1* and tumor protein 53 gene (*TP53*), as well as amplification of platelet-derived growth factor receptor A gene (*PDGFRA*) [7]. The Neural subtype is characterized by expression of neuron markers [7]. The Classical subtype is characterized by amplification of epidermal growth factor receptor gene (*EGFR*) and the absence of *TP53* mutations [7]. The Mesenchymal subtype is characterized by deletions or mutations of the neurofibromin 1 gene (*NF1*) [7]. It was noted that patients with the Classical tumor subtype experienced the greatest response to therapy, while no response was observed for patients with the Proneural subtype [7].

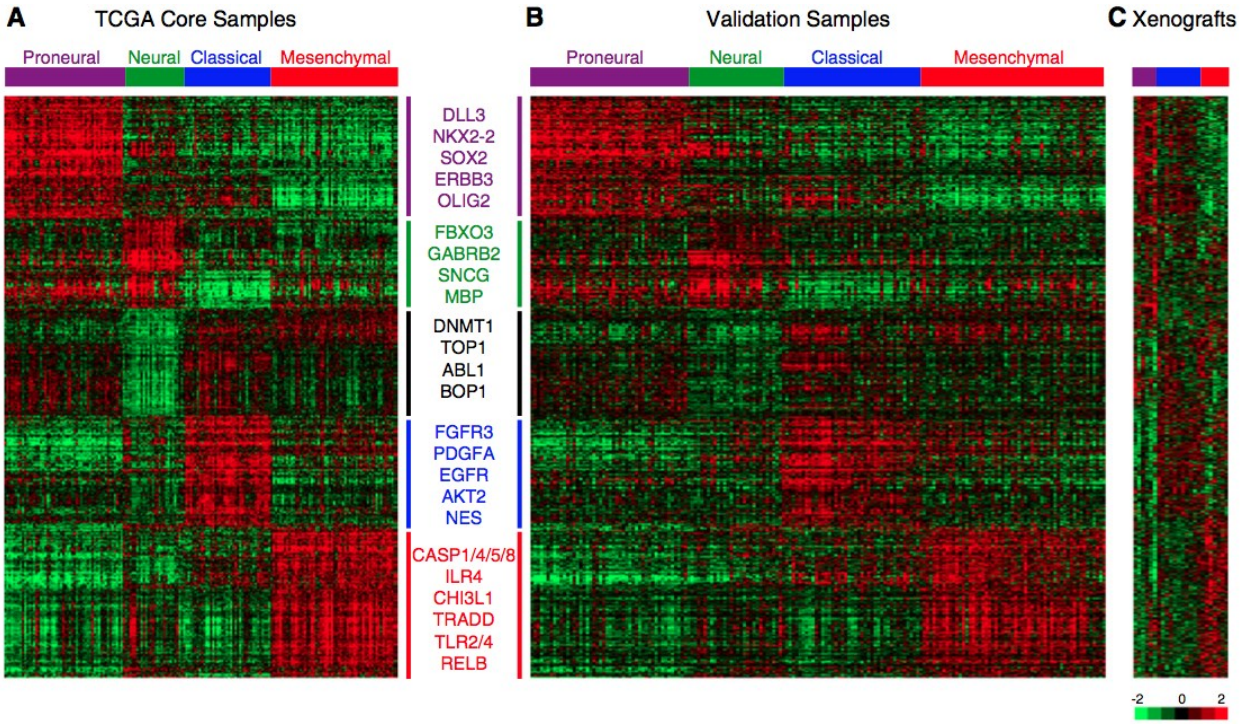

Figure 1.3 Four GBM subtypes generated from gene expression data. **(A)** A set of 173 samples from The Cancer Genome Atlas (TCGA) were ordered and clustered using a predictive gene list. **(B)** Gene order from (A) was maintained in the validation set of 260 GBM samples. **(C)** Gene expression for 24 xenograft samples. Figure adapted from Verhaak *et al.*, 2010 [7].

In 2012, Sturm *et al*. identified six epigenetic GBM subgroups using DNA methylation patterns, distinct mutations, DNA copy-number alterations, and transcriptome patterns [9]. They used a cohort of 210 GBMs to determine genome-wide DNA methylation patterns, followed by consensus clustering across the dataset in combination with mutational status, DNA copy-number aberrations, and gene expression signatures, in order to identify six subgroups which also incorporate those created by Verhaak *et al*. [7]: IDH, K27, G34, RTK I (PDGFRA), Mesenchymal, and RTK II (Classic) (Figure 1.4) [9]. Interestingly, there appeared exclusive relationships between some subgroups: H3 Histone, Family 3A coding gene (*H3F3A*) K27 and G34 mutations were limited to the K27 and G34 subgroups, respectively; 88% of *IDH1*-mutated tumors were contained within the IDH subgroup. Furthermore, the IDH subgroup displayed widespread hypermethylation while the G34 subgroup showed widespread hypomethylation. Researchers also noted *H3F3A* and *IDH1* mutations were mutually exclusive, further verifying this finding using targeted sequencing analysis of 460 GBM patient samples from which they

determined associations between age and mutation (*H3F3A* K27 was observed in GBM in children, *H3F3A* G34 in adolescents, and *IDH1* in young adults). Subgroups also presented different overall survivals, with the IDH and K27 subgroups exhibiting the longest survival times [9].

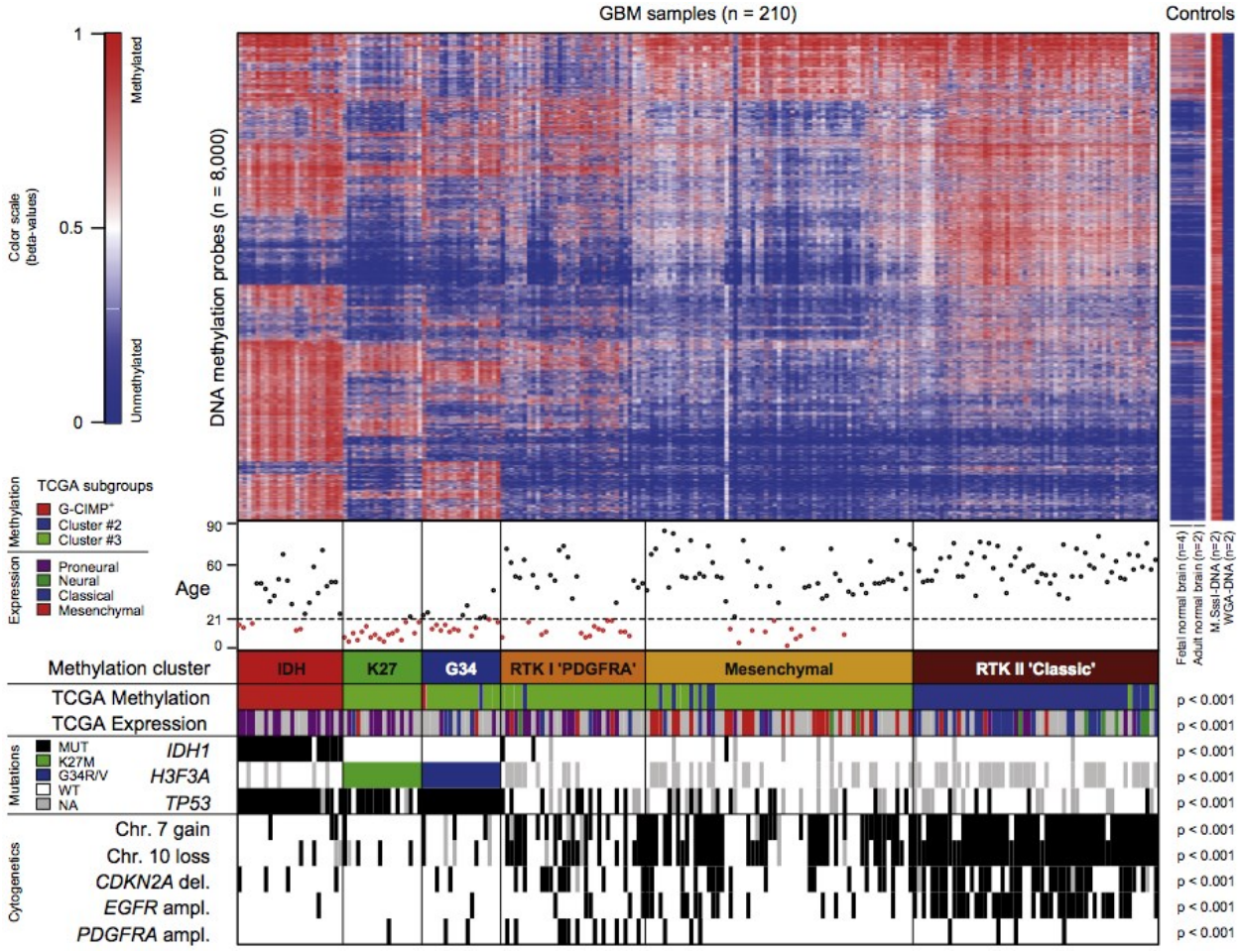

Figure 1.4 Six epigenetic GBM subgroups defined using DNA methylation patterns, distinct mutations, DNA copy-number alterations, and transcriptome patterns. Methylation heat map of all samples (n=210) and control samples clustered according to the six subgroups. Figure adapted from Sturm *et al.* (2012) [9].

To further emphasize the heterogeneous nature of GBM and perhaps illustrate the risk of "over-categorizing" any cancer type, when Hegi *et al.* classified the subset of GBMs that display an oligodendroglioma-like component they found that these patient samples could be classified

into two subtypes, the Proneural (36%) and Classical (43%) GBM subtypes defined by Verhaak *et al*., but could not identify any prognostic significance after adjusting for O^6-methylguanine-DNA methyltransferase gene (*MGMT*) status [10].

Each development in categorization illustrates the heterogeneity of GBM and the intensely complicated systems involved in this cancer. While studies often try to build off the classification systems created by other groups, there are still gaps within and between the various systems that emphasize the need for a more unified classification system to create integrated tumor profiles that are translatable to the clinical setting.

1.1.3 Treatment of GBM

Despite research efforts, GBM remains one of the most challenging cancers to treat and there is currently no curative therapy. Malignant gliomas are highly invasive and GBM is particularly aggressive; most patients die within one year of diagnosis with only 5.1% surviving for more than 5 years [1]. While there have been advances in understanding the pathways that contribute to GBM tumor biology, translation of this knowledge to patient treatment is slow and there has been limited success in the application of new therapies over the past decade [11].

Currently, standard treatment comprises maximal safe resection of the tumor mass or biopsy (if possible), followed by radiation therapy (RT) (6 weeks with a typical dose of 60 Gy) and chemotherapy with temozolomide (TMZ) (concomitant with 75 mg/m^2 daily, followed by at least 6 months adjuvant with 150-200 mg/m^2 for 5 days every 28 days) [12]. Unfortunately, there are significant limitations in each area of treatment. In newly diagnosed patients, the extent of resection bears prognostic significance, however, some tumor locations are not suitable for surgical intervention and these patients suffer a worse prognosis [11]. For patients who are eligible for surgery, complete surgical resection and RT are extremely challenging due to the diffuse and invasive nature of the cancer, which forms tentacle-like structures through the parenchyma of the brain [13]. Furthermore, choice of chemotherapy agent is limited, as many therapeutics cannot cross the blood-brain barrier and are thus unsuitable [11]. For these reasons, recurrence is common and treatment is essentially used to slow cancer progression, but may have only palliative effects [13].

While treatment of GBM is challenging, there are some prognostic factors that predict effectiveness. Hegi *et* al. showed that *MGMT* promoter methylation correlates favourably with treatment response to TMZ and these patients have a better prognosis [14]. However, despite this finding, standard of care is commonly very similar between patients and does not take into account molecular or epigenetic signatures of individual patient's tumors, as such testing may be uncommon in routine clinical settings outside of clinical trials [11].

There is no standard of care for recurrent GBM and therapeutic options are limited and generally not effective. If possible, patients may undergo a second surgery, which can be helpful diagnostically as many patients develop areas of necrosis from RT that may falsely register as tumor formation on MRI [11]. During surgery, physicians may have the opportunity to introduce chemotherapy wafers, such as Carmustine impregnated wafers, which modestly extend time to progression in patients with recurrent GBM [11]. However, re-irradiation, particularly with fractionated stereotactic RT is more common and has been shown to extend survival [11]. There are a few chemotherapy agents that may be employed, such as TMZ, nitrosoureas, platinoids, topoisomerase inhibitors, procardacine in combination with vincristine, and tamoxifen, but effectiveness is limited [11]. TMZ is used primarily in newly diagnosed GBM patients but has been assessed in treatment of recurrent tumors with varying dosing schedules and has been shown to improve survival for some patients [11]. Bevacizumab is an anti-vascular endothelial growth factor (VEGF) inhibitor that was approved by the Food and Drug Administration (FDA) for use in recurrent GBM in 2009. There is debate as to whether it should be used independently or in combination with cytotoxic drugs and additional studies are needed to clarify this issue and determine the appropriate duration of therapy, as patients may be kept on the drug indefinitely [11]. While it appears more clinical trials are necessary to optimize even current GBM treatments, testing potential treatments in a large cohort of patients is difficult for a disease that is relatively rare.

There are many features of GBM that make it an extremely difficult disease to treat and despite continued characterization of GBM heterogeneity, customized therapies are limited in availability and effectiveness. However, there is continued effort to define GBM subtypes that can be more easily translated to the clinical setting and to develop and test novel therapeutic agents for the treatment of GBM.

1.2 Radiation Therapy

1.2.1 Radiation Therapy Overview

Radiation therapy (RT), surgery, and chemotherapy are the three modalities commonly used in the treatment of cancer. RT involves the use of ionizing radiation for the treatment of malignant neoplasms. X-rays, gamma rays, and charged particles are used in RT and may be delivered via external-beam radiation, brachytherapy (internal RT), or systemic RT [15].

Ionizing radiation exerts its effects by removing electrons from their orbitals, causing the associated atoms to become charged or ionized. The energy exposure of tissues is referred to as the absorbed dose, with 1 Gy equivalent to 1 Joule/kg. Common dosages range from 60-80 Gy for treatment of solid tumors and 20-40 Gy for treatment of lymphomas.

The direct and indirect effects of RT are used to induce non-repairable cellular damage leading to necrosis, apoptosis, or senescence resulting in the destruction of cells and tissues [16]. In cancer treatment, RT aims to deliver a precise dose of radiation to a specific tumor volume while limiting damage to surrounding tissue. When successful, RT can shrink and potentially eradicate a tumor mass, improve quality of life, and prolong survival [17].

1.2.2 Radiation-Induced Cell Death

In the treatment of malignancies with RT, the principal goal is to inhibit cancer progression through the destruction of tumor cells. The major types of cell death induced by RT are apoptosis and mitotic catastrophe (Figure 1.5). Apoptosis is induced in cells with intact mechanisms for programmed cell death; however, most solid tumor cells have lost their pro-apoptotic mechanisms (for example, *TP53* mutations are observed in over half of all cancers). In such cases, apoptosis plays a minimal role in the destruction of malignant tissue and radiation-induced senescence and mitotic catastrophe are more significant in inducing cell death [18].

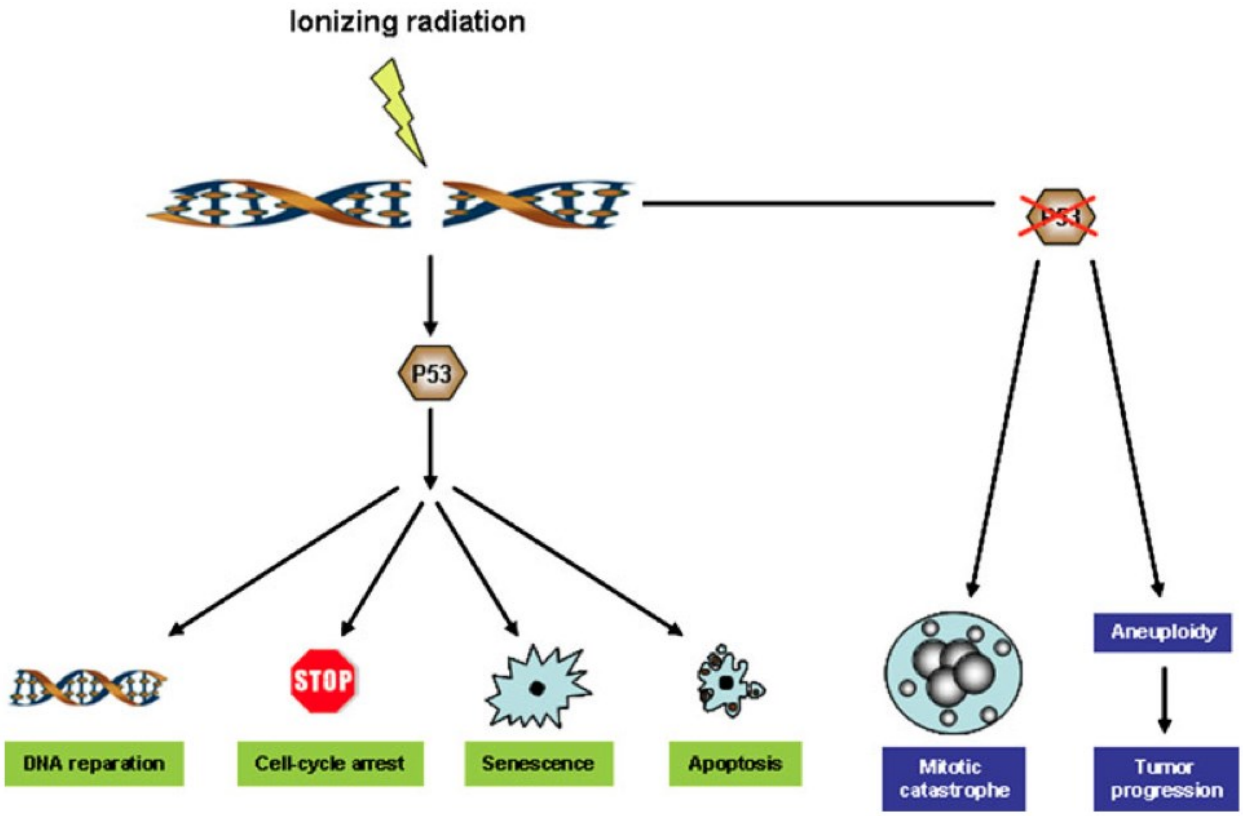

Figure 1.5 Cellular responses to ionizing radiation used in RT. Figure adapted from Halperin *et al.*, 2008 [18].

The p53 transcription factor is one of the most widely studied proteins in cancer biology. Its target genes play important roles in cell cycle arrest, DNA repair, apoptosis, and senescence. In cells possessing wild-type *TP53*, irradiation activates p53 resulting in growth cycle arrest and initiation of DNA repair mechanisms. In cells that experience extensive DNA damage, p53 may initiate apoptosis or senescence. Cells lacking functional p53 may bypass these processes and progress through the cell cycle, enabling cancer progression [18].

Radiation-induced apoptosis is the dominant form of cell death in lymphoid and myeloid derived cells. Successful induction of apoptosis requires the activation of caspase proteases 3, 6, and 7, which can be activated by radiation through either the intrinsic or extrinsic caspase activation cascades. However, radiation-induced damage may alternatively result in cell death via mitotic catastrophe [18].

Mitotic catastrophe is observed in most non-hematopoietic tumor cells and is the main mechanism of cell death observed in solid tumors in response to RT. Mitotic catastrophe refers to

cell death resulting from aberrant mitosis, which generates abnormal chromosome distribution and cell division, leading to the development of giant cells and unusual nuclear morphology. Mitotic catastrophe may result as a consequence of DNA damage in combination with deficient cell cycle checkpoints or the hyperamplification of centrosomes. Tumor cells often undergo mitotic catastrophe in response to DNA damage due to deficiencies in apoptotic signaling pathways (often the result of impaired p53 function) and ineffective cell cycle checkpoint signaling. Mitotic catastrophe results in a delay in mitosis and is followed by activation of the caspase cascade, which ultimately results in cell death [18].

A third possible response to irradiation is cellular senescence. The term senescence denotes a state of permanent cell cycle arrest and is induced by cellular stress resulting from DNA damage. Senescence is a normal part of the cell life cycle and occurs in cells that have come to the end of their proliferative life span. In response to low dose irradiation, the DNA damage response (DDR) is activated and pushes the cell into reversible cell cycle arrest in order to repair DNA damage. As stated previously, extensive DNA damage may result in cell death via apoptosis or mitotic catastrophe, or may alternatively drive the cell into a deeper state of repair and prolonged DDR signaling resulting in senescence. This form of senescence has a protective effect by preventing the transmission of DNA mutations to daughter cells. In tumor cells, induction and maintenance of the senescent state is promoted by p53 and typically complemented by p21 expression; however, radiation-induced senescence has been observed in the absence of both. While senescent cells do not proliferate, they stay metabolically active and are known to secrete both pro- and anti-tumorigenic factors. These opposing observations are of interest in cancer research and understanding whether senescent cells are promoting or inhibiting tumor growth remains controversial [18].

RT has a number of implications on cell functioning and exerts its toxic effects through various mechanisms. For this reason, it is clear that knowledge of the genetic profiles of tumors may help in predicting tumor response to ionizing radiation. Ultimately, a greater understanding of the molecular mechanisms involved in radiation-induced cell death and resistance will aid in the development of more efficacious RT treatments for patients.

1.2.3 Radioresistance in GBM

RT is an important arm of treatment in GBM and has been shown to extend survival in GBM patients [19]. However, radioresistance remains a significant barrier in successful response to treatment. Research in this area has focused on understanding the molecular mechanisms that regulate radiosensitivity and the identification of related markers that may translate to the clinical setting.

As previously discussed, p53-mediated apoptosis is an important mechanism of radiation-induced cell death [18]. Such response is observed in medulloblastoma, which is sensitive to RT and has a much better prognosis than GBM. When comparing wild-type *TP53* medulloblastoma cell lines to wild-type *TP53* GBM cell lines, Shu *et al.* observed radiation induced apoptosis in the medulloblastoma lines but not the GBM lines [20]. The source of this disparity was the inability of p53 to induce $p21^{BAX}$ expression in the GBM cell lines, as expression of $p21^{BAX}$ in these cell lines was sufficient to cause apoptosis. Limited $p21^{BAX}$ expression was linked to a lack of p53-mediated *BAX* promoter activation, which is inhibited in GBM cells possessing wild-type *TP53* [20]. It was proposed that this pathway could be exploited as a potential target to improve radiosensitivity in GBM cells [20].

In keeping with the above findings, *TP53* expression and mutation status showed no significant relationship with response to radiation in a study examining 170 GBM patients [21]. However, epidermal growth factor receptor (*EGFR*) expression was shown to be a predictor of poor radiation response [21]. Amplification of *EGFR* is observed in approximately 40% of GBMs and in about one third of these cases *EGFR* is also mutated [21]. This observation was an important characteristic of Verhaak *et al.*'s Classical GBM subgroup, 97% of which included GBMs with *EGFR* amplification but lacking *TP53* mutations. This subgroup was shown to have longer survival in response to radiation and chemotherapy treatment [7]. Conversely, a study in an orthotopic xenograft model found that *EGFR* amplification was not a predictor of GBM response to RT [22], suggesting there may be further intricacies mediating the role of *EGFR* in radiation response.

In vitro experiments have been used to examine the response of GBM cell lines to radiation. In a panel of six human GBM cell lines (A172, GB-1, T98G, U251MG, U373MG, U87MG) researchers discovered that the most radiosensitive cell line (measured by reduced proliferation) displayed transient increased p21 and p27 expression, corresponding with G_1 cell-

cycle arrest, while the most resistant cell line showed the inverse expression profile and did not arrest [23]. While researchers saw a dose-dependent reduction in cell proliferation, apoptosis was not observed in any of the cell lines, regardless of p53 status; instead, all cell lines formed acidic vesicular organelles as a likely defense mechanism against radiation-induced cellular toxins [23]. However, extended assays showed that even the most radiosensitive cell line returned to its normal proliferative state, which parallels the clinical response in patients who undergo RT only to have tumor reformation 6 to 8 months later [23].

More recently, micro RNAs (miRNAs) have been implicated in the resistance of GBMs to ionizing radiation. Xiao *et al*. showed that the expression of miR-135b confers radioresistance, with the discovery of its expression in U87R cells (a radioresistant cell line derived from U87MG) protecting cells from radiation, its knockdown reversing this protection, and its induction enhancing radioresistance in the parental U87MG cell line [24]. Glycogen synthase kinase 3 beta (GSK3β) was shown to be a direct target of miR-135b and this finding was reflected in GBM patient samples; increased miR-135b and reduction of GSK3β expression were observed compared to normal brain tissue, a trend that was even more pronounced in recurrent tumor samples developed after radiation [24].

Despite improvements in our understanding of the mechanisms that regulate response to ionizing radiation, there are still many unknowns and radioresistance remains a significant obstacle in GBM treatment. While research tends to look at specific pathways and proteins that are aberrantly expressed in radioresistant cells, it is likely the concerted effect of many processes that is modulating this resistance. Due to the varied response of different tissues and cancers to RT, it is reasonable to consider that there are additional unknown factors specific to GBM that modulate these response pathways.

1.3 O^6-methylguanine-DNA methyltransferase (MGMT)

1.3.1 MGMT Overview

Repair of DNA damage is vital in the prevention of cancer and many proteins and pathways have been implicated in the identification and repair of DNA damage. One such protein is O^6-methylguanine-DNA methyltransferase (MGMT), which is ubiquitously expressed in normal human tissues and is highly conserved phylogenetically [25, 26]. The essential conserved function of MGMT is alkyl transfer [27]. MGMT has been shown to act independently to remove alkyl adducts, specifically *O6*-methylguanine (*O6*-MeG), *O4*-methylthymine (*O4*-MeT), *O6*-ethylguanine, and *O6*-chloroethylguanine [27], via a stoichiometric reaction that transfers bound alkyl groups to a cysteine residue in its active site, irreversibly inactivating MGMT (Figure 1.6) [28]. *O6*-MeG and *O4*-MeT are the more carcinogenic adducts and their repair is critical in the maintenance of cellular integrity. Left unrepaired, these lesions are mutagenic, leading to incorrect base insertion at a rate of 90% [27].

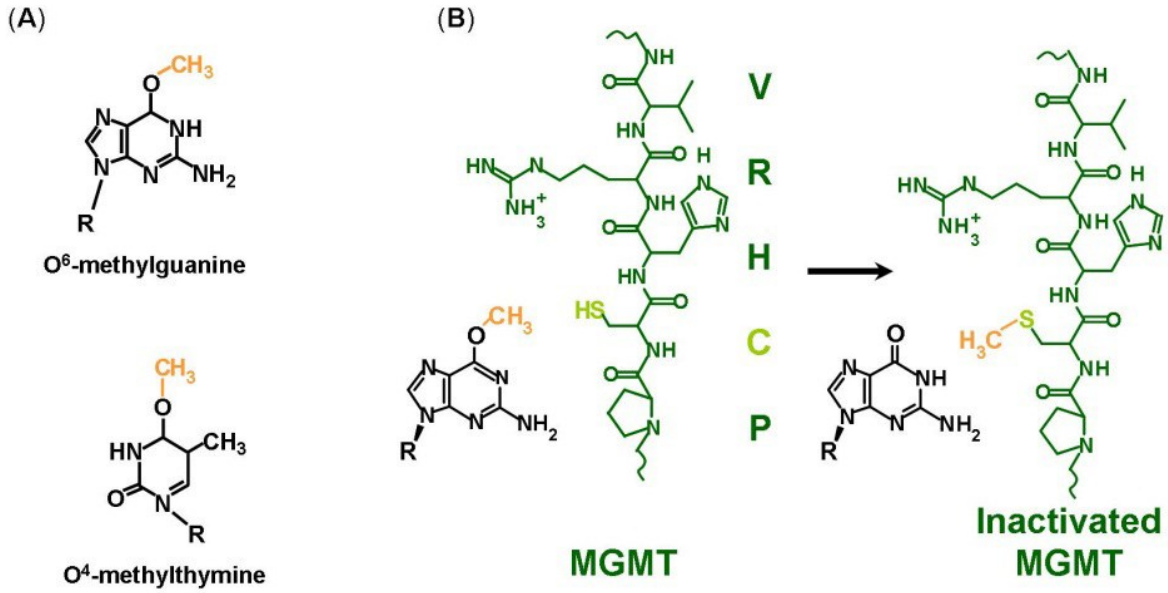

Figure 1.6 Mechanism of MGMT-mediated DNA repair. **(A)** The most carcinogenic MGMT DNA repair substrates, *O6*-MeG and *O4*-MeG **(B)** MGMT mediated repair of methylated DNA via transfer of the methyl adduct to Cys145 in the MGMT protein. Figure adapted from Nay *et al.* (2013) [29].

MGMT is unique among other DNA repair proteins in that it acts autonomously to carry out its function [27]. All other DNA base repair systems require the involvement of multiple enzymes: Nucleotide Excision Repair (NER), Base Excision Repair (BER), and Mismatch Repair (MMR) all require multiple proteins or protein complexes [27]. These repair systems have well characterized functions in proliferating cells while MGMT presents an anomaly; it is expressed at far greater levels than necessary to repair endogenous DNA lesions and is not necessary for DNA replication or cell survival [27]. However, MGMT expression is also down-regulated in many cancers suggesting a role in malignant transformation [27]. Fully understanding the role of MGMT is of interest in cancer research because of its protective function against carcinogens and alkylating chemotherapy agents and thus its potential as a target for inhibition.

MGMT is intrinsically able to independently mediate all aspects of its DNA repair function. Research has identified conserved structural motifs of MGMT and its protein homologs in other species, as well as conserved amino acid residues within MGMT, that mediate DNA recognition, selectivity and binding, alkyltransferase activity, and degradation [30]. The act of DNA repair begins with DNA-bound MGMT moving across double-stranded DNA, flipping each base into its active site, permitting transfer of an alkyl group from the DNA to the active Cys145 residue [27]. Mutation of this or surrounding residues reduces or eliminates alkyltransferase activity through protein destabilization [31, 32]. Transfer of an alkyl group results in a conformational change in the MGMT protein, which releases it from the DNA and prepares the protein for ubiquitylation and degradation [27]. MGMT is comprised of two domains, with residues involved in DNA binding, nucleotide flipping, and the active site pocket located in the C-terminal domain. The N-terminal domain is involved in maintaining the C-terminal domain in an active configuration and may have other functions beyond this structural role [33]. For example, residues Glu45 to Gly55 are believed to form a "hydrophobic handle" that may anchor MGMT to other proteins [34]. Although MGMT has been mostly described as a nuclear protein, its cytoplasmic localization has also been reported [35, 36]. Additionally, the presence of MGMT has been shown to affect the localization of other proteins. For example, the MGMT enhancer binding protein (MEBP) is normally present in both the nucleus and the cytoplasm of MGMT-expressing cells; however, in MGMT-deficient cells, MEBP is present in the cytoplasm only [37].

MGMT levels have been shown to correlate to resistance to treatment. For example, increased MGMT expression is observed in tamoxifen-resistant breast cancer cells and has been shown to be significantly elevated in patients who have undergone failed tamoxifen treatment [38]. Additionally, MGMT expression has been reported to have an inverse relationship with angiogenesis and invasion in GBM cells [39]. Specifically, MGMT positive GBM cells have been shown to express increased levels of vascular endothelial growth factor receptor 1 (VEGFR-1) and decreased levels of VEGFR-2, compared to MGMT negative GBM cells [40]. While the DNA repair of MGMT is well characterized, the possible involvement of MGMT in cellular functions other than DNA repair has not been fully investigated.

1.3.2 Regulation of MGMT

There are several mechanisms of *MGMT* regulation beyond the self-regulation of protein activity previously described.

It is well established that *MGMT* promoter methylation is a cause of *MGMT* transcriptional silencing [41-49]. Interestingly, *MGMT* promoter methylation is observed only in tumors and is seen in tandem with aberrant promoter methylation of other cancer-related genes, such as *CDKN1A* (encodes p21/WAF1), *MLH1*, and *CDKN2A* (encodes p16/INK4A) [27]. While the causes of *MGMT* promoter methylation are unclear, the resulting lack of MGMT protein activity may permit the mutagenesis of *O6*-MeG adducts and contribute to malignant transformation [27]. In support of this, Gilliland *et al*. showed an increased risk of *MGMT* and *p16* promoter methylation in the respiratory epithelium of individuals at high risk of developing lung cancer, suggesting it to be an important step in respiratory carcinogenesis [50].

MGMT expression is also suppressed by expression of wild-type *TP53*. This was shown to occur through p53 binding of the Sp1 transcription factor, preventing its interaction with the *MGMT* promoter in HCT116 cells, an affect that was reversed with overexpression of Sp1 [51].

Alternatively, *MGMT* expression may be induced through promoter interaction. For example, tricostatin A, a specific inhibitor of histone deacetylase, has been shown to increase *MGMT* mRNA and protein expression in promoter methylated cells through chromatin remodeling, increasing binding of the transcriptional co-activator cyclic AMP response element-binding protein (CBP)/p300 to the *MGMT* promoter and inducing gene expression [52]. Several

recognition sequences within the *MGMT* promoter permit the induction of *MGMT* expression by glucocorticoids, cyclic AMP, and protein kinase C activators [27]. Additionally, *MGMT* expression can be induced by DNA damage; for example, *MGMT* mRNA expression is increased up to 4.5 fold with cellular exposure to *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) or ionizing radiation [53]. However, increases in *MGMT* expression do not seem to correlate directly to DNA alkylation, suggesting that *MGMT* transcriptional activation occurs in response to multiple signals of DNA damage [27].

MGMT protein expression correlates poorly with mRNA expression, suggesting mechanisms of protein regulation beyond promoter methylation [54]. Post-translation mechanisms of regulation have been described, specifically the role of miRNAs—which operate independently of promoter methylation—in the regulation of MGMT protein expression. In 2013, Quintavalle *et al*. showed that miR-221 and miR-222 target MGMT mRNA and are upregulated in GBM patients. Researchers observed a negative correlation between miR-221/222 expression and *MGMT* mRNA and protein expression, showing that miR-221/222 negatively regulated *MGMT* expression through direct targeting of the *MGMT*-3'UTR in a panel of glioma and GBM cells. However, they also reported an increase in *MGMT* expression in U87MG cells (MGMT negative cell line) with transfection of miR-221/222, which was not addressed [55]. Kreth *et al*. showed that there are two *MGMT* transcripts produced with varying 3'-UTR length, the longer of the two containing an additional alternative poly(A) site. GBM samples with "medium" and "low" MGMT expression exhibited a 3.2 and 5.1 fold increase of the longer transcript variant, respectively, compared to "high" expressing MGMTs, which had an expression profile similar to that of normal brain. The longer transcript was shown to lower MGMT protein expression due to the inclusion of specific miRNA binding sites within the elongated 3'-UTR, which were acted upon by miR-181d, miR-767-3p, and miR648 to negatively mediate *MGMT* expression via mRNA degradation or translation inhibition in GBM cells [56].

MGMT protein is also present as a phosphoprotein under physiological conditions [57]. Protein phosphorylation was shown to reduce the activity of recombinant MGMT by 30%-65% while protein dephosphorylation stimulated activity, suggesting that phosphorylation may act as a control mechanism of MGMT activity [57]. The ubiquitin-proteasome pathway further mediates MGMT activity, with phosphorylated MGMT proteins targeted for degradation through this pathway [58]. MGMT contains a nuclear localization signal and an accessory protein enables

its movement to the nucleus. Additionally, phosphorylation has also been shown to increase the nuclear localization of MGMT [27].

The existence of various mechanisms of *MGMT* regulation, which act in addition to the self-deactivating stoichiometric reaction MGMT undergoes upon DNA repair, suggests there are pathways exerting effects on MGMT activity in response to cellular events outside of DNA alkylation.

1.3.3 Role of MGMT in Chemo- and Radioresistance in GBM

Elucidating the molecular mechanisms that control *MGMT* expression is of major clinical relevance. *MGMT* expression has conclusively been shown to prevent cancer in various mouse models—including the MNU-induced thymic lymphoma mouse model, MNNG33-induced two-stage skin tumors, and NNK-induced lung tumors—by removing induced *O6*-MeG adducts [27]. *MGMT* overexpression has also been shown to have a protective effect in mice with DNA mismatch repair deficiencies or loss of wild-type p53.

The DNA repair function of MGMT is significant not only for the removal of adducts induced by alkylating environmental agents but also because it influences the effectiveness of alkylating molecules used in chemotherapy treatment of various cancers, including GBM.

The current standard of care chemotherapy agent used in the treatment of GBM is temozolomide (TMZ). TMZ is a monofunctional DNA alkylating agent that methylates DNA at the *N7* position of guanine (*N7*-MeG), the *N3* position of adenine (*N3*-MeA), and the *O6* position of guanine (*O6*-MeG) residues. TMZ imparts a significant therapeutic benefit to GBM patients, improving median survival time from 12.2 months to 14.6 months for patients receiving surgery, RT, and TMZ, opposed to RT alone (Figure 1.7) [12, 59].

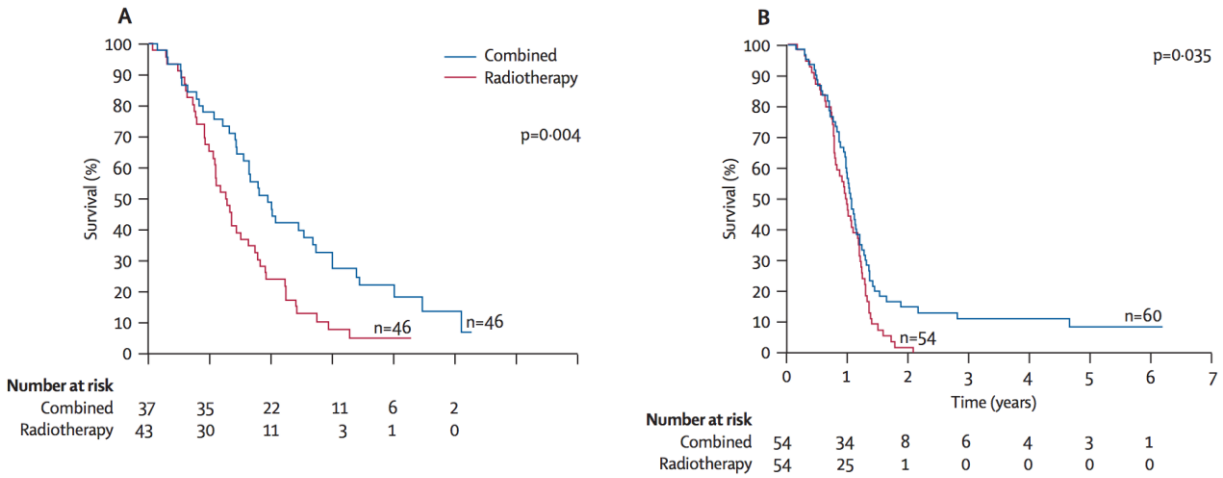

Figure 1.7 Role of MGMT promoter methylation in GBM patient survival with respect to TMZ treatment. Kaplan-Meier estimates of overall survival of patients with methylated *MGMT* promoters **(A)** and unmethylated *MGMT* promoters **(B)**. Figure adapted from Stupp *et al*. (2009) [59].

The cytotoxicity caused by TMZ is predominantly attributed to the *O6*-MeG lesion. While *N7*-MeG and *N3*-MeA lesions are rapidly restored to their native forms by BER, the *O6*-MeG lesion is directly repaired by MGMT. However, if the *O6*-MeG lesion is not repaired it will mispair with thymine (instead of cytosine) causing a GC→AT transversion. This mismatch may be recognized by MMR, which will excise the mispaired thymine residue on the daughter strand of the DNA but will not repair the modified *O6*-MeG on the template strand. This will result in continuous thymine mismatching and excision, eventually causing the replication fork to collapse which should push the cell into cell cycle arrest and trigger apoptosis [60]. Because of this, TMZ treatment is most effective at inducing cell death in cells with low levels of MGMT and functioning MMR [60].

It is established that *MGMT* promoter methylation is a cause of *MGMT* transcriptional silencing, which translates clearly to patient survival times in GBM [61]. Approximately 50% of patients diagnosed with GBM have a methylated *MGMT* promoter and *MGMT* promoter methylation status is a positive predictive marker for response to treatment: there is a 46% 2 year survival of those with promoter methylation compared to 14% for those without promoter methylation [61]. When MGMT protein is present, it is essentially able to reverse the cytotoxic damage potential of the *O6*-MeG lesion induced by TMZ treatment, rendering chemotherapy

much less effective. However, TMZ resistance may develop and an MGMT-dependent mechanism of resistance has been observed *in vitro* [62]. GBM cell lines (SNB19VR and U373VR) exposed to increasing TMZ doses generated TMZ-resistant cell lines. U373VR resistant cells showed increased expression of MGMT protein but become re-sensitized to TMZ with pharmacological depletion of MGMT [62].

There is limited information regarding the mechanistic relationship between *MGMT* expression and response to ionizing radiation in GBM. However, induction of *MGMT* mRNA expression and activity have been observed in human cell lines in response to radiation and other DNA-damaging agents, suggesting general genotoxic induced upregulation [63, 64]. Exposure of H4IIE rat hepatoma cells to ionizing radiation has been shown to increase *MGMT* mRNA levels up to 4.5 fold through *MGMT* promoter induction [53]. Elevated *MGMT* mRNA was noted 6 hours after ionizing radiation treatment, reaching peak expression levels at the 24 hour time point. Notably, induction times varied with mutagenic treatment (alternatively treated with MNNG), indicating different mechanisms of induction in response to different damaging treatments [53]. It has been shown that radiation-induced *MGMT* expression may be influenced by hydroxyl radical-mediated DNA damage, as the addition of a hydroxyl radical scavenger (DMSO) abolished radiation-induced MGMT response but did not impact protein function [64]. Furthermore, addition of H_2O_2 (source of activated oxygen species with radiation exposure) was shown to increase MGMT activity after radiation exposure [64]. While there is limited information regarding the details of radiation-induced MGMT expression and activity, it is evident that tissue or cancer-specific responses could have significant implications for patient care; particularly in GBM, which exhibits varied MGMT expression and incorporates RT as a key modality of treatment.

In GBM, MGMT has clear implications on patient survival and thus understanding the processes that regulate MGMT expression is extremely important.

1.3.4 MGMT-Binding Partners

Owing to the clinical significance of MGMT as a DNA repair protein involved in resistance to alkylating agents, many studies have focused on the structural and functional aspects of how MGMT binds to DNA and exerts its alkyltransferase activity. While MGMT is

known to exercise its DNA repair function independently of other proteins [26, 27, 65, 66], increasing evidence suggests the role of MGMT as an ancillary protein mediating other functions through interactions with binding partners in other cancer types.

In the nucleus, MGMT localizes both diffusely and in concentrated small foci or "speckles" at active sites of transcription [67]. These speckles are depleted when cells are exposed to alkylating agents, as MGMT is converted to its inactive form after correcting mutagenic *O6*-MeG lesions [67]. While MGMT inherently possesses the ability to repair such damage, support of a co-factor may aid in repair efficiency. In breast cancer cell lines, active MGMT co-precipitates with the CBP/p300-containing complex known to activate the transcription activities of nuclear receptors. As a component of the CBP/p300-containing complex, MGMT couples DNA repair events and transcription regulation [68]. Additionally, MGMT has been shown to bind BRCA2, with increased binding in response to alkylation [69]. The E6 human papillomavirus oncoprotein has been shown to bind MGMT and form a complex with the cellular ubiquitin-ligase E6-associated protein (similar to its targeting of tumor suppressor genes such as p53) for inactivation through the ubiquitin-proteasome pathway [58]. Proteomic analysis of MGMT-binding partners in HT29 human colon cancer cells enabled the identification of 60 MGMT-interacting partners, which may integrate DNA damage and repair signals with replication, cell cycle progression, and genomic stability [70].

A novel functional role of MGMT is likely not exclusive of its primary function of DNA repair, but may integrate DNA repair with an additional function to produce other responses (as hypothesized in human colon cancer cells [70]). Multiple functions of MGMT could work in tandem to signal cellular pathways, having major implications in the understanding of how MGMT affects cellular functions on a larger scale.

The potential for novel MGMT functions and MGMT interactors has been examined, though in limited contexts. MGMT is a downstream effector of the transcription factor zinc finger E-box binding homeobox 1 (ZEB1), which influences invasion, chemotherapy resistance, and tumorigenesis in invasive GBM cells [71]. In human pancreatic cancer cells, decreased MGMT expression through depletion with O^6-benzyl guanine (O^6BG) is correlated with decreased p53 activation, increased cell apoptosis, and significantly reduced tumor growth *in vivo* [72]. In addition to the increased motility and invasiveness of MGMT-deficient esophageal cancer cells compared to MGMT-proficient, MGMT-deficient and MGMT-proficient cells have

been shown to differentially express 19 proteins, most notably Ezrin, the membrane-cytoskeleton linker protein [73].

The potential for MGMT-binding partners in GBM represents an area of research that has not been investigated. However, due to the clinical significance of MGMT and the inverse relationship discovered between MGMT expression and GBM angiogenesis and invasion [40, 74], the elucidation of MGMT-binding partners and their functional significance would aid in the understanding of GBM biology and may translate to patient care through the development of targeted chemotherapy agents.

1.4 Heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1)

1.4.1 hnRNPA1 Overview

RNA binding proteins (RBPs) regulate the maturation of RNA through control of the capping, splicing, polyadenylation, nuclear export, stability, and translation of messenger RNA (mRNA) through binding of specific sequences or secondary structures within transcripts [75]. Heterogeneous nuclear ribonucleoproteins (hnRNPs) are RBPs that have important roles in the maturation of mRNA [76]. hnRNP proteins are expressed in all tissue types, though their abundance and relative stoichiometry vary [75].

mRNAs are produced by post translational modification of primary transcripts produced by RNA polymerase II; these primary transcripts are called heterogeneous nuclear RNAs (hnRNAs). (While the terms hnRNA and pre-mRNA are often used synonymously, only a subset of hnRNAs actually form mRNA and the remainder are recycled [77].) The hnRNP designation is one of exclusion: they are the class of protein which bind hnRNA but are not stable components of other ribonucleoprotein complexes [77]. Through direct binding, hnRNP proteins affect the structure of hnRNA and permit or block their interactions with other ribonucleoproteins. These interactions affect the processing of pre-mRNA and thus also influence protein expression [77].

hnRNP proteins are identified alphabetically, with at least 20 major proteins (A1 to U) and other less abundant minor proteins [77]. hnRNPA1 is a member of the A/B subfamily of hnRNP proteins, along with A2/B1, A3, A0, B2, and AB [75]. The A/B proteins share a similar structure of two RNP-motif RNA binding domains and a glycine-rich auxiliary domain at the carboxyl terminus, which mediates protein interaction [77]. The A/B proteins have important functions in pre-mRNA processing and are known to shuttle between the nucleus and the cytoplasm with a role in mRNA transport [76]. The M9 sequence in hnRNPA1 acts both as its nuclear export and localization signal [75]. hnRNPA1 expression varies in relation to cell cycle and state of proliferation [77].

It was originally thought that hnRNP proteins bound non-preferentially to hnRNA but it is now known that binding affinity functions on a spectrum [77]. hnRNP proteins are abundant in the nucleus and it has been proposed that non-specific binding may facilitate the search for higher affinity binding sites [77] or that local concentration of hnRNP proteins influence binding

accessibility [75]. The hnRNPA/B family have been shown to bind transcripts both non-specifically and with distinct preference for specific sequences; however, the molecular basis for this binding dichotomy is poorly understood [75]. hnRNP proteins A1, C, and D have been shown to preferentially bind intron sequences at or near the 3' splice site and the majority of RNA molecules bound by A1 contain sequences the resemble 5' and 3' splice sites [77].

hnRNPA1 is one of the most prevalent and ubiquitously expressed hnRNP proteins and has significant impact on gene expression through proposed actions in splicing (as an exonic repressor), mRNA transport, and telomere biogenesis [75, 76]. There are two experimentally validated hnRNPA1 transcript variants: the full length isoform, A1-B, and the shorter variant, A1-A [75]. The A1-A isoform is over 20 times more plentiful than A1-B in normal tissues [75]. The A1 protein can interact with both single-stranded and double-stranded DNA, as well as RNA; however, hnRNPA1 shows a clear affinity for AUUA-rich sequences found in 3'-untranslated region and the UAGA(G) motif [75]. hnRNPA1 undergoes post-translational modification in the form of methylation, phosphorylation, and SUMOylation [75]. Protein kinase C (PKC) and Mitogen-Activated Protein Kinase (MAP)-Interacting Kinases (MNKs) phosphorylate hnRNPA1, reducing its capacity to bind certain RNA sequences and localize properly [75].

hnRNPA1 is known to regulate transcriptional events through interaction with various promoter sequences [75]. It has been shown to both reduce (by binding to promoter genes for thymidine kinase, γ-fibrinogen, and vitamin D receptor) and activate transcription (ApoE) [75]. hnRNPA1 is also known to bind specific DNA structures and may facilitate transcription by unwinding G-quadruplex DNA structures within promoter regions. Specifically, human KRAS and c-Myc promoters contain these types of structures and are located close to hnRNPA1 binding sites [75]. hnRNPA1 may also exert control over transcriptional events through protein-protein interactions with transcription factors. For example, hnRNPA1 can bind the inhibitory subunit of NF-κB alpha (IκBa), activating NF-κB [75].

hnRNPA1 participates in spliceosome assembly and is a modulator of alternative splicing via splicing repression [75]. hnRNPA1 is a key regulator of expression for several genes related to development and response to stimuli and disease in human and viral genes, as shown in Table 1.1 [75], and may exert one of multiple mechanisms to modulate splicing. hnRNPA1 may compete with members of the serine-arginine-rich (SR) family of splicing activators for mutual

binding sites in alternatively spliced exons, regulating the proportion of inclusion or exclusion of the exon [75]. In the absence of mutual binding sites, hnRNPA1 may bind cooperatively along an exon, even displacing other bound proteins, to physically block the binding sites of other splicing factors and repress splicing [75]. There are also specific intron binding sites for hnRNPA/B proteins, which may prevent binding of splicing regulators on intron areas [75]. hnRNPA1 is proposed to mediate its own expression through alternative splicing of the *hnRNPA1* exon 7B via a "looping out mechanism" that suggests two hnRNPA1 proteins bind either side of the exon and interact with each other through their Gly-rich domains and "loop out" the exon [75]. hnRNPA1 is also proposed to interact with other splicing regulators to exert splicing control and act cooperatively with other hnRNP proteins to regulate the same set of genes [75]. Continuous research reveals the extensive impact of hnRNPA1 on alternative splicing across the human transcriptome and the various modalities used to exert this function [75].

Gene	Organism	Splicing Event
Medium-chain acyl-CoA dehydrogenase (MCAD)	Human	Exon 11 skipping
Myelin-associated glycoprotein (MAG)	Human	Exon 12 skipping
Interferon regulatory factor-3 (IRF-3)	Human	Exons 2 and 3 skipping
TNF Receptor Superfamily Member 6 (Fas)	Human	Exon 6 skipping
Ras-related C3 botulinum toxin substrate 1 (Rac 1)	Human	Exon 3b skipping
Insulin receptor gene (INSR)	Human	Exon 11 skipping
Breast cancer 1 (BRCA1)	Human	Exon 18 skipping
Breast cancer 1 (BRCA1)	Human	Exon 6 skipping
Homeodomain interacting protein kinase 3 (HIPK3)	Human	Testis-specific Exon skipping
Bovine growth hormone (BGH)	Bovine	Exon 5 skipping
Survival of Motor Neuron 2, (SMN2)	Human	Exon 7 skipping
Fibroblast growth factor receptor 2 (FGFR2)	Human	K-SAM exon skipping
Amyloid precursor protein (APP)	Human	Exon 7 and 8 skipping
Dystrophin	Human	Exon 31 skipping
β-tropomyosin	Chicken	Exon 6B skipping
pX region	Human T-cell leukemia virus type 1 (HTLV-1)	Exon skipping
V-Ha-ras Harvey rat sarcoma viral oncogene homolog (C-H-ras)	Human	Exon IDX skipping
Proto-oncogene tyrosine-protein kinase Src (c-Src)	Human	Exon N1 skipping
Trans-activator of transcription (Tat)	Human immunodeficiency virus type 1 (HIV-1)	Exon 3 3' ss respression
Trans-activator of transcription (Tat)	Human immunodeficiency virus type 1 (HIV-1)	Exon 2 3' ss respression
Carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM1)	Human	Exon 7 skipping
heterogeneous ribonucleoprotein A1 (hnRNPA1)	Human	Exan 7B skipping
Puryvate kinase (PKM)	Human	Exon 9 skipping
Viral protein R (VPR)	Human immunodeficiency virus type 1 (HIV-1)	Repression 3' splice site A2
E6/E7	Human papillomavirus type-16 (HPV-16)	E6 exon skipping

Table 1.1 Role of hnRNPA1 in alternative splicing. Figure adapted from Jean-Philippe *et al.*, 2013 [75].

Beyond splicing and localization, hnRNPA1 is also involved in regulating mature mRNA expression through the modulation of mRNA turnover and translation [75]. hnRNPA1 has been

observed in cap-dependent and -independent translation mechanisms with the effect of selecting for specific translation initiation sites and in a few cases, inhibiting translation [75].

While the full range of hnRNPA1 functions has not been elucidated, its presence has a significant impact on gene expression, which has been shown to be important to multiple cellular processes.

1.4.2 Role of hnRNPA1 in Cancer

Because of their extensive binding potential, the repercussions of hnRNP protein expression ripple through all areas of cellular function and play key roles in human disease. hnRNPA1 is often overexpressed in a variety of cancers, including breast, colorectal, lung, and gliomas [75], and is known to promote tumor invasion in hepatocellular carcinoma [78]. In keeping with this role, hnRNPA1 knockdown in cancer cells results in apoptosis [79], while its expression has anti-apoptotic effects, potentially mediated through splicing of caspase 2 pre-mRNA [80].

Alternative splicing of mRNA precursors provides an opportunity for the creation of multiple protein isoforms with potentially diverse functions, a process commonly co-opted by cancer cells as a means to produce proteins that promote growth and survival. For example, hnRNPA1 is known to modulate the alternative initiation and enhance translation of the pro-angiogenic human fibroblast growth factor 2 (FGF-2) mRNA, with short interfering RNA-mediated knockdown of hnRNPA1 inhibiting translation [81].

hnRNPA1 has also been shown to negatively regulate expression of human Let-7a miRNA, which has known functions in cancer and pluripotency [82]. Unfortunately, there is limited knowledge regarding hnRNP-targeted miRNAs, but their description may reveal additional pathways modulated by hnRNPA1 [75].

Finally, hnRNPA1 has been implicated in larger scale processes vital to cellular transformation. Cancerous cells [83] and noncancerous cells induced to proliferate [84] preferentially undergo aerobic glycolysis, a process believed to provide many of the precursors necessary for growth [85]. The change from oxidative phosphorylation to aerobic glycolysis includes a shift in pyruvate kinase isoform expression, switching to production of the embryonic pyruvate kinase isoform (PKM2), which appears universally expressed in tumor cells [85].

hnRNPA1 is one of three hnRNP proteins (along with polypyrimidine tract binding protein [PTB/hnRNPI] and hnRNPA2) that have been shown to promote the mutually exclusive alternative splicing that results in the formation of PKM2 (the embryonic isoform) over PKM1 (the adult isoform). Additionally, the oncogenic transcription factor c-Myc upregulates production of these three hnRNP proteins, maintaining the increased ratio of PKM2 to PKM1 in cancer cells [86]. In a panel of human gliomas, overexpression of these three hnRNP proteins correlated with increased PKM2 mRNA expression, with highest overexpression observed in GBM samples, implicating these proteins in a regulatory pathway essential to tumor cell proliferation [86]. Upregulation of hnRNPA1 and A2 proteins is broadly observed in cancer, implying common pathways may support this expression profile across many cancer types [85].

Alternative splicing has an extensive impact on cell functioning, with metabolism, apoptosis, cell cycle control, invasion, metastasis, and angiogenesis of tumor cells all affected by changes in alternative splicing [85]. Due to its implications in mRNA processing, targeting the hnRNPA1 protein may prove useful in the development of novel cancer therapeutics for a variety of cancers [75].

1.5 Annexin A2

1.5.1 Annexin A2 Overview

Annexins are a family of calcium-dependent, membrane-bound proteins characterized by a unique "annexin core" domain, which enables their docking on negatively charged phospholipid membranes [87]. Each annexin protein includes a highly variable N-terminal domain that contains binding sites for cytoplasmic proteins, which can be directed to the membrane via the core domain [87].

While the structures of annexin proteins are well characterized, their exact functions have not been fully defined [87]. Membrane-bound annexins can form self-assemblies to affect the organization of membrane lipids, affecting membrane-domain organization, endocytosis, and exocytosis [87]. As membrane-binding requires Ca^{2+}, Ca^{2+} levels have implications on the membrane functions of annexin proteins [87]. Furthermore, annexins are known to bind a variety of cytosolic proteins via their N-terminal domains, which may be the target of post-translational modification by phosphorylation. Annexin A2 tyrosine phosphorylation is catalyzed by the Src-family of tyrosine kinases and results in altered Ca^{2+} affinities of the protein, with implications on membrane functions [87].

S100A10 (P11) can bind two annexin A2 monomers on different membranes bringing into contact and creating a connection between membrane surfaces. Annexins also have the ability to form lateral assemblies on model membranes. Different annexin proteins appear to form different assemblies and annexin A2 forms monolayer clusters that coincide with the presence of certain membrane lipids. These formations may be involved in annexin mediated or supported formation of some phospholipid domains. Annexin A2 can bind to phosphatidylinositol-4,5-bishosphate ($PtdIns(4,5)P_2$), which is important in the development or stabilization of actin assemblies at cellular membranes [87].

In addition to binding membranes based on composition, some annexins, including A2, can bind membranes in a Ca^{2+}-dependent manner. It is believed that two or more annexin cores bound to different membranes interact with each other to foster interaction between membranes. The annexin-A2−S100A10 complex described earlier is a heterotetrameric complex with the S100 protein acting as a link between the membrane-bound annexin molecules. *In vitro* data and in *vivo* experiments with *Caenorhabditis elegans*, provide support for a role of annexins in

membrane aggregation, as opposed to membrane fusion. While this induced membrane interaction is an interesting part of annexin A2 function, the mechanisms involved are unknown [87].

Annexins have been shown to be involved in membrane domain formation *in vitro* and *in vivo*. Annexin A2 is involved in membrane lipid aggregation at locations of actin cytoskeleton attachment. Support for annexin A2 involvement came from studies showing annexin A2 as a component of the filamentous (F)-actin-rich comet tails that drive endocytic vesicles from the plasma membrane to the interior of the cell, with annexin A2 mutation inhibiting this action. Annexin 2 is also a component of the F-actin formations observed at sites of membrane attachment of enteropathogenic *Escherichia coli*. Actin reorganization at PtdIns(4,5)P_2 enriched membrane sites is an important characteristic in both of the above processes. Additionally, Annexin A2 binds actin directly and may act as a platform to permit F-actin interaction. These roles suggest the importance of annexin A2 in cellular signaling and maintenance of cell-to-cell contact [87].

There are also factors outside of Ca^{2+} concentration that affect annexin transport and localization. The annexin A2 N-terminal domain contains a nuclear export signal that is recognized by the leptomycin-sensitive nuclear transport machinery, which limits its presence in the nucleus. However, in the presence of tyrosine-protein phosphatase inhibitors, heightened levels of annexin A2 are observed in the nucleus, suggesting that tyrosine phosphorylation may result in a conformational change which permits annexin A2 entry into the nucleus [87].

Annexin A2 also has a contested role in Ca^{2+}-mediated exocytosis. Addition of annexin A2 was shown to block the secretory ability of permeabilized chromaffin cells but a dominant-negative mutation in annexin A2 did not impact exocytosis in neuroendocrine PC12 cells. More recent work in endothelial cells suggests that annexin A2 may only be involved in a subset of Ca^{2+}-dependent exocytosis, as downregulation of annexin A2 and disruption of the annexin-A2−S100A10 complex was shown to inhibit exocytosis of Weibel-Palade bodies specifically, without impacting other forms of Ca^{2+}-regulated exocytosis [87].

However, annexin A2 has a more established role in endocytosis and is known to possess an endosome targeting sequence. Overexpression of dominant interfering mutants has a direct consequence on endosome morphology and transport. Downregulation of annexin A2 has been shown to impede internalization of EGFR and depletion results in the irregular appearance of

perinuclear Rab11-positive recycling endosomes. Annexin A2 is proposed to be a membrane scaffold, necessary for the establishment of endosomal tubules [87].

Annexins can also act as regulators of ion channels. The annexin-A2−S100A10 complex has a proposed role in the regulation of Cl^- channels. S100A10 is known to interact with various ion channels and, typically in complex with annexin A2, is necessary for the transport of various ion channels to the plasma membrane. Annexins themselves may also function as Ca^{2+} channels, though this role is heavily disputed [87].

Annexin A2 also possesses extracellular functions and one of its most well characterized roles is in the plasminogen activation system, which is important in thrombolysis, wound healing, and cancer progression [88]. Annexin A2 is a component of the cell surface complex that binds tissue plasminogen activator (tPA) and plasminogen, bringing them into close proximity, catalyzing the activation of inactive zymogen plasminogen to active serine protease plasmin and protecting the reaction from inhibitors [88].

Reflective of its abundance and locations in the cell, annexin A2 expression is significant in both intracellular and extracellular activities. While the full extent of annexin A2 activity is unknown, its importance is reflected in the changes observed in *in vitro* and *in vivo* knockout models.

1.5.2 Role of Annexin A2 in Cancer

Annexin A2 has roles in a range of cellular processes, including exocytosis, endocytosis, membrane trafficking, and plasmin activation [87]. While the full extent of these functions has not been exposed, annexin A2's importance in normal cell functioning is apparent as its knockdown negatively regulates cell division and proliferation [89], which may bear significance in the context of cancer cells. Furthermore, annexin A2 is radiosensitive, revealing itself as a potentially important protein in the treatment of patients using RT. Increased annexin A2 protein levels and translocation to the nucleus are observed upon radiation exposure in human skin organotypic culture and murine epidermal cells [90]. Whole genome expression profiling of annexin A2 positive and negative (shRNA knockdown) cells showed changes in the radioresponsive transcriptome (namely, enrichment of NF-κB, STAT1, EGFR, p63, PPAR-g, and c-FOS signaling networks in annexin A2 positive cells and enrichment of EGR1, OCT1,

GATA-1, SRF, AP-2, and SP3 signaling networks in knockdown cells), suggesting a role for annexin A2 in radioresistance. [90]. Consistent with this observation, annexin A2 has been shown to move to the nucleus in response to additional genotoxic agents and when depleted, cells showed increased levels of phospho-histone H2AX and p53, suggesting a role for annexin A2 in DNA repair [91].

Elevated annexin A2 expression has been observed in GBM and positively correlates with the histological grade of gliomas and central nervous system dissemination [92-94]. Overexpression of annexin A2 correlates with increased plasmin activity on the tumor cell surface, which mediates degradation of the extracellular matrix and promotes neoangiogenesis [92]. Annexin A2 has been shown to localize to the pseudopodia of invasive glioma cells [95] and knockdown of annexin A2 in glioma cells decreased tumor size and slowed tumor progression *in vivo* by reducing invasion, angiogenesis, and proliferation, while increasing apoptosis [92]. Recently, annexin A2 was shown to regulate the angiogenesis and invasion phenotypes of malignant gliomas, with expression positively mediating the adhesion of glioma cells to vascular endothelial cells and promoting the expression of the proangiogenic factors vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) [96].

Together, the above information signifies an important role for annexin A2 in cancer progression and potentially in mediating tumor response to RT, which could be relevant in patient treatment.

1.6 Connecting Text: Research Rationale and Objectives

Our interest in MGMT activity in GBM stems from multiple sources but revolves around the potential for translational applications of MGMT in the treatment of GBM patients. As previously discussed, MGMT expression is of clinical relevance in GBM, as MGMT protein expression counteracts the effects of the standard of care chemotherapy agent used in treatment by repairing the alkyl lesions it induces. In addition, our group has observed an inverse relationship between MGMT protein expression and the angiogenic and invasive characteristics of GBM [74]. MGMT overexpression decreased migration, the spindle-shape phenotype, and the expression and activation of focal adhesion kinase (FAK) in GBM (Figure 1.8) [74]. MGMT positive cells were shown to express a higher ratio of vascular endothelial growth factor receptor 1 (VEGFR-1) to VEGFR-2 compared to MGMT negative cells, indicating negative regulation of VEGF-mediated angiogenesis [40]. MGMT expression and overexpression increased invasive potential compared to MGMT negative cells and MGMT cells pharmacologically depleted of MGMT using O^6BG. Consistent with this concept, our group developed MGMT knockdown cell lines that exhibited increased invasion compared to their parental lines and empty vector controls. These characteristics were further displayed in MGMT positive and MGMT negative patient derived cells.

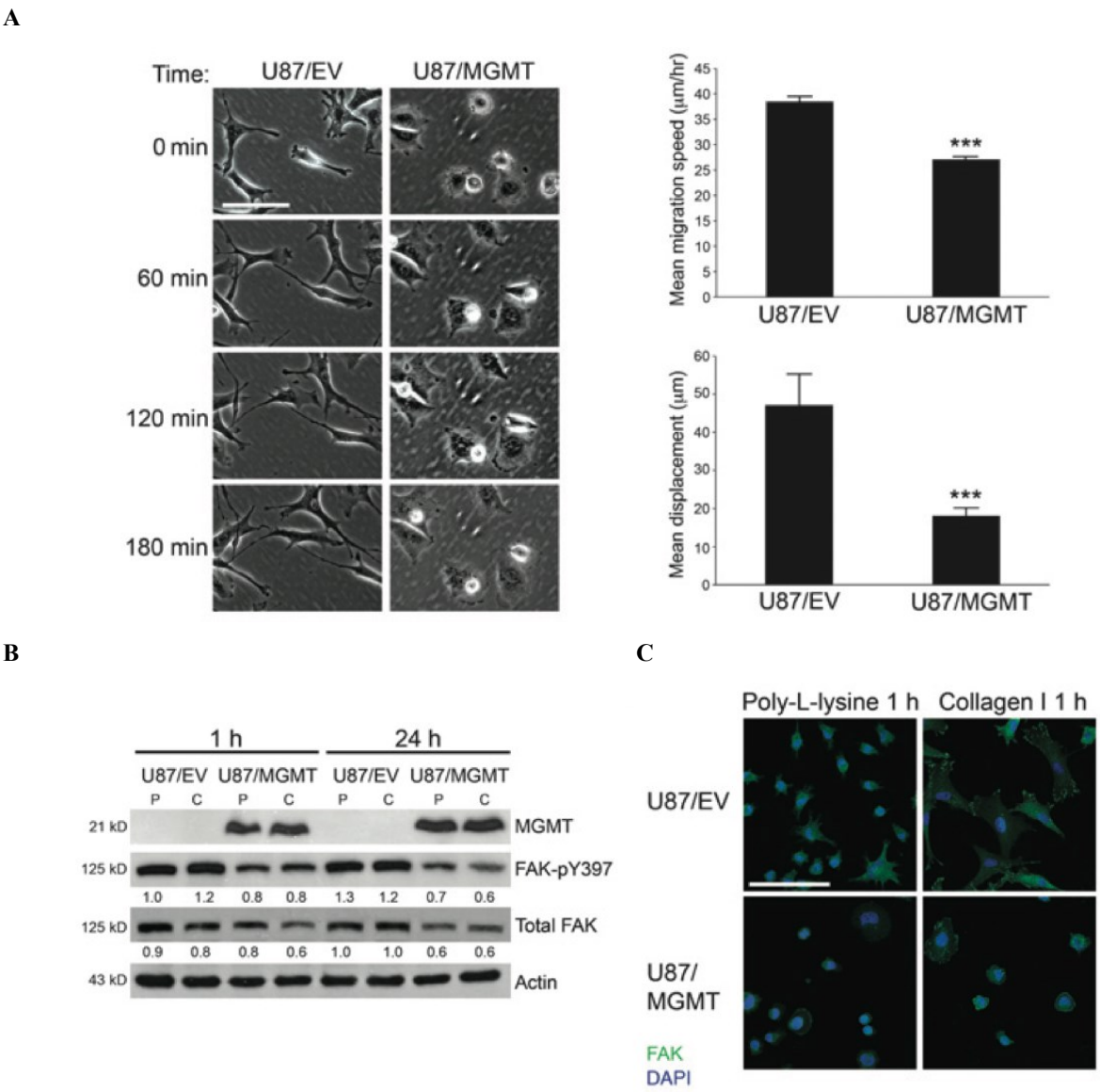

Figure 1.8 Effects of overexpression of MGMT in U87MG cells. **(A)** U87/MGMT cells migrating on collagen-I show loss of migratory phenotype compare to U87/EV cells (left). Mean migration speed and displacement is slower and lower, respectively, in U87/MGMT cells compared to U87/EV cells migrating on collagen I (right). **(B)** Lower levels of FAK-pY397 and total FAK proteins observed in U87/MGMT cells compared to U87/EV cells plated on collagen-I (C) or PLL (P) by Western blot. **(C)** Immunofluorescence staining of FAK-pY397 (green) and nuclear DNA (blue) reflective of the reduced FAK expression observed in U87/MGMT cells compared to U87/EV in Western blotting. Figure adapted from Chahal *et al.*, 2012 [74].

Our previous studies revealed an inverse relationship between MGMT expression and GBM angiogenesis and invasion, challenging the paradigm that MGMT exerts its function in GBM solely as a DNA repair protein. However, the mechanisms by which MGMT affects angiogenesis and invasion at the molecular level remain unknown. We hypothesized that interaction of MGMT with binding partners may confer novel functions to MGMT beyond its known role as a DNA repair protein. We aimed to validate the existence of binding partners with the ultimate goal of understanding how such interactions influence GBM angiogenesis and invasion. Furthermore, we are interested in investigating the relationship between MGMT expression and response to ionizing radiation in GBM cells to determine whether MGMT expression influences this response system as it does invasion and angiogenesis.

Currently, there exists limited information regarding MGMT interactions outside those of DNA repair. The few papers that have reported such interactions use a limited number of human cell types: HT29 human colon cancer cells [70]; BxPC3 and Capan-1 human pancreatic adenocarcinoma cell lines [69]; MCF7, T47D, BT549, MDA-231 breast cell lines, and virus-infected HeLa and human fetal lung fibroblast MRC5.SV40 cells [68]; and HT29 and CEM human lymphoblastic leukemia cell lines [97]. This is the first investigation of binding partners of endogenously expressed MGMT in GBM.

2. Results

2.1 Introduction

GBM is the most common primary malignant glioma in adults and is one of the most lethal human cancers, with an average survival of less than one year from the time of diagnosis [4]. The poor prognosis and high mortality of GBM can be attributed to its highly vascularized and invasive nature, making complete surgical resection and treatment difficult [4]. Though research has focused on elucidating the pathways involved in the increased invasiveness characteristic of GBM, the mechanisms by which these pathways are activated are not fully understood. However, it is known that angiogenesis plays an important role in the progression of GBM and is inversely correlated with survival [4]. MGMT expression has been reported to have an inverse relationship with angiogenesis, with MGMT overexpression in GBM cells reducing invasion, the presence of invasion-related genes, and the spindle-shape cellular phenotype common to migrating GBM cells [39].

Repair of DNA damage is vital in the prevention of cancer and many proteins and pathways have been implicated in the identification and repair of such damage. One such protein is MGMT, which is ubiquitously expressed in normal human tissues and is highly conserved phylogenetically [25, 26]. The essential conserved function of MGMT is alkyl transfer [27]. MGMT is believed to act independently to remove alkyl adducts, with preference for those at the O6 position of guanine [27], via a stoichiometric reaction that transfers bound alkyl groups to a cysteine in its active site and irreversibly inactivates MGMT [28]. Repair of such lesions is critical in the maintenance of cellular integrity and without such repair these lesions are mutagenic, leading to incorrect base insertion at a rate of 90% [27].

Owing to the clinical significance of MGMT as a DNA repair protein involved in resistance to alkylating agents, most studies focus on the structural and functional aspects of how MGMT binds to DNA and exerts its alkyltransferase activity. Though MGMT is believed to act independently [26, 27, 65, 66] without the support of a complex of proteins as is common for many DNA repair proteins, previous studies have shown that MGMT does interact with other binding partners in various cancer types, suggesting MGMT may possess a functional role in addition to DNA repair.

The possible involvement of MGMT in cellular functions other than DNA repair has not been fully investigated, though MGMT expression has been reported to have an inverse relationship with angiogenesis and invasion in GBM cells [39]. Specifically, MGMT positive GBM cells have been shown to express higher increased levels of vascular endothelial growth factor receptor 1 (VEGFR-1) and decreased levels of VEGFR-2, compared to MGMT negative GBM cells [40].

Building off of the concept that MGMT may possess novel functions in addition to alkyl transfer or may interact with other proteins to affect its alkyl function, we used immunoprecipitation to capture potential MGMT protein binding partners in two cell lines and identified them using mass spectrometry. We first used human embryonic kidney cells (HEK-293T) overexpressing FLAG-tagged MGMT followed by mass spectrometry analysis using 293T-Flag/MGMT and control Flag-tagged empty vector (293T-Flag/EV) to identify potential binding partners with functional relevance in angiogenesis and invasion, as is a common initial strategy for IP experiments due to the ease of transfection for the overexpression of desired proteins [98]. As a second step, we performed direct immunoprecipitation of endogenous MGMT from T98G cells followed by mass spectrometry analysis to identify MGMT-binding partners specific to GBM.

A protein network was created in MetaCore (GeneGO) and pathway analysis was used to provide functional context for each binding partner and identify proteins with the greatest potential for impacting cellular processes related to cancer proliferation. We then used immunofluorescence staining and confocal microscopy to analyze MGMT colocalization with select potential binding partners and attempted co-immunoprecipitation (co-IP) of MGMT to verify binding interaction.

Finally, we examined a possible role for MGMT expression in response to ionizing radiation in GBM cells to determine whether MGMT expression influences this response system as it does invasion and angiogenesis.

Identification of MGMT-binding partners unique to specific cell lines (stated above) warrants the search for MGMT interactions specific to GBM, which may unlock an unknown role of MGMT specific to GBM progression.

2.2 Materials and Methods

2.2.1 Cell Culture and Irradiation

Human Glioblastoma cell lines T98G, U87MG, A172, U138, LN18 and human embryonic kidney cell line 293T were obtained from the American Type Culture Collection (ATCC). U87MG cells transfected with MGMT (U87/MGMT) and empty vector control (U87/EV) were previously established in the laboratory of Dr. Rolando Del Maestro. 293T cells were modified to overexpress Flag-tagged MGMT (293T-Flag/MGMT) or Flag-tagged empty vector control (293T-Flag/EV). Cells were grown at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin.

Relevant protein expression of listed cell lines:

	T98G	U87MG	U87/EV	U87/MGMT	A172	U138	LN18
MGMT	+	-	-	+	-	+	+
p53	+ mutant	+ wild-type	+ wild-type	+ wild-type	+ wild-type	+ wild-type	+ wild-type

Cell irradiation was performed using a Faxitron X-ray machine (Faxitron X-ray Corporation, Wheeling, IL). Cells in 10cm cell culture dishes or 24-well plates were placed on a rotating turntable to ensure dose homogeneity. Irradiation was performed at a tube voltage of 160 kVp, with a current of 6.3 mA, and at a dose rate of 1450 R/min. Cells were exposed to a single dose of 6 Gy.

2.2.2 Cell Lysis and Western Blotting

Cells were exposed to 6 Gy radiation or left untreated (0 Gy control) and lysed 24 or 72 hours post treatment.

Cells were washed twice with 1X phosphate-buffered saline (PBS) and lysed with RIPA-based lysis buffer (1X RIPA lysis and extraction buffer, 1X protease inhibitor cocktail, 0.2 mM sodium orthovanadate). Protein concentration was determined using a BCA protein assay kit (Pierce) according to manufacturer instructions. 30 μg of protein were loaded per well and separated by electrophoresis on a 12% SDS-PAGE gel under reducing conditions. Proteins were then transferred onto polyvinylidene difluoride membranes.

Membranes were blocked with 5% milk or BSA solution then probed with primary antibodies directed against MGMT (Santa Cruz Biotechnology Inc., sc-33674), hnRNPA1 (Abcam, ab177152), annexin A2 (Cell Signaling Technology, 8235), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Cell Signaling Technology, 9101), p44/42 MAPK (Erk1/2) (Cell Signaling Technology, 9102), phospho-Akt (Ser473) (Cell Signaling Technology, 4060), Akt1/2/3 (Santa Cruz Biotechnology Inc., sc-8312), p53 (Santa Cruz Biotechnology Inc., sc-126), p21 Waf1/Cip1 (12D1) (Cell Signaling Technology, 2947), and β-actin (Sigma-Aldrich, A1978) for 1 hour or overnight, at room temperature or at 4°C, respectively. Membranes were washed in Tris-Buffered Saline with 1% Tween-20 (TBST) solution then incubated with appropriate secondary antibody solutions (prepared in the blocking solution) for 40 minutes. Membranes were washed in TBST then exposed using enhanced chemiluminescence (ECL) western blotting substrate on a digital Omega Lum C Imaging System (Aplegen, Gel Company).

Protein densitometry analysis was performed using ImageJ as described in the *ImageJ User Guide* [99].

2.2.3 Immunoprecipitation for Proteomic Analysis

Pre-cleared 293T-Flag/MGMT and 293T-Flag/EV lysates were subjected to affinity purification using an anti-Flag monoclonal antibody covalently attached to agarose resin (FLAG M Purification Kit, Sigma) according to the manufacturer's instructions. The affinity bound Flag fusion proteins were eluted from the resin through competition with Flag peptides.

Direct immunoprecipitation of endogenous MGMT from T98G cells was performed using anti-MGMT antibody or isotype control antibody non-covalently linked to Dynabeads Protein G (Life Technologies) according to the manufacturer's instructions.

Eluted samples and whole cell lysates were separated on SDS-PAGE.

2.2.4 Peptide Identification by Liquid Chromatography-Tandem Mass Spectrometry

Gels containing the immunoprecipitation lysates were stained with Coomassie blue and bands were excised is a clean room. Excised bands were subjected to trypsin digestion and the resulting tryptic peptides were purified and identified by liquid chromatography (LC)-tandem mass spectrometry (MS/MS) using a microcapillary reversed-phase high pressure liquid

chromatography-coupled LTQ-Orbitrap XL Hybrid Ion Trap-Orbitrap Mass Spectrometer (Thermo Scientific) with a nanospray interface (Institut de recherches cliniques de Montréal, Director Proteomic platform) [100].

The resultant MS/MS spectra were searched against a proteome database for peptide matching and protein identification (Uniref100 human protein database released May 2012). Proteins were identified with high confidence (Scaffold software: minimum protein identity set at 99.9% and at least 2 unique peptides identified with peptide probability more than 95% for each of the two peptides).

2.2.5 Proteomic Analysis Using MetaCore

Proteins identified by mass spectrometry from the T98G cell line were uploaded to MetaCore trial software (GeneGo, Inc. Thomson Reuters). MetaCore is a manually curated database of directional protein, DNA, RNA, drug, and biomolecule interactions that includes transcription factors, receptors, ligands, kinases, drugs, endogenous metabolites, and other molecules. Of the proteins uploaded, 172 GI numbers were recognized and added to the system. A Pathway Map was created using "significant interactions within sets" to identify known interactions between the recognized proteins in our set. Our protein list was overlaid against the MetaCore Glioblastoma network to identify proteins from our list that are known to be modified or play important roles in GBM. An Enrichment Analysis Report was generated to identify the pathways of strongest relevance to our uploaded dataset from the MetaCore database of Pathway Maps based on greatest overlap.

2.2.6 Immunofluorescence Staining, Confocal Microscopy, and Image Analysis

For immunofluorescence staining, cells were plated on glass coverslips at varying densities and allowed to adhere overnight. Cells were fixed with 4% paraformaldehyde solution for 15 minutes at room temperature and then permeabilized with 0.1% Triton X-100 solution for 10 minutes at room temperature. Coverslips were blocked with 5% normal goat serum is PBS for 1 hour at room temperature then incubated with primary antibodies against MGMT, hnRNPA1, and/or annexin A2 overnight at 4°C. Coverslips were then washed with 100 mM glycine / 1XPBS solution, then incubated with fluorescence-conjugated secondary antibodies Alexa Fluor

488 (Life Technologies) and/or Alexa Fluor 555 (Life Technologies) diluted in 1% NGS / 1XPBS solution in darkness for 1 hour at room temperature. Coverslips were washed and mounted to glass slides using ProLong Diamond Antifade Mountant with DAPI (Life Technologies) and left to dry in darkness overnight.

Images were captured using a Zeiss LSM 780 laser scanning microscope (Carl Zeiss MicroImaging, Göttingen, Germany) with an oil objective at 63X magnification. Colocalization analysis was performed using the Zeiss-associated Zen software according to the Carl Zeiss produced *Colocalization Analysis in AIM and Zen* guidelines [101]. Protein distribution analysis was performed using ImageJ software using the Intensity Ratio Nuclei Cytoplasm Tool Image as part of the Cell Image Analyzer developed by Montepellier RIO Imaging [102].

2.2.7 Cell Lysis and Co-Immunoprecipitation

T98G, U87/EV, and U87/MGMT cells were lysed using non-denaturing IP Lysis Buffer (Pierce) supplemented with protease inhibitor cocktail according to manufacturer instructions. Protein concentration was measured and immunoprecipitation performed using Dynabeads Proteins G (Life Technologies) covalently cross-linked to primary MGMT antibody, isotype control antibody, or beads alone. Target proteins were eluted into SDS-PAGE sample loading buffer (heated to 95°C) for 3 minutes and collected for SDS-PAGE. Western blotting was completed as described.

2.3 Results

2.3.1 Identification of MGMT-Binding Partners in 293T and GBM Cell Lines

As a general strategy to investigate MGMT protein interactions, we generated a 293T cell line overexpressing Flag-tagged MGMT (293T-Flag/MGMT) to identify MGMT-binding partners through affinity purification of Flag-tagged MGMT followed by mass spectrometry analysis. A Flag-tagged empty vector (293T-Flag/EV) served as a control (Figure 2.1A). Pre-cleared 293T-Flag/MGMT and 293T-Flag/EV lysates were subjected to affinity purification using an anti-Flag monoclonal antibody covalently attached to agarose resin. The affinity bound Flag fusion proteins were eluted from the resin by two methods: incubation with SDS solution or competition with Flag peptides. The resulting eluates were separated by SDS-PAGE and Coomassie Blue staining enabled the identification of 6 bands including Flag-MGMT in the 293T-Flag/MGMT elution but not in the 293T-Flag/EV control elution (Figure 2.1B). Bands were excised from the gel, then subjected to trypsin digestion and identified by liquid chromatography-tandem mass spectrometry (MS/MS).

The resultant MS/MS spectra of the excised bands were searched against a proteome database for peptide matching and identification. Our analysis provided evidence for binding of MGMT to a total of 120 binding partners, which were not identified in the 293T-Flag/EV control cell line (Table 2.1). We removed common background contaminants typically present in affinity-purified protein samples using the Flag affinity approach in 293T cells [103, 104]. We used the gene ontology (GO) database [105] to search for functional categories and identified binding partners involved in DNA repair, ubiquitin pathway, DNA replication and transcription, RNA metabolism and processing, protein biosynthesis and trafficking, cellular metabolism, cell cycle and division, and response to stress and cell death (Table 2.1). Strikingly, as shown in Table 2.1, we also identified proteins directly involved in cell motility and/or angiogenesis, including cytoskeletal-related proteins, small GTPases family and their regulators, and direct regulators of angiogenesis.

This preliminary data provided some mechanistic insights into the relationship between MGMT and effector proteins involved in angiogenesis and invasion. To identify specific MGMT-binding partners in the context of GBM, we used T98G, a human GBM cell line with constitutive expression of MGMT. MGMT-binding partners were identified via

immunoprecipitation (IP) of endogenous MGMT, followed by mass spectrometry analysis. IP was performed using monoclonal antibodies against MGMT and an isotype control. Similarly to the 293T cell lines, eluted proteins were resolved by SDS-PAGE and then stained with Coomassie Blue. With Western blotting analysis we observed precipitation of MGMT with use of the MGMT antibody and noted its absence with the use of the isotype control (Figure 2.2).

The T98G MGMT and isotype control bands were excised from the gel and subjected to trypsin digestion for identification by liquid chromatography MS/MS. The resultant spectra were searched against a proteome database for peptide matching and protein identification. Analysis identified 186 proteins that specifically interacted with endogenous MGMT. Using the GO database [105] to search for functional categories, we identified proteins involved in mitochondrial metabolism, DNA repair and replication (DNA-binding proteins), ubiquitin pathway (selective proteolysis related proteins), transcription regulators (nuclear membrane proteins), RNA post-transcriptional processing (pre-mRNA-binding proteins, transcriptional splicing regulation factors), protein biosynthesis and trafficking (ribosomal proteins), cellular metabolism (cell enzyme and its regulators), cell cycle and division (cell cycle regulators), response to stress and cell death (molecular chaperons), cell migration and invasion (cytoskeleton-associated proteins, cell adhesion molecules, extracellular matrix proteins). These proteins have been grouped by their broad functional categories along with their names, Genbank Accession numbers, UniProt Accession numbers, mass, and number of peptides identified, which served as the basis for their designation (Table 2.2).

2.3.2 MetaCore Analysis of MGMT-Binding Partners

Using MetaCore software from Thomson Reuters, we were able to identify interactions of several proteins from our list of GBM MGMT-binding partners that have known functional roles in GBM. When our protein set is overlaid with MetaCore's GBM interactions report (which includes 817 interactions), there are 31 interactions that each includes one protein from our set. These interactions are shown in Table 2.3, which include transcription factors and activation binding or RAS superfamily proteins.

To understand the biological significance of our proteomics results and to visualize the newly identified GBM MGMT-binding partners in the context of the cell, we performed an

enrichment analysis report using the pathway maps function to identify pathways that share the most significant homology with our list (Table 2.4). The most relevant pathways identified, specifically cytoskeleton remodeling and cell adhesion, have clear implications in angiogenesis and invasion. Table 2.4 displays the enrichment pathway analysis of GBM MGMT-binding partners, providing the pathways of greatest significance that include members of our list. This analysis aids in understanding the potential implications of each binding interaction in the greater context of cellular processes. These data reinforce the idea that MGMT interactions with binding partners may mediate an upstream role of MGMT in angiogenesis and invasion.

The MetaCore interactions report (Table 2.3) and the enrichment pathway analysis (Table 2.4) revealed notable proteins from our list of T98G MGMT-binding partners that are members of important pathways or interactions in GBM. We saw a high level of redundancy of ras-related C3 botulin toxin substrate 1 (Rac1), cell division control protein 42 homolog (CDC42), CD44, and Filamin A, meaning that these proteins appeared multiple times across both reports, indicating known significance in GBM biology.

An MGMT protein interactions report (expanded by one interaction and including canonical pathways) was used to compare known MGMT interactions with our list of potential MGMT binding partners. This report shared a single directional protein interaction overlap with our uploaded protein set from the T98G cell line. This interaction is with ubiquitin, which has been shown to bind to MGMT upon its inactivation, resulting in the degradation of MGMT through the ubiquitin proteolytic pathway [70, 97]. We see very little overlap when comparing the protein sets created from mass spectrometry analysis of the 293T and T98G cells lines, with only two identical matches, Histone H1.3 and Histone H1.4. Furthermore, comparison of both of our protein sets to the STRING database [106] of predicted functional partners for MGMT showed no overlap from experimental data or text mining. Comparison to the Protein Interaction Network Analysis (PINA) platform [107] matched six of our determined proteins (annexin A2 [70], rRNA 2'-O-methyltransferase fibrillarin [70], heterogeneous nuclear ribonucleoproteins A2/B1 [70], proliferating cell nuclear antigen [70], polyubiquitin-C [108-111]) from a list of 78 binding partners. We also looked at several other databases including BioGRID3.4 [112], Ensembl (release 83) [113], The Human Protein Atlas [114, 115], and the Human Protein Reference Database (HPRD) [116], but did not find any additional matches to our experiments or any other MGMT interactions beyond those identified in the articles already cited. The lack of

continuity between MGMT-binding partners in different cell lines indicates specific interactions in each cellular context, potentially conferring distinct MGMT functions in different cancers.

Figure 2.3 illustrates the biological network analysis of GBM MGMT-binding partners constructed using the Build Network tool in MetaCore. The network was generated to show significant interactions within our dataset, how these proteins interact with one another, and their subcellular locations. It can be noted that the dominant source of interactions within the set revolves around ubiquitin. It has been shown that the E6 human papillomavirus oncoprotein binds MGMT to form a complex with the cellular ubiquitin-ligase E6-associated protein, similar to its targeting of tumor suppressor genes such as p53, for inactivation through the ubiquitin-proteasome pathway [58]. It is also important to note that cellular protein localization varies greatly, with a strong presence in the cytoplasm, indicating potential translocation of MGMT out of the nucleus, as suggested in earlier reports [35, 36].

2.3.3 Validation of Select Identified Binding Partners

Annexin A2 and hnRNPA1 were among the proteins identified with high confidence in our proteomic analysis (Scaffold software: minimum protein identity set at 99.9% and at least 2 unique peptides identified with peptide probability more than 95% for each of the two peptides). As discussed, both proteins play important roles in cancer and may hypothetically mediate an upstream role of MGMT in angiogenesis and invasion. Knockdown of annexin A2 in glioma cells has been shown to decrease tumor size and slow tumor progression *in vivo* by reducing invasion, angiogenesis, and proliferation, while increasing apoptosis. hnRNPA1 is involved in packaging of pre-mRNA and alternative splicing of angiogenic factors, including human fibroblast growth factor 2 (FGF-2).

We looked at basal MGMT, hnRNPA1, and annexin A2 protein expression in T98G, U87MG, A172, U138, and LN18 GBM cell lines as shown in Figure 2.4. While all cell lines examined expressed hnRNPA1, LN18 appeared to have the highest expression. Annexin A2 appeared to have strong expression in the MGMT-positive cell lines (T98G, U138, and LN18) while the MGMT-negative U87MG cell line appears to have reduced expression and the MGMT-negative A172 cell line has very low expression.

In order to verify hnRNPA1 and annexin A2 as MGMT-binding partners, we completed immunofluorescence staining with confocal analysis to assess their colocalization and aimed to perform co-IP of MGMT followed by western blotting to verify binding interaction.

Using immunofluorescence staining, MGMT was shown to colocalize with both hnRNPA1 and annexin A2 in MGMT-transfected GBM cells (U87/MGMT) (Figure 2.5). Manders Colocalization Coefficients [117] for hnRNPA1 with MGMT and annexin A2 with MGMT were determined using ZEN Microscope and Imaging Software (Carl Zeiss Microimaging, LLC) in accordance with the *Colocalization Analysis in AIM and Zen* guidelines [101]. The Manders Colocalization Coefficient is a value ranging from 0 to 1, which represents the proportion of one protein that is colocalized with a second protein (equal to colocalized pixels divided by non-colocalized pixels of the same channel, with each pixel possessing a value of 1). A Manders value of 0 would represent no colocalization while a value of 1 would represent complete colocalization. These coefficients allow us to understand the proportion of one protein population that is colocalized with a second protein population and vice versa, producing two values for each protein pair analyzed.

Analysis of our images revealed hnRNPA1 almost completely colocalized with MGMT (mean colocalization coefficient of 0.98) with far less of the MGMT protein population colocalizing with hnRNPA1 (0.33) (Figure 2.5.B). This difference is likely due to the high abundance and predominantly nuclear cellular location of hnRNPA1, while MGMT was observed in both the nucleus and the cytoplasm (Figure 2.5.A).

A similarly high proportion of the annexin A2 protein population colocalized with MGMT (0.91), while a large proportion of the MGMT population also colocalized with annexin A2 (0.72) (Figure 2.5.B). In contrast to hnRNPA1, colocalization occurred in the cytoplasm, as annexin A2 is observed on or near the cell membrane or in the cytoplasm, with limited presence in the nucleus (Figure 2.5.A).

The colocalization analysis indicates that MGMT does colocate with hnRNPA1 and annexin A2, signifying that they are in close proximity and that binding interactions are feasible. However, we were unable to successfully pull down MGMT with hnRNPA1 or annexin A2 in co-IP experiments. While we did not show binding, these results do not exclude the existence of MGMT binding hnRNPA1 or annexin A2, as the failure may lie in being unable to completely optimize the experiment to permit and maintain binding interaction.

2.3.4 GBM Cellular Response to Irradiation and Effect on MGMT Localization

MGMT expression may be induced in response to ionizing radiation [118] and other genotoxic stress through transcriptional activation [63]. To investigate the impact of ionizing radiation in GBM cells, we examined MGMT protein expression and localization in response to irradiation. We also looked at the expression of our two potential MGMT-binding partners, as well as proliferation and cell survival markers, to see if any radiation induced changes in expression correlated with MGMT expression.

MGMT positive (T98G and U87/MGMT) and MGMT negative (U87MG) GBM cells were irradiated with a dose of 6 Gy or not exposed to radiation (0 Gy) as a control. Cells were fixed 2 or 24 hours post irradiation and immunofluorescently stained for MGMT protein expression to determine if there was a change in MGMT localization with exposure to low dose irradiation. Confocal images were analyzed using the Intensity Ratio Nuclei Cytoplasm Tool plugin in ImageJ, developed by Montepellier RIO Imaging [102]. While there were slight increases in MGMT nuclear localization post irradiation (particularly in irradiated T98G cells fixed at the 2 hour time point) these increases were modest and not statistically significant (Figure 2.6).

When looking at protein expression, we did not see a dramatic increase in MGMT expression in irradiated cells; only in T98G cells lysed 24 hours after irradiation did we see a 1.2 fold increase in MGMT expression compared to the non-irradiated control (Figure 2.7).

We also examined the impact of radiation on the activation of protein kinase B (Akt) and extracellular signal-regulated kinase-1 and -2 (Erk1/2); p53 and p21 expression; and hnRNPA1 and annexin A2 expression (Figure 2.7).

Akt is a known proto-oncogene and plays an essential role in regulating diverse cellular functions including metabolism, growth, proliferation, survival, transcription, and protein synthesis. Activating phosphorylation of Akt (pAkt) permits full enzymatic activity and dysregulation of this activation is common in cancer, permitting unregulated growth and anti-apoptotic signaling. We observed slight variations in pAkt and Akt expression, though not consistent with condition or MGMT expression (Figure 2.7).

The p53 tumor suppressor protein regulates cell cycle and its native form is anti-tumorigenic. p53 is commonly mutated in cancer to inhibit its DNA-binding ability, rendering it ineffective, permitting uncontrolled cell division. p21 is a cyclin-dependent kinase inhibitor and

is known to interact with p53 as an intermediate in p53-mediated inhibition of proliferation in response to DNA damage. We saw tandem induction of p53 and p21 expression at our later time point in irradiated U87MG and U87/MGMT cells, in keeping with the knowledge that U87MG cells possess wild-type *TP53* (Figure 2.7). However, we observed a greater induction of p53 and p21 expression in U87/MGMT cells compared to U87MG cells: 2.5-fold increase in p53 expression in U87/MGMT relative to control compared to 1.5-fold increase in U87MG, and 5-fold increase in p21 expression in U87/MGMT relative to control compared to 2.3 fold increase in U87MG. T98G cells are known to express mutant *TP53* and thus expectedly showed high expression of p53 protein in all conditions (Figure 2.7).

The Erk1/2 signaling cascade has important roles in proliferation, differentiation, and survival. Erk1/2 is activated through phosphorylation (pErk) and inappropriate activation is common in cancer. Western blotting analysis showed an increased ratio of pErk to total Erk1/2 in U87MG cells (1.7) compared to U87/MGMT cells (1) and a decrease in T98G cells (0.7) 24 hours after irradiation (Figure 2.7). Ionizing radiation induced a drop in the ratio of pErk to total Erk1/2 (0.6) with an increase in total Erk1/2 expression (2) in irradiated U87MG cells at the 72 hour time point, while expression in U87/MGMT and T98G cells remained relatively stable (Figure 2.7).

Protein expression analysis of potential MGMT-binding partners showed a decreased annexin A2 and hnRNPA1 expression at the 24 hour time point in irradiated U87MG and U87/MGMT cell lines, followed by increased expression of both proteins at the 72 hour time point (Figure 2.7). This was contrasted by the reverse expression pattern in irradiated T98G cells (Figure 2.7).

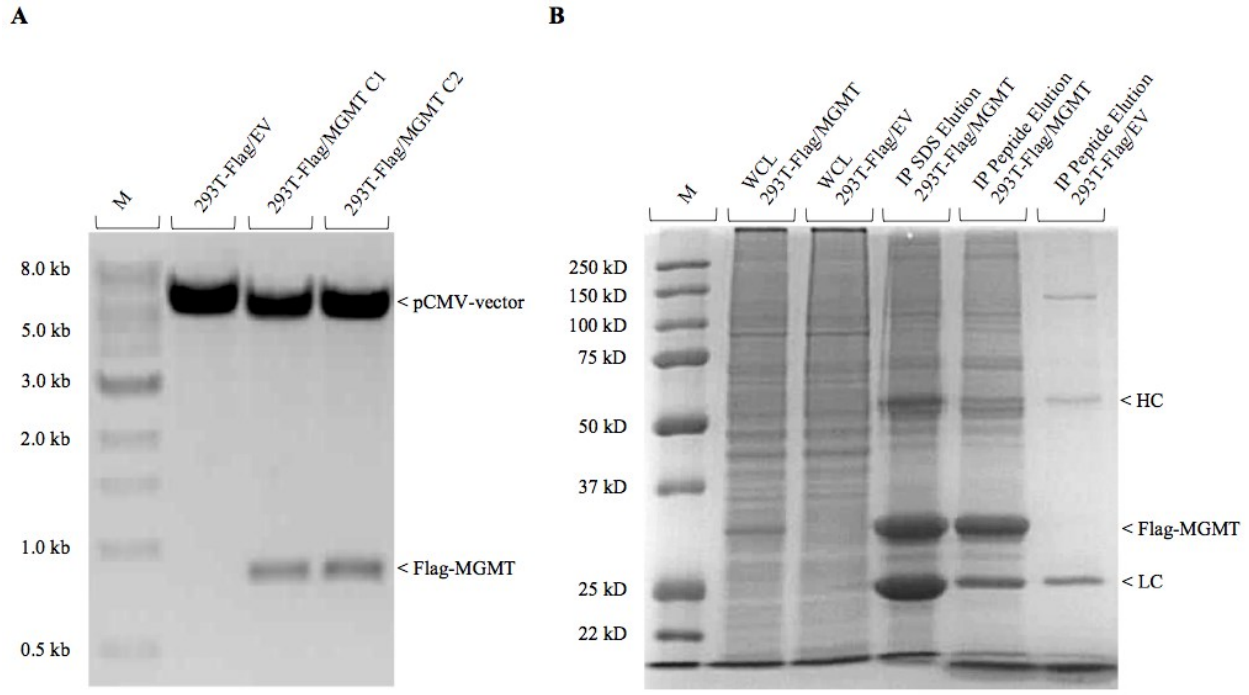

Figure 2.1 Expression and affinity purification of Flag-MGMT in 293T-Flag/MGMT and 293T-Flag/EV. **(A)** Flag-MGMT expression in 293T Flag-tagged MGMT cells lines and empty vector control. Lane 1 shows the 293T Flag-tagged empty vector (EV) lysate; Lane 2 shows 293T Flag-tagged overexpressing MGMT (293T-Flag/MGMT) Clone 1; and Lane 3 shows a 293T-Flag/MGMT Clone 2. Gel stained with Coomassie Blue. **(B)** Coomassie Blue staining following affinity purification of Flag-MGMT in 293T-Flag/MGMT and Flag-tagged EV control. Lane 1 shows whole cell lysate of 293T-Flag/MGMT; Lane 2 shows whole cell lysate of 293-Flag/EV; Lane 3 shows SDS elution of Flag-tagged MGMT from 293T-Flag/MGMT; Lane 4 shows competitive Flag peptide elution of Flag-tagged MGMT from 293T-Flag/MGMT; Lane 5 shows competitive Flag peptide elution of Flag-tagged EV control.

Function	Uniprot Accession #	Uniprot Name	Molecular Weight (kDa)
DNA Replication and Translation	Q9H0D6	5'-3' exoribonuclease 2	109
	Q8N9N2	Activating signal cointegrator 1 complex subunity 1	45
	P06733	Alpha-enolase	47
	Q96SN8	CDK5 regulatory subunit-associated protein 2	215
	Q8WVB6	Chromosome transmission fidelity protein 18 homolog	107
	Q9BPX3	Condensin complex subunit 3	114
	P61201	COP9 signalosome complex subunit 2	52
	Q86VP6	Cullin-associated NEDD8-dissociated protein 1	136
	Q14181	DNA polymerase alpha subunit B	66
	P24928	DNA-directed RNA polymerase II subunit RPB1	217
	P51530	DNA2-like helicase	120
	Q5VTR2	E3 ubiquitin-protein ligase BRE1A	114
	Q96AE4	Far upstream element-binding protein 1	68
	Q12789	General transcription factor 3C polypeptide 1	239
	Q8WUA4	General transcription factor 3C polypeptide 2	101
	P49915	GMP synthase [glutamine-hydrolyzing]	77
	P11142	Heat shock cognate 71 kDa protein	71
	O14929	Histone acetyltransferase type B catalytic subunit	49
	P16402	Histone H1.3	22
	P10412	Histone H1.4	22
	Q9BTE3	Mini-chromosome maintenance complex-binding protein	73
	P22234	Multifunctional protein ADE2	47
	P52948	Nuclear pore complex protein Nup98-Nup96	198
	Q13416	Origin recognition complex subunit 2	66
	O43913	Origin recognition complex subunit 5	50
	Q92841	Probable ATP-dependent RNA helicase DDX17	50
	Q9UQ80	Proliferation-associated protein 2G4	44
	Q9HCE1	Putatuve helicase MOV-10	114
	P40937	Replication factor C subunity 5	73
	Q96PK6	RNA-binding protein 14	69
	Q98XP5	Serrate RNA effector molecule homolog	101
	Q7KZF4	Staphylococcal nuclease domain-containing protein 1	102
	A6HNR9	Structural maintenace of chromosomes flexible hinge domain-containing protein 1	226
	Q8WXI9	Transcriptional repressor p66-beta	65
	Q9H5H4	Zinc finger protein 768	60
Cytoskeletal-Related	P61158	Actin-related protein 3	47
	P55196	Afadin	207
	Q9H1A4	Anaphase-promoting complex subunit 1	216
	O60716	Catenin delta-1	108
	O14578	Citron Rho-interacting kinase	231
	Q14008	Cytoskeleton-associated protein 5	226
	P47756	F-actin-capping protein subunit beta	31
	Q7Z4H7	HAUS augmin-like complex subunity 6	109
	Q9H0B6	Kinesin light chain 2	69
	Q9NSK0	Kinesin light chain 4	69
	P33176	Kinesin-1 heavy chain	110
	Q15334	Lethal(2) giant larvae protein homolog 1	115
	Q9H0A0	N-acetyltransferase 10	116
	P13797	Plastin-3	71
	Q15262	Receptor-type tyrosine-protein phosphatase kappa	162
	P16615	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	115
	P23258	Tubulin gamma-1 chain	54
Small GTPases Family and Regulators	Q96NW4	Ankyrin repeat domain-containing protein 27	117
	Q5JSL3	Dedicator of cytokines protein 11	238
	Q9HBB9	Gem-associated protein 4	119
	Q92538	Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1	206
	Q9BX10	GTP-binding protein 2	66
	Q38SD2	Leucine-rich repeat serine/threonine-protein kinase 1	73
	P50395	Rab GDP dissociation inhibitor beta	51
	Q6GYQ0	Ral GTPase-activating protein subunity alpha-1	230
	Q92974	Rho guanine nucleotide exchange factor 2	112
Angiogenesis	Q9UPY3	Endoribonuclease Dicer	219
	P13489	Ribonuclease inhibitor	50

Table 2.1 Mass spectrometry identified MGMT-binding partners in 293T-Flag/MGMT cell line organized by function.

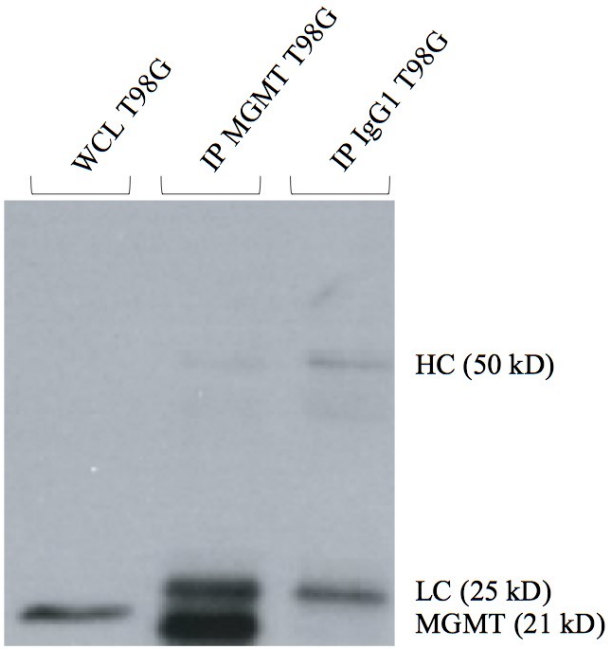

Figure 2.2 Immunoprecipitation of endogenous MGMT in T98G, a human GBM cell line. Western blot analysis of T98G total lysate (Lane 1), immunoprecipitation of MGMT using an anti-MGMT antibody (Lane 2), and isotype control antibody (Lane 3).

Functional Category	Protein Name	Genbank Accession #	UniProt Accession #	Closest Uniprot Protein Match	Mass (kDa)	Peptide Match
Cell Differentiation	Transgelin 2 (**TAGLN2**)	GI:12803567	P37802	Transgelin-2	22.39	4
	Transgelin-2 isoform b	GI:4507357	P37802		22.41	4
	Chain A, **Cdc42** (T35a)	GI:270063471	P60953	Cell division control protein 42 homolog	19.72	4
	Cell division control protein 42 homolog isoform 1 precursor	GI:4757952	P60953		21.26	4
	Chain A, Cdc42ack Gtpase-Binding Domain Complex	GI:5542168	P60953		21.42	4
	Chain A, Cdc42hs-Gdp Complex.	GI:4389379	P60953		21.11	4
Cell-Cell Interactions	Collagen type VI, alpha 3 chain (COL6A3)	GI:3127926	P12111	Collagen alpha-3(VI) chain	343.55	16
	Collagen alpha-3(VI) chain isoform 1 precursor	GI:55743098	P12111		343.67	16
	Collagen alpha-3(VI) chain isoform 5 precursor	GI:55743106	P12111		321.35	16
	L1 cell adhesion molecule **(L1CAM)**	GI:33354077	P32004	Neural cell adhesion molecule L1	139.77	7
	Neural cell adhesion molecule L1 isoform 1 precursor	GI:4557707	P32004		140.00	7
	Neural cell adhesion molecule L1 isoform 3 precursor	GI:221316760	P32004		138.91	7
	Non-neural L1CAM	GI:145652526	P32004		138.75	7
	Neural cell adhesion molecule L1 isoform 2 precursor	GI:13435353	P32004		139.52	7
	L1 cell adhesion molecule, isoform CRA_c	GI:119593195	P32004		140.14	7
	Annexin A2 isoform 2	GI:4757756	P07355	Annexin A2	38.61	39
	Annexin A2 isoform 1	GI:50845388	P07355		40.41	39
	Annexin A2 (**Annexin II**)	GI:73909156	P07355		40.53	39
	CD44 molecule (Indian blood group)-**CD44**	GI:45501213	P16070	CD44 antigen	76.63	5
	cell surface glycoprotein CD44	GI:950418	P16070		81.55	5
	CD44 antigen (Indian blood group), isoform CRA_b	GI:119588551	P16070		46.26	5
	CD44 antigen (Indian blood group), isoform CRA_c	GI:119588552	P16070		75.96	5
	CD44 antigen (Indian blood group), isoform CRA_d	GI:119588553	P16070		81.57	5
	CD44 antigen (Indian blood group), isoform CRA_f	GI:119588556	P16070		76.64	5
	CD44 antigen (Indian blood group), isoform CRA_h	GI:119588559	P16070		73.15	5
	CD44 antigen (Indian blood group), isoform CRA_i	GI:119588560	P16070		43.01	5
	CD44 antigen (Indian blood group), isoform CRA_j	GI:119588561	P16070		58.03	5
	cell adhesion molecule	GI:180130	P16070		39.49	5
	CDw44 antigen precursor	GI:180197	P16070		39.56	5
	CD44E (epithelial form)	GI:29801	P16070		53.60	5
	epican	GI:31191	P16070		76.57	5
	CD44 antigen isoform 6 precursor	GI:321400138	P16070		46.57	5
	CD44 antigen isoform 7 precursor	GI:321400140	P16070		37.28	5
	CD44R5	GI:435700	P16070		43.00	5
	CD44 antigen isoform 2 precursor	GI:48255937	P16070		76.61	5
	CD44 antigen isoform 3 precursor	GI:48255939	P16070		53.41	5
	CD44 antigen isoform 4 precursor	GI:48255941	P16070		39.42	5
	CD44R4	GI:7705157	P16070		46.25	5
	CD44 antigen isoform 1 precursor	GI:48255935	P16070		81.54	5
Chaperone	T-complex polypeptide 1 **(TCP1,** also known as **TCP-1-alpha)**	GI:36796	P17987	T-complex protein 1 subunit alpha	60.40	7
	T-complex protein 1 subunit alpha isoform a	GI:57863257	P17987		60.35	7
	Chain A, human heart L-lactate dehydrogenase H Chain, Ternary complex with Nadh and Oxamate	GI:13786847	P00338	L-lactate dehydrogenase A chain	36.51	6
	L-lactate dehydrogenase B chain **(LDHB)**	GI:4557032	P07195	L-lactate dehydrogenase B chain	36.64	7

Cytoskeletal-Related Proteins	**Plectin (PLEC) isoform 1**	GI:41322916	Q15149	Plectin	531.78	32
	Plectin isoform 1a	GI:41322923	Q15149		516.19	32
	Plectin isoform 1b	GI:41322919	Q15149		516.27	32
	plectin isoform 1c	GI:47607492	Q15149		518.47	32
	Plectin isoform 1d	GI:41322910	Q15149		512.60	32
	Plectin isoform 1e	GI:41322908	Q15149		513.70	32
	Plectin isoform 1f	GI:41322912	Q15149		514.77	32
	Plectin isoform 1g	GI:41322914	Q15149		516.47	32
	Filamin-A isoform 1	GI:116063573	P21333	Filamin-A	280.01	28
	Filamin-A isoform 2	GI:160420317	P21333		280.73	28
	FLJ00343 protein	GI:260268505	P21333		281.46	28
	Unnamed protein product	GI:28243	P21333		280.75	28
	Filamin A	GI:53791221	P21333		278.22	28
	Myosin-9 (**MYH9**)	GI:12667788	P35579	Myosin-9	226.54	14
	Emerin	GI:4557553	P50402	Emerin	29.00	3
DNA Replication, Transcription, & Translation, Chromatin Structure	60S ribosomal protein L21	GI:18104948	P46778	60S ribosomal protein L21	18.57	22
	Heterogeneous nuclear ribonucleoproteins A2/B1 isoform B1 (hnRNP A2 or hnRNP A2/B1 isoform B1)	GI:14043072	P22626	Heterogeneous nuclear ribonucleoproteins A2/B1	37.43	22
	Heterogeneous nuclear ribonucleoproteins A2/B1 isoform A2 (hnRNP A2 or hnRNP A2/B1 isoform A2)	GI:4504447	P22626		36.01	22
	Ribosomal protein L17	GI:42542645	P18621	60S ribosomal protein L17	21.42	18
	40S ribosomal protein S9	GI:14141193	P46781	40S ribosomal protein S9	22.59	21
	Neuroblast differentiation-associated protein AHNAK isoform 1 (**AHNAK** isoform 1)	GI:61743954	Q09666	Neuroblast differentiation-associated protein AHNAK	629.1	28
	Heterogeneous nuclear ribonucleoprotein A1 isoform a	GI:4504445	P09651	Heterogeneous nuclear ribonucleoprotein A1	34.20	13
	Heterogeneous nuclear ribonucleoprotein A1 isoform b	GI:14043070	P09651		38.75	13
	Heterogeneous nuclear ribonucleoprotein A1 **(hnRNPA1)**	GI:47939618	P09651		34.18	13
	40S ribosomal protein S11	GI:4506681	P62280	40S ribosomal protein S11	18.43	14
	60S ribosomal protein L24	GI:4506619	P83731	60S ribosomal protein L24	17.78	10
	Ras-related protein **Rab-34**, isoform NARR (**NARR**)	GI:372266180	P0DI83	Ras-related protein Rab-34, isoform NARR	21.12	9
	histone H2A type 2-A (**HIST2H2AA3**)	GI:4504251	Q6FI13	Histone H2A type 2-A	14.10	5
	histone H2A type 2-C **(HIST2H2AC)**	GI:24638446	Q16777	Histone H2A type 2-C	13.99	5
	scar protein	GI:337930	P62701	40S ribosomal protein S4, X isoform	27.41	8
	40S ribosomal protein S4, X isoform X isoform	GI:4506725	P62701		29.60	8
	Ribosomal protein S4, X-linked X isoform variant	GI:62896517	Q53HV1	Ribosomal protein S4, X-linked X isoform	29.58	8
	Histone H1b **(HIST1H1B)**	GI:356168	P16401	Histone H1.5	21.74	6
	Histone H1.2	GI:4885375	P16403	Histone H1.2	21.37	6
	Histone H1.3	GI:4885377	P16402	Histone H1.3	22.35	6
	Histone H1.4	GI:4885379	P10412	Histone H1.4	21.87	6
	Histone cluster 1, H1e **(HIST1H1E)**	GI:66365795	Q4VB24	Histone cluster 1, H1e	21.89	6
	60S ribosomal protein L18a	GI:11415026	Q02543	60S ribosomal protein L18a	20.76	7
	60S ribosomal protein L4	GI:16579885	P36578	60S ribosomal protein L4	47.70	5
	Ribosomal protein L4 variant	GI:62087534	P36578		49.00	5
	60S ribosomal protein L9	GI:15431303	P32969	60S ribosomal protein L9	21.86	6
	Chain C, Human PCNA	GI:2914385	P12004	Proliferating cell nuclear antigen	28.75	6
	Proliferating cell nuclear antigen **(PCNA)**	GI:4505641	P12004		28.77	6
	60S ribosomal protein L5	GI:14591909	P46777	60S ribosomal protein L5	34.36	6
	Ribosomal protein L5 variant	GI:62896767	P46777		34.36	6

DNA Replication, Transcription, & Translation, Chromatin Structure	hnRNP 2H9B (**hnRNP H3**)	GI:7739445	P31942	Heterogeneous nuclear ribonucleoprotein H3	31.53	5
	Heterogeneous nuclear ribonucleoprotein H3 isoform a	GI:14141157	P31942		36.93	5
	Heterogeneous nuclear ribonucleoprotein H3 isoform b	GI:14141159	P31942		35.24	5
	Heterogeneous nuclear ribonucleoprotein H3 isoform a variant	GI:62898443	P31942		36.93	5
	rRNA 2'-O-methyltransferase fibrillarin (**fibrillarin**, FBL)	GI:12056465	P22087	rRNA 2'-O-methyltransferase fibrillarin	33.78	4
	FBRL_HUMAN	GI:3399667	P22087		33.43	4
	60S ribosomal protein L6 (RPL6)	GI:16753227	Q02878	60S ribosomal protein L6	32.73	5
	Ribosomal protein, large, P0 (RPLP0)	GI:12654583	P05388	60S acidic ribosomal protein P0	34.28	4
	Ribosomal protein P0 variant	GI:62896495	P05388	60S acidic ribosomal protein P0	34.30	4
	Ribosomal protein L13	GI:42490910	P26373	60S ribosomal protein L13	24.27	3
	Serine/arginine-rich splicing factor 3 (SRSF3, SRP20, also known as **SFRS3**)	GI:4506901	P84103	Serine/arginine-rich splicing factor 3	19.33	3
	Splicing factor, arginine/serine-rich 3, isoform CRA_c	GI:119624305	P84103	Serine/arginine-rich splicing factor 3	14.75	3
	Chromobox protein homolog 3 **(HP1-gamma**, also known as CBX3, or HP1Hs-gamma)	GI:15082258	Q13185	Chromobox protein homolog 3	20.81	3
	60S ribosomal protein L10	GI:13592053	P27635	60S ribosomal protein L10	24.61	3
	Ribosomal protein L10 variant	GI:62898179	P27635	60S ribosomal protein L10	24.54	3
	60S ribosomal protein L26	GI:4506621	P61254	60S ribosomal protein L26	17.26	3
	40S ribosomal protein S23	GI:4506701	P62266	40S ribosomal protein S23	15.81	3
GTPase	Guanine nucleotide binding protein (G protein), beta polypeptide 1 **(GNB1)**	GI:91992949	P62873	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	36.30	4
	Ras-related protein Rap-1b isoform 1 precursor	GI:7661678	P61224	Ras-related protein Rap-1b	20.83	8
	Ras-related protein Rap-1b isoform 3 (**Rap-1b** isoform 3)	GI:354459354	P61224		18.78	8
	Ras-like protein	GI:190875	P63000	Ras-related C3 botulinum toxin substrate 1	21.34	5
	Ras-related C3 botulinum toxin substrate 1 isoform Rac1 **(Rac1)**	GI:9845511	P63000		21.45	5
	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1), isoform CRA_d	GI:119575445	P63000		24.18	5
Metabolic Processes	Malate dehydrogenase, mitochondrial precursor	GI:21735621	P40926	Malate dehydrogenase, mitochondrial	35.50	20
	Malate dehydrogenase precursor	GI:2906146	P40926		35.53	20
	Unknown	GI:41472053	P40926		33.23	20
	Malate dehydrogenase 2, NAD (mitochondrial)-**MDH2**	GI:49168580	P40926		35.56	20
	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	GI:31645	P04406	Glyceraldehyde-3-phosphate dehydrogenase	36.05	9
	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1 **(ATP5A1)**	GI:127798841	P25705	ATP synthase subunit alpha, mitochondrial	59.71	9
	ATP synthase subunit alpha, mitochondrial isoform a precursor	GI:4757810	P25705		59.75	9
	ATP synthase subunit alpha, mitochondrial isoform c	GI:50345982	P25705	ATP synthase subunit alpha, mitochondrial	54.50	9
	Dihydrolipoamide branched chain transacylase E2 **(DBT)**	GI:16741763	P11182	Lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex, mitochondrial	53.52	5
	Branched chain acyltransferase precursor	GI:179354	P11182		53.96	5
	Lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex, mitochondrial precursor	GI:392494079	P11182		53.49	5
	Dihydrolipoyltransacylase	GI:736675	P11182		52.92	5

Metabolic Processes	L-lactate dehydrogenase A chain isoform 1 (LDHA isoform 1)	GI:5031857	P00338	L-lactate dehydrogenase A chain	36.69	4
	Lactate dehydrogenase A variant (**LDHA** variant)	GI:62897717	P00338		36.69	4
	L-lactate dehydrogenase A chain isoform 3 (LDHA isoform 3)	GI:260099723	P00338		39.84	4
	Chain A, human muscle L-lactate dehydrogenase M Chain, Ternary complex with Nadh and Oxamate	GI:13786849	P00338		36.56	4
	Fumarate hydratase, mitochondrial (**FH** also known as **FUMH)**	GI:19743875	P07954	Fumarate hydratase, mitochondrial	54.64	3
	Fumarate hydratase, isoform CRA_a	GI:119590496	P07954		52.65	3
	Fumarate hydratase, isoform CRA_b	GI:119590497	P07954		54.70	3
	Fumarate hydratase, isoform CRA_d	GI:119590499	P07954		46.41	3
	Cytochrome c oxidase subunit II -mitochondrion (**COX2** also known as MT-CO2)	GI:110083932	P35354	Prostaglandin G/H synthase 2	25.55	3
	Aldo-keto reductase family 1, member B1 (aldose reductase)-**AKR1B1**	GI:13529257	P15121	Aldose reductase	35.82	3
	Aldo-keto reductase family 1, member B1 variant (AKR1B1 variant)	GI:62089178	P15121		29.16	3
	Ubiquinol-cytochrome c reductase core I protein (**UQCRC1**)	GI:515634	P47985	Cytochrome b-c1 complex subunit 1, mitochondrial	52.62	3
	Cytochrome b-c1 complex subunit 1, mitochondrial precursor	GI:46593007	P47985		52.65	3
	SHMT2 protein	GI:60552225	P34897	Serine hydroxymethyltransferase, mitochondrial	52.91	3
	Serine hydroxymethyltransferase 2 (mitochondrial) variant	GI:62898842	P34897		55.98	3
	Serine hydroxymethyltransferase (**SHTM**)	GI:703093	P34896	Serine hydroxymethyltransferase, cytosolic	52.46	3
	Serine hydroxymethyltransferase, mitochondrial isoform 3	GI:261862348	P34897	Serine hydroxymethyltransferase, mitochondrial	53.46	3
	Serine hydroxymethyltransferase 2 (mitochondrial), isoform CRA_a	GI:119617399	P34897		27.13	3
	Serine hydroxymethyltransferase 2 (mitochondrial), isoform CRA_d	GI:119617402	P34897		55.21	3
	Serine hydroxymethyltransferase 2 (mitochondrial), isoform CRA_g	GI:119617405	P34897		44.26	3
	Serine hydroxymethyltransferase 2 (mitochondrial), isoform CRA_h	GI:119617407	P34897		44.62	3
	Serine hydroxymethyltransferase, mitochondrial isoform 1 precursor	GI:19923315	P34897		56.00	3
	Chain A, Human Mitochondrial Serine Hydroxymethyltransferase 2.	GI:310689962	P34897		54.15	3
Miscellaneous	ADM precursor	GI:4501945	P35318	ADM	20.42	11
	Preproadrenomedullin (**adrenomedullin**)	GI:599559	P35318		20.61	11
	Unnamed protein product	GI:193786694	O43852	Calumenin	37.02	3
	Calumenin isoform b precursor	GI:194578885	O43852		37.14	3
	Calumenin	GI:2809324	O43852		37.07	3
	Calumenin isoform 5	GI:295848251	O43852		26.86	3
	Calumenin isoform 9	GI:295848259	O43852		17.58	3
	Calumenin isoform c precursor	GI:314122177	O43852		38.05	3
	Calumenin isoform d precursor	GI:314122179	O43852		38.08	3
	Calumenin isoform a precursor	GI:4502551	O43852		37.11	3
	CALU	GI:49456627	O43852		37.01	3
	crocalbin-like protein	GI:8515718	Unknown		34.99	3
	Transmembrane protein 109 precursor (**TMEM 109** precursor)	GI:13129092	Q9BVC6	Transmembrane protein 109	26.21	5

Miscellaneous	Annexin A1 (**Annexin I**)	GI:4502101	P04083		38.72	3
	Annexin A1, isoform CRA_b	GI:119582950	P04083	Annexin A1	40.22	3
	Annexin A1, isoform CRA_c	GI:119582952	P04083		40.08	3
	T-complex protein 1 subunit zeta isoform a	GI:4502643	P40227		58.03	5
	Chaperonin containing TCP1, subunit 6A isoform a variant	GI:62089036	P40227	T-complex protein 1 subunit zeta	57.76	5
	Acute morphine dependence related protein 2	GI:14517632	P40227		58.01	5
	p180/ribosome receptor	GI:338221409	A7BI36	p180/ribosome receptor	165.75	18
	Leucine-rich repeat-containing protein 59 (**LRRC59**)	GI:40254924	Q96AG4	Leucine-rich repeat-containing protein 59	34.93	11
	Ornithine aminotransferase (OAT)	GI:1168056	P04181		48.54	3
	Ornithine aminotransferase, mitochondrial isoform 1 precursor	GI:4557809	P04181	Ornithine aminotransferase, mitochondrial	48.53	3
	Chain A, Ornithine Aminotransferase Mutant Y85i	GI:78101702	P04181		48.49	3
	Cytochrome b5	GI:2662291	O43169	Cytochrome b5 type B	16.33	3
	Cytochrome b5 type B (**CYB5B**)	GI:83921614	O43169		16.70	3
Ubiquitin proteolysis	**Ubiquitin**	GI:229532	P0CG48		8.45	6
	Polyubiquitin	GI:2627129	P0CG48		68.49	6
	UBC protein	GI:38114661	P0CG48		34.31	6
	Ubiquitin C splice variant	GI:54300702	P0CG48	Polyubiquitin-C	17.15	6
	Ubiquitin C variant	GI:62089150	P0CG48		147.34	6
	Polyubiquitin-C (**UBC**)	GI:67191208	P0CG48		77.03	6
	Polyubiquitin-B precursor	GI:11024714	P0CG48		25.76	6
Unknown	Aging-associated gene 9 protein	GI:54303910	Unknown		36.05	9
	Unnamed protein product	GI:189053924	Unknown		21.90	6
	Unnamed protein product	GI:189069163	Unknown		37.30	4
	Unnamed protein product	GI:189054116	Unknown		26.20	5
	Unnamed protein product	GI:194374129	Unknown		40.37	3

Table 2.2 Mass spectrometry identified MGMT-binding partners in T98G cell line grouped by function.

From		To		Effect	Mechanism	Details of Interaction
Protein	Protein Type	Protein	Protein Type			
FPR	GPCR	G-protein beta/gamma	G beta/gamma	Activation	Binding	FPR physically interacts with G-protein beta/gamma and increases its activity.
DVL-3	Generic binding protein	Rac1	RAS - superfamily	Activation	Binding	Dvl-3 physically interacts with Rac1 and increases its activity
CREB1	Transcription factor	LRRC59	Generic binding protein	Unspecified	Transcription regulation	PRO1855 promoter has a putative CREB1-binding site.
Ubiquitin	Generic binding protein	TAK1 (MAP3K7)	Protein kinase	Activation	Binding	Ubiquitin binds TAK1.
PAK1	Protein kinase	Rac1	RAS - superfamily	Activation	Binding	PAK1 interacts with an unspecified isoform of Rac. This interaction was modeled on a demonstrated interaction between PAK1 from an unspecified species and human Rac.
CREB1	Transcription factor	COL6A3	Generic binding protein	Unspecified	Transcription regulation	COL6A3 promoter has a putative CREB1-binding site.
IL8RB	GPCR	G-protein beta/gamma	G beta/gamma	Activation	Binding	IL8RB interacts with G-protein beta/gamma and increases its activity.
Ubiquitin	Generic binding protein	NFKBIA	Generic binding protein	Inhibition	Binding	In the context of an IkBa/NF-kB complex, efficient IkBa ubiquitination is dependent on phosphorylation by IkK.
CREB1	Transcription factor	TCP1-zeta-1	Generic binding protein	Unspecified	Transcription regulation	CREB1 regulates transcription of TCP1-zeta-1.
CREB1	Transcription factor	RPS9	Generic binding protein	Unspecified	Transcription regulation	RPS9 promoter has a putative CREB1-binding site.
CREB1	Transcription factor	PCNA	Generic binding protein	Activation	Transcription regulation	CREB1 binds to promotor of PCNA gene and induces its transcription.
TRAF2	Generic binding protein	Ubiquitin	Generic binding protein	Activation	Binding	TRAF2 is E3 ligase that mediates Lys63-linked TAK1 polyubiquitination
IL8RA	GPCR	G-protein beta/gamma	G beta/gamma	Activation	Binding	IL8RA interacts with G-protein beta/gamma and increases its activity.
VAV-1	Regulators (GDI, GAP, GEF)	Rac1	RAS - superfamily	Activation	Transformation	VAV-1 acts as GEF for Rac1.
CDC42	RAS - superfamily	PAK1	Protein kinase	Activation	Binding	Cdc42 interacts with Pak1.
Kalirin	Regulators (GDI, GAP, GEF)	Rac1	RAS - superfamily	Activation	Transformation	Overexpression of Kalirin GEF1 led to activation of endogenous Rac.
VAV-2	Regulators (GDI, GAP, GEF)	Rac1	RAS - superfamily	Activation	Transformation	Vav2 is guanine nucleotide exchange factor for Rac1.
Rac1	RAS - superfamily	p67-phox	Generic binding protein	Activation	Binding	Activation of Rac1 induces interaction of Rac1 with p67-phox and reactive oxygen species (ROS) formation.
Ubiquitin	Generic binding protein	RIPK1	Protein kinase	Activation	Binding	Upon TNFR1 activation, RIP1 is recruited to the receptor where Lys 377 serves as an acceptor site for K63-linked polyubiquitin chains. To attenuate TNF signalling, A20 targets RIP1 for inactivation by removing the K63-linked ubiquitin chains and adding K48-linked ubiquitin chains to promote degradation
CD44	Generic receptor	VIL2 (ezrin)	Generic binding protein	Activation	Binding	CD44 binds to and activates ezrin.
BETA-PIX	Regulators (GDI, GAP, GEF)	CDC42	RAS - superfamily	Activation	Transformation	Binding of Cdc42 to beta-PIX was determined using GST pull-downs and then quantification of coprecipitated myc-beta-PIX.
Rac1	RAS - superfamily	p47-phox	Generic binding protein	Activation	Binding	Rac1 physically interacts with p47-phox and increases its activity.

Ubiquitin	Generic binding protein	IKK-gamma	Generic binding protein	Activation	Binding	PolyUb chains activate IKK by direct binding to NEMO.
Rac1	RAS - superfamily	MLK3 (MAP3K11)	Protein kinase	Activation	Binding	The association between Rac1 and MLK3 and the autophosphorylation of MLK3 are simultaneously inhibited at ischemia reperfusion.
PREX1	Regulators (GDI, GAP, GEF)	Rac1	RAS - superfamily	Activation	Transformation	PREX1 is a guanine nucleotide exchange factor GEF for Rac1.
G-protein beta/gamma	G betta/gamma	PREX1	Regulators (GDI, GAP, GEF)	Activation	Binding	PREX1 is directly activated by the beta/gamma subunits of heterotrimeric G proteins and by the lipid second messenger PtdIns(3,4,5)P3, which is generated by PI3K.
Annexin I	Generic binding protein	FPR	GPCR	Activation	Binding	Annexin I binds to and activates FPR.
Rac1	RAS - superfamily	NOX1	Generic enzyme	Activation	Binding	Rac1 binds to NOX1 and activates it.
VAV-3	Regulators (GDI, GAP, GEF)	Rac1	RAS - superfamily	Activation	Transformation	Vav3 mediate the rapid activation of Rac1 through its direct phosphorylation by the EGFR kinase.
VAV-2	Regulators (GDI, GAP, GEF)	CDC42	RAS - superfamily	Activation	Transformation	Vav2 acts as GEF for Cdc42, and its knockdown significantly suppressed EGF-induced Cdc42 activation.
ARF6	RAS - superfamily	Rac1	RAS - superfamily	Activation	Binding	Rac1 associates with ARF6.

Table 2.3 Protein interactors from our list of GBM MGMT-binding partners (blue cells) that have known functional roles with other proteins in GBM. Created using MetaCore's GBM interactions report.

Enrichment by Pathway Maps						
#	Maps	Total # Pathway Members	pValue	Min FDR	Number of Proteins in Pathway from T98G MGMT-Binding Partners	
1	Cytoskeleton remodeling_Regulation of actin cytoskeleton by Rho GTPases	23	1.153E-06	2.997E-04	4:	CDC42, Rac1, Filamin A, MyHC
2	Hyaluronic acid/ CD44 signaling in cancer	58	5.057E-05	4.383E-03	4:	CDC42, CD44, Rac1, Filamin A
3	Development_Regulation of cytoskeleton proteins in oligodendrocyte differentiation and myelination	58	5.057E-05	4.383E-03	4:	hnRNP A2, CDC42, L1CAM, Rac1
4	Cytoskeleton remodeling_RalA regulation pathway	30	1.593E-04	1.036E-02	3:	CDC42, Rac1, Filamin A
5	Cell adhesion_Alpha-4 integrins in cell migration and adhesion	34	2.325E-04	1.099E-02	3:	CDC42, Rac1, Filamin A
6	Role of Tissue factor in cancer independent of coagulation protease signaling	35	2.537E-04	1.099E-02	3:	CDC42, Rac1, Filamin A
7	Cell adhesion_Role of tetraspanins in the integrin-mediated cell adhesion	37	2.997E-04	1.113E-02	3:	CDC42, Rac1, Filamin A
8	Cell adhesion_Chemokines and adhesion	100	4.204E-04	1.146E-02	4:	CDC42, CD44, Rac1, Filamin A
9	Cytoskeleton remodeling_Cytoskeleton remodeling	102	4.534E-04	1.146E-02	4:	CDC42, Rac1, Filamin A, MyHC
10	Cytoskeleton remodeling_Hyaluronic acid/ CD44 signaling pathways	43	4.691E-04	1.146E-02	3:	CDC42, CD44, Rac1
11	Development_S1P1 signaling pathway	44	5.022E-04	1.146E-02	3:	CDC42, CD44, Rac1
12	High shear stress-induced platelet activation	46	5.729E-04	1.146E-02	3:	RAP-1B, L1CAM, Filamin A
13	wtCFTR and deltaF508 traffic / Membrane expression (normal and CF)	46	5.729E-04	1.146E-02	3:	Ubiquitin, Rac1, Filamin A
14	Transport_The role of AVP in regulation of Aquaporin 2 and renal water reabsorption	50	7.325E-04	1.360E-02	3:	Annexin II, MYH9, MyHC
15	Blood coagulation_GPVI-dependent platelet activation	55	9.686E-04	1.679E-02	3:	CDC42, RAP-1B, Rac1
16	Transcription_Sirtuin6 regulation and functions	64	1.505E-03	2.445E-02	3:	Ubiquitin, LDHA, LDHB
17	Urea cycle	70	1.948E-03	2.980E-02	3:	MDH2, OAT, FUMH
18	Development_FGF2-dependent induction of EMT	20	2.272E-03	3.282E-02	2:	CDC42, Rac1
19	Cell cycle_Sister chromatid cohesion	22	2.750E-03	3.544E-02	2:	Histone H1, PCNA
20	Apoptosis and survival_NO signaling in apoptosis	23	3.005E-03	3.544E-02	2:	G3P2, Rac1

Table 2.4 Top 20 most significant pathological processes involving MGMT-binding partners identified in T98G GBM cell line.

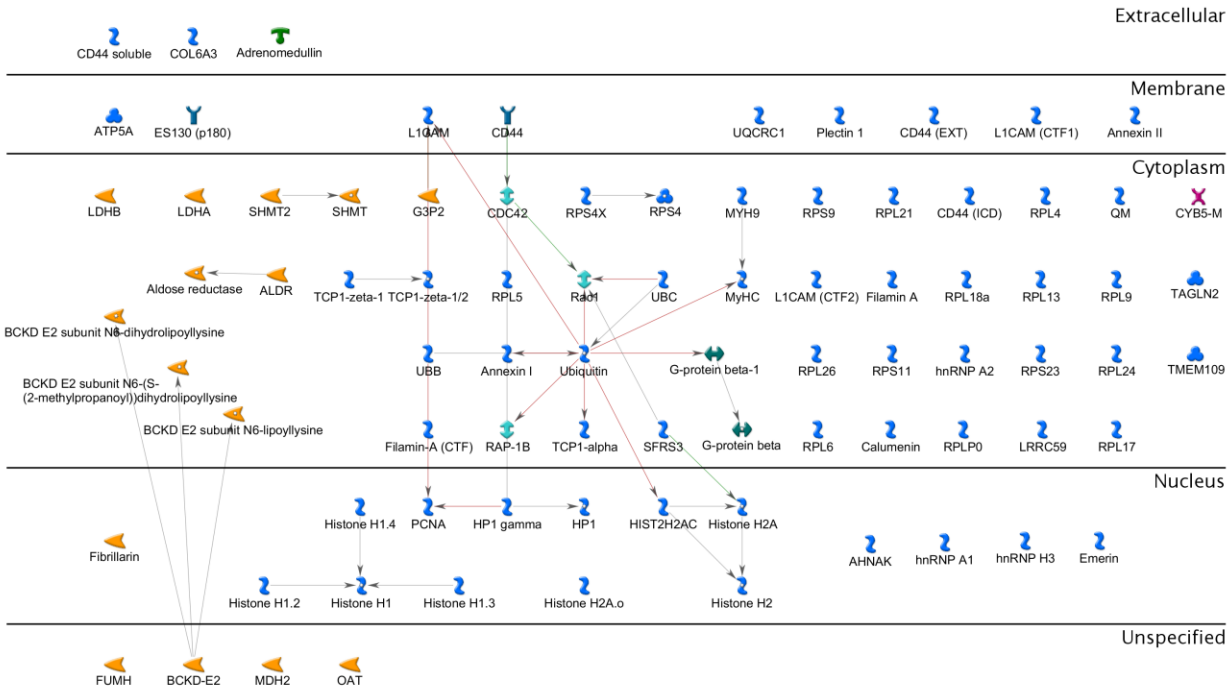

Figure 2.3 Network of potential GBM MGMT-binding partners created using the Build Network tool in MetaCore.

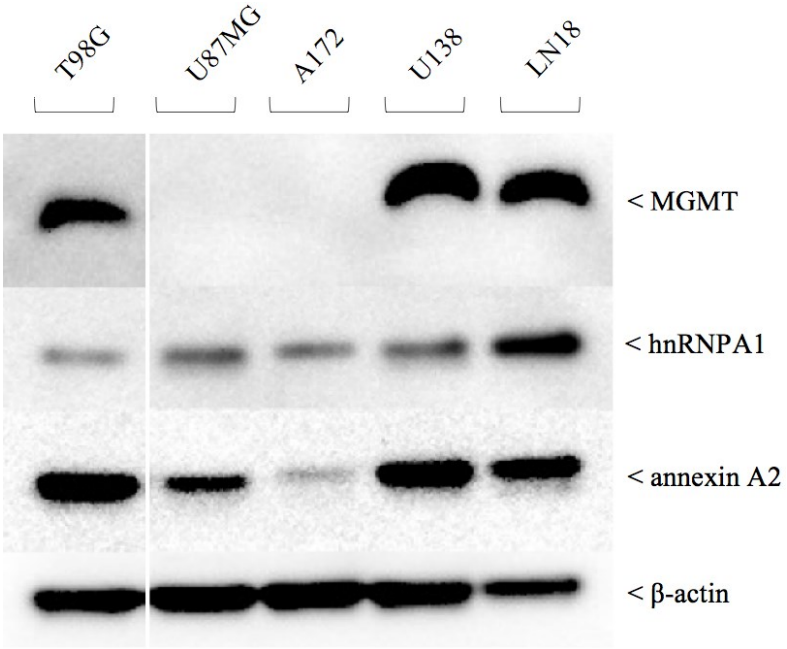

Figure 2.4 Basal MGMT, hnRNPA1, and annexin A2 expression in T98G, U87MG, A172, U138, and LN18 GBM cell lines.

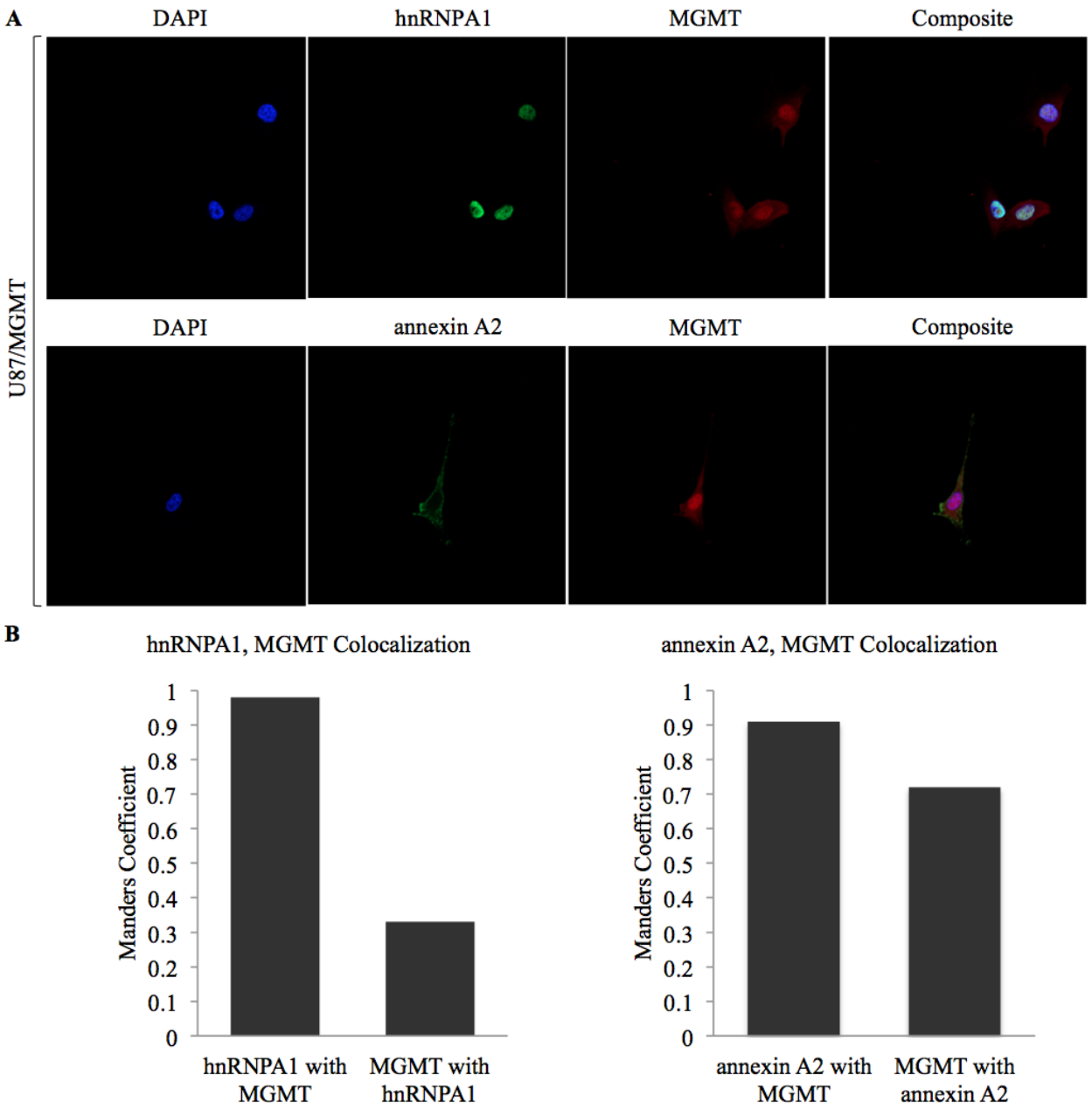

Figure 2.5. MGMT, hnRNPA1, and annexin A2 expression and colocalization in U87/MGMT transfected cells. (A) Representational images of hnRNPA1, annexin A2, and MGMT expression and localization in U87/MGMT transfected cell line. (B) MGMT colocalization with hnRNPA1 and annexin A2 represented using Manders Coefficients.

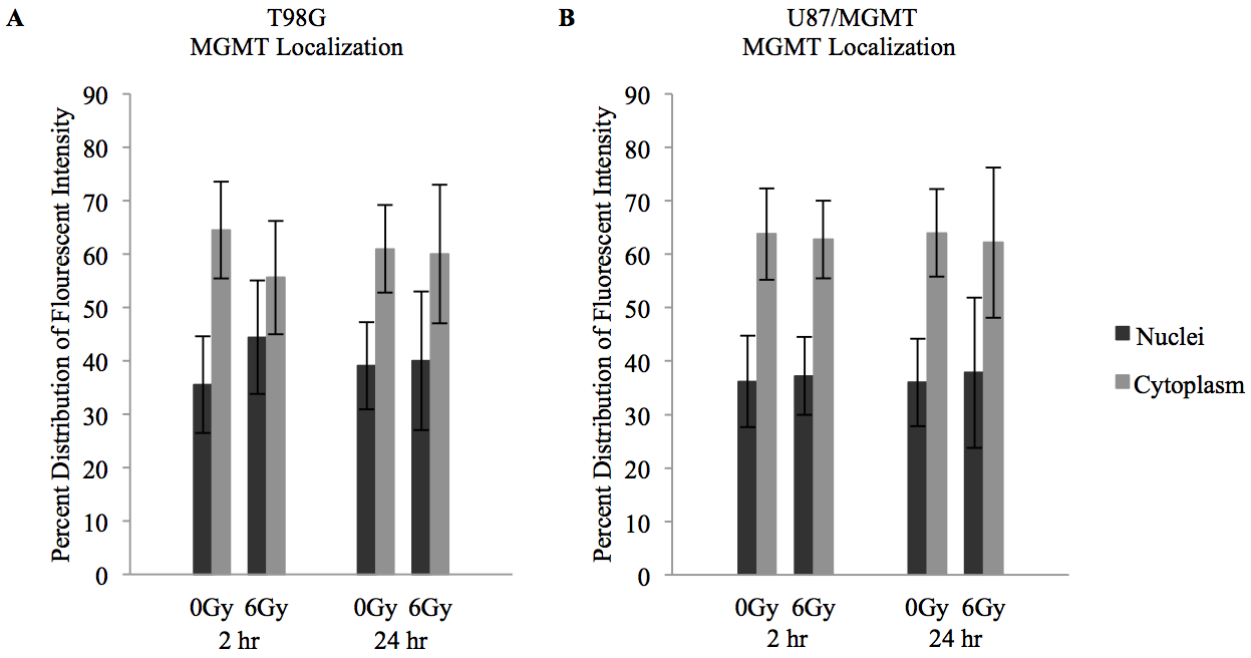

Figure 2.6 Percent nuclear versus cytoplasmic MGMT intensity in T98G (A) and U87/MGMT (B) cells 2 hour and 24 hours post 6 Gy radiation exposure compared to 0 Gy control. U87MG cells (negative for MGMT expression) were used as a negative control.

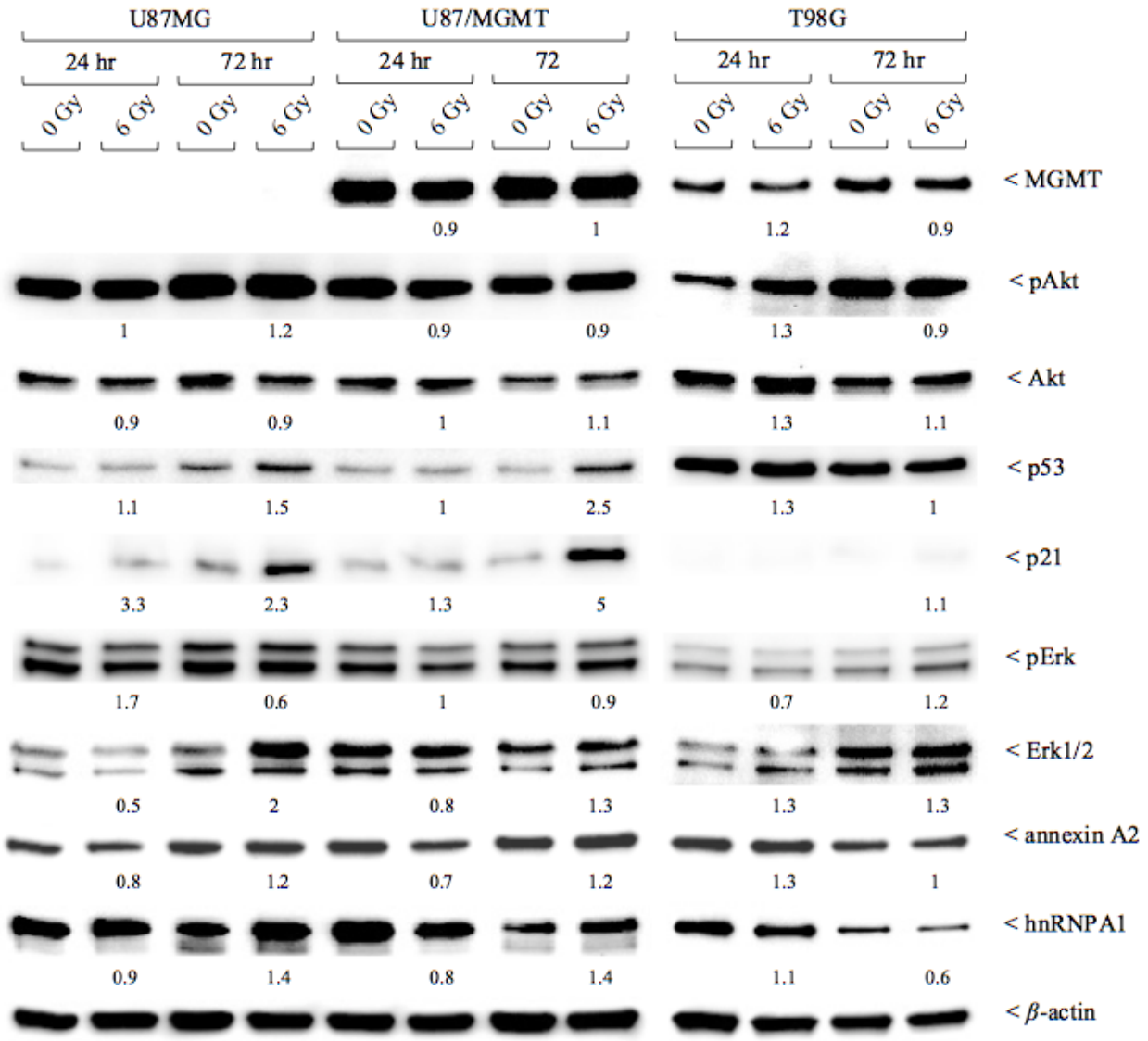

Figure 2.7 Western blotting analysis showing protein levels in U87MG, U87/MGMT, and T98G cell lines irradiated with 6 Gy or 0 Gy (control) lysed 24 or 72 hours post treatment. Protein expression of each cell line is normalized to actin and then to the 24 0 Gy control (first lane of each cell line). pAkt and pErk expression are normalized to total Akt and total Erk1/2 expression, respectively. Total Akt and total Erk1/2 expression are normalized to Actin. It should be noted that U87MG cell lines possess wild-type *PT53*, which T98G cell line is known to possess mutant *PT53*.

2.4 Discussion

MGMT is commonly described as having the sole function of DNA repair; however, our study challenges the paradigm that MGMT acts only in this capacity in GBM. A novel functional role of MGMT is likely not exclusive of its primary function of alkyl transfer, but may integrate DNA repair with an additional function to produce other responses in the cell (as hypothesized in human colon cancer cells [70]). Multiple functions of MGMT could work in tandem to signal other cellular pathways, having major implications in cellular function on a larger scale. The potential for novel MGMT functions through protein interactions has been previously examined, though in limited contexts [68-70, 97]. In this study, we support the existence of MGMT-binding partners by revealing a diverse set of binding proteins unique to GBM with defined roles in important processes that potentially contribute to GBM invasion and angiogenesis.

Identifying novel functional activities specific to the context of GBM may prove clinically relevant, considering that MGMT activity is higher in a significant proportion of human brain tumors, including GBM, compared to regular brain tissue [119] and that MGMT inhibits the effectiveness of alkylating chemotherapy agents used in cancer treatment. We were able to identify 186 potential MGMT-binding partners in human T98G GBM cells. These binding partners represent a wide range of cellular proteins and suggest a potential role for MGMT in mitochondrial metabolism, DNA repair and replication, the ubiquitin pathway, transcription regulation, RNA post-transcriptional processing, protein biosynthesis and trafficking, cellular metabolism, cell cycle and division, response to stress and cell death, cell migration, and invasion (Table 2.2).

Using MetaCore software to analyze our results, we produced a report of MGMT interactions (Table 2.3) and an enrichment pathway analysis (Table 2.4), which revealed notable proteins from our list of T98G MGMT-binding partners that were identified as members of pathways or interactions important to GBM disease progression. Proteins Rac1, CDC42, CD44, and Filamin A appeared multiple times across both reports, indicating their known significance in GBM biology.

Rac1 is a GTPase of the Ras superfamily of small GTP-binding proteins, which mediate a range of activities, including cell growth, cytoskeletal reorganization, and the activation of protein kinases. CDC42 is a GTPase of the Rho-subfamily, which have characterized involvement in cell morphology, migration, endocytosis and cell cycle progression. CDC42 is

known to regulate actin polymerization, and Rac1 and CDC42 are known drivers of GBM invasion, with their hyperactivation blocking GBM cell invasion [120]. Furthermore, pharmacological inhibition of Rac1 combined with erlotinib treatment (HER1/EGFR–targeted therapy) showed an increased antiproliferative effect in GBM cells *in vitro* [121].

CD44 is a cell surface antigen involved in cell-to-cell interactions, migration, and adhesion. CD44 has been shown to play a significant role in GBM invasion [122] and has been proposed as a therapeutic target for GBM [123].

Filamin A is an actin binding protein important in the branching and linking of actin to membrane glycoproteins, is known to act as a scaffold for various cytoplasmic signaling proteins, and plays a role in the development of blood vessels. Filamin A was recently identified as a downstream effector of rapamycin complex 2 (mTORC2), governing GBM cell motility and invasion [124]. Furthermore Rho and Rac GTPases are known to bind filamin and induce filopodia, which are integral structures in cell motility.

Collectively, our MetaCore analysis identified several proteins and pathways from our MGMT-binding partners with importance in cytoskeletal remodeling and cell adhesion, which support our previous findings for an inhibitory role of MGMT in GBM angiogenesis and invasion [40, 74]. This analysis further supports the role of MGMT in these processes and indicates likely MGMT-binding partners with significance in these pathways.

When comparing our binding partner results to those of other cell lines and proteomics databases, there was a marked lack of overlap. For example, Niture *et al*. identified 60 MGMT-binding partners with broad functional roles using extracts of HT29 human colon cancer cells [70]. However, there exists very little overlap between these binding partners and those revealed in our experiments. Though there are some similarities in identified interactions (for example, MGMT binds different H2A histones in both cell lines), there were only four exact matches with our set of MGMT-binding partners in GBM: ubiquitin, ribonucleoproteins A2/B1 (hnRNPA2/hnRNPB1), annexin A1, and annexin A2 [70]. (It is also important to note that in addition to the difference in cell type used, interacting partners were identified using affinity chromatography with human recombinant MGMT protein coupled to activated Sepharose [70], while our investigation used direct immunoprecipitation of Flag-tagged and endogenous MGMT protein.) Furthermore, there were only two exact matches between the MGMT-binding partners we identified in 293T and T98G cells: histones H1.3 and H1.4. This lack of continuity between

MGMT-binding partners may indicate specific interactions in each cellular context, potentially conferring different functional roles for MGMT in different cancers. This suggests the existence of GBM-specific MGMT-binding partners that may confer specific function to MGMT in addition to its role in DNA repair or may aid or inhibit this function. It is also important to consider that protein interactions described for one cell type and set of conditions may provide only part of the total interactions, representing a snapshot of activity, as only a portion of the cellular protein pool is active at any given time [125]. Because of this, other MGMT-binding partners may exist and the set of binding partners may fluctuate given various cellular conditions, such as stress. It may be interesting to further investigate the existence of GBM-specific MGMT-binding partners in cells that have been irradiated to see if there are changes in the interaction profile after such exposure.

Furthermore, there is structural evidence to support the existence of MGMT-binding interaction with other proteins. Structural and biochemical studies tend to focus on about 25 highly conserved residues, most of which are now known to be involved in DNA binding, alkyl transfer, and upholding protein structure [26]. However, some studies have produced more questions than answers in the pursuit of understanding the roles of these residues in MGMT. For example, while repair of *O6*-MeG residues is efficacious, repair of *O6*-MeT residues by MGMT was discovered to be extremely slow and actually impede repair of these lesions by NER, increasing mutations and cell death [126, 127]. In terms of tertiary structure, MGMT is composed of two distinct lobes, one containing the N-terminal domain and the other the C-terminal. Originally, the N-terminal domain seemingly had no function [33], but it was later determined that a portion of the N-terminal lobe contains a distinct lack of polar residues described to form a "hydrophobic handle," which may anchor MGMT to other proteins [34]. Overall, structural studies have focused on the mechanistic steps involved in MGMT-mediated alkyltransferase but have discovered structural regions that may impart additional functions, supporting the concept of MGMT-binding proteins.

Annexin A2 and the hnRNPA1 were among the proteins identified with high confidence in our proteomic analysis and represent potential MGMT interacting partners. As discussed, both proteins play important roles in cancer and may hypothetically mediate an upstream role of MGMT in angiogenesis and invasion. While we were able to observe protein colocalization, we were unable to definitively validate binding interaction between MGMT and hnRNPA1 or

annexin A2. These results may be due in part to the use of co-IP for binding studies; co-IP experiments are challenging to optimize and are not conducive to transient protein interactions, which may be the true source of our negative results. One additional downfall in the use of co-IP is that it does not permit us to distinguish between proteins that have bound directly to MGMT versus those that have bound indirectly through an intermediate or the formation of protein complexes. Moving forward, chemical crosslinking of MGMT to potential interacting proteins during incubation may prove an effective improvement to experimental design. Alternatively, other methods of detecting protein interaction, such as fluorescence resonance energy transfer (FRET) may provide more definitive answers to address the question of MGMT-binding partners.

We also investigated the role of ionizing radiation exposure in GBM protein expression in order to investigate whether such stress induces varied protein expression in MGMT-positive versus -negative GBM cells. We assessed expression of MGMT, hnRNPA1, annexin A2, and signaling pathways involved in proliferation and survival. While hnRNPA1 and annexin A2 expression were affected, these changes did not appear to correlate with MGMT expression and were not consistently influenced by exposure to ionizing radiation. We also did not observe significant changes in MGMT expression or localization in irradiated cells, although *MGMT* promoter activation and *MGMT* mRNA expression have been shown to increase linearly in response to exposure up to 8 Gy in H4IIE rat hepatoma cells [53]. However, we noticed a low pErk to Erk1/2 expression ratio in irradiated MGMT-positive cell lines compared to negative, indicating a lack of Erk activation in the MGMT-positive cells. Interestingly, suppressors of cytokine signaling 1 (SOCS1) and 3 (SOCS3) have been shown to be abnormally expressed in GBM cell lines and primary tissues resulting in amplified Erk-MAPK pathway signaling, potentially increasing resistance to ionizing radiation [128]. As we observed low Erk activation in MGMT-positive cells compared to -negative, this may suggest increased radiosensitivity of MGMT-positive cells and presents an avenue for further investigation.

We also observed increased p53 expression, mirrored by increased p21 expression, in irradiated U87/MGMT cells compared to U87MG cells. Wild-type p53 expression is known to down-regulate MGMT through interaction with the Sp1 transcription factor [51] and overexpression of p53 protein in *TP53*-null *MGMT*-proficient cells inhibits the transcription of endogenous *MGMT*, altering sensitivity to alkylating agents [129]. The reason for this *MGMT* regulation is unknown but our results indicate that increased MGMT expression may be inducing

increased wild-type p53 and subsequent increased p21 expression, hypothetically mediating a pro-apoptotic response to ionizing radiation. Additional studies are needed to further assess the effects of radiation on cell cycle, clonogenic survival, apoptosis, and whether ionizing radiation affects colocalization and co-IP of MGMT with its binding partners, in addition to any functional consequences of these changes.

Despite the need for additional experiments to verify the potential MGMT-binding partners, we have identified the functional significance of their interactions through pathway analysis. Our results support the concept that MGMT-binding partners do exist in the context of GBM, providing an alternative to the common perception that MGMT functions solely as a DNA repair protein in an independent fashion. The use of a proteomic-based approach provides cellular context to these binding partners, allowing us to understand the potential effects of these interactions and their implications in the greater context of cancer development and treatment. Identifying pathways that integrate MGMT activity will provide a more comprehensive understanding of MGMT functions and may lead to new strategies in the development of drug therapeutics or improve the application of combination therapies in the treatment of GBM patients.

3. Conclusions and Perspectives

Elucidating how MGMT is involved in novel functional activities beyond its known role is crucial in determining how MGMT affects GBM angiogenesis and invasion at the molecular level and may reveal potential novel targets for treatment. We hypothesized that MGMT may be exerting a functional role beyond DNA repair through binding interactions with other proteins. Using a mass-spectrometry-based proteomic approach, we were able to identify potential MGMT-binding partners, representing a wide range of cellular proteins with diverse functions. While at this stage, we have not validated MGMT interaction with hnRNPA1 or annexin A2, further investigation is necessary to investigate interactions with other binding partners, and determine if cellular stress (such as ionizing radiation exposure) influences these interactions. Alkylating agents such as TMZ are also known to affect MGMT expression, and thus MGMT protein interactions in cells that have been stimulated by exposure to TMZ may reveal new MGMT-interaction profiles not detected in the absence of external stress induced by chemotherapy. Moving forward, using TMZ-resistant cells would aid in understanding GBM invasion and angiogenesis in response to TMZ beyond the known role of MGMT as a DNA repair protein primarily involved in removal of alkyl adducts. Furthermore, the use of alternative models such as an MGMT CRISPR knock-out with matched MGMT knock-in cell lines would prove valuable in defining the direct and indirect mechanisms of MGMT action.

Thus far, our results provide the first evidence supporting the interaction of MGMT with binding partners in the context of GBM. Our proteomic-based strategy would be further strengthened through phosphoproteomic studies to reveal unphosphorylated and phosphorylated MGMT-binding partners, which might bare functional significance and influence downstream mechanisms of action. Elucidating how MGMT is involved in novel functional activities will unravel how MGMT affects GBM angiogenesis and invasion at the molecular level. This strategy may ultimately lead to the development of new drugs targeting the interaction of MGMT with relevant binding partners in combination with other treatments, such as angiogenic inhibitors currently used for treatment of GBM patients with recurrent tumors.

Overall, our study will improve our understanding of the full scope of MGMT function in GBM. In GBM, MGMT is of valuable clinical significance in the assessment of response to chemotherapy, but beyond this role as a negative prognostic marker, MGMT expression may

contribute to other areas of cell functioning, specifically GBM invasion and angiogenesis, making it a logical focus for further studies.

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