Supplemental Methods and Figure Legends for the Following Manuscript:

MARCKS Regulates Growth, Radiation Sensitivity and is a Novel Prognostic Factor for Glioma

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Running Title: MARCKS Regulates GBM Growth and is Prognostic in Patients

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS

Immunoblotting

Protein concentration was determined using the Pierce BCA protein assay kit (Thermo Scientific). All samples were electrophoresed through reducing SDS-polyacrylamide gels and electroblotted onto PVDF membranes (Immobilon). Membranes were blocked in phosphate buffered saline (PBS) containing 2% BSA and 0.1 % Tween-20 for one hour at 25°C and incubated with primary antibodies at 1:1000 dilution. Once washed, membranes were incubated with secondary antibody at 1:5000 (donkey anti-rabbit IgG-HRP, Jackson ImmunoResearch Laboratories, catalog # 711-035-152) and reactivity was detected using ECL chemiluminescent kits (Pierce, Rockford, IL) according to manufacturer's directions. The developed films were scanned using an Epson Perfection V500 Photo Scanner. Densitometry was performed using the program UN-SCAN-IT Gel using logarithmic analysis.

Plasmid constructs

The pCMV-MARCKS-WT-GFP plasmid (Origene # RG210277) and the pLenti4/V5-DEST plasmid (Invitrogen # V498-10) were digested with NdeI (catalog # R0111, New England Biolabs) and MluI (catalog # R0198, New England Biolabs) to generate the insert containing the MARCKS coding sequence and the appropriate vector backbone. The vector was then treated with Antarctic Phosphatase (catalog # M0289, New England Biolabs) overnight. The digested fragments were gel purified (catalog # 28704, Qiagen) and the MARCKS insert was ligated into the plenti4/V5-DEST vector. The resulting lentiviral transfer vector was designated pLenti4-MARCKS. The empty vector control plasmid was generated by digesting pLenti4/V5-DEST with XbaI (catalog # R0145, New England Biolabs) to remove the attR1-CmR-ccdB-attR2 sequence. The digested fragments were treated with Klenow fragment (catalog # M0120, New England

Biolabs), gel purified, and the resulting vector was ligated closed to generate the pLenti4-DELTA plasmid. Ligations were performed with T4 DNA Ligase (catalog # 15224-017, Invitrogen) at 16°C overnight. The ligation reactions were transformed into OneShot Stbl3 Chemically Competent Cells (catalog # C7373-03, Invitrogen) and plated onto LB agar containing 100µg/mL ampicillin. DNA Miniprep (catalog # 27104, Qiagen) and Maxiprep (catalog # 12262, Qiagen) kits were used for plasmid purification. Constructs were sequenced at the UAB Center for AIDS Research DNA Sequencing Core.

Lentiviral Vector Production

293FT cells were cultured to 90% confluence, harvested and counted with a hematocytometer. 27µL of Lipofectamine 2000 (catalog # 11668, Invitrogen) was mixed in 1.5mL of Opti-MEM (catalog # 11058, Invitrogen) and incubated at room temperature for 5 minutes. 4µg each of psPAX2, pCMV-VSV-G, and the appropriate lentiviral vector transfer plasmid were mixed in 1.5mL of Opti-MEM. The plasmid and lipofectamine media was mixed and allowed to incubate for 20 minutes at room temperature. $15x10^6$ cells were plated in 10mL of DMEM supplemented with 10% FBS without antibiotics onto a 10cm dish coated with poly-D-lysine (catalog # P7886, Sigma). The Lipofectamine-plasmid complexes were added in a drop-wise fashion to the cells prior to adherence and the cells were allowed to attach overnight. The next morning the media was replaced with fresh DMEM supplemented with 10% FBS and 1% Pen-Strep. Lentiviral supernatants were harvested at 24 hours, filtered through a 0.45µm filter, aliquoted and stored at -80°C. Aliquots of lentivirus were quantified using the QuickTiter p24 ELISA (Cell Biolabs, Inc.).

Cell Culture and Stable Cell Line Selection

$1x10^5$ U251 and $2x10^5$ U87 cells were plated in 6 well plates and allowed to adhere overnight. The next morning, 75ng of p24 quantified lentiviral particles containing MARCKS shRNA, or 94ng containing a wild type MARCKS expression construct and their appropriate control viruses were used to infect U251 or U87, respectively, in a 500µL volume for 2 hours at 37°C in 5% CO_2. After 2 hours the media containing the lentiviral particles was aspirated and replaced with 2mL of fresh media. The cells were allowed to incubate for 72 hours then re-plated into a 10cm dish. After adherence, the media on the infected U251 cells was replaced with 1µg/mL Puromycin and allowed to select for 2 passages before frozen stocks were prepared. The infected U87 cells were expanded to three 10cm dishes and a dose titration of 25, 50, and 100µg/mL of Zeocin was used to select for the highest over-expressing cells. A plate of U251 and a plate of U87 wild-type cells were used as a control to ensure a sufficient antibiotic concentration. After selection of the transformed U87 cells, the 10cm dish containing cells selected with 25µg/mL Zeocin were harvested for the proliferation assay and western blot. To increase MARCKS levels, the infected cells were selected with higher levels of Zeocin (100µg/mL), were imaged, and quantified as described below.

Cell size quantification

20 random fields were captured at 10x magnification on an EVOS fl digital inverted microscope (AMG). The size of 20 cells was determined using ImageJ by tracing the border of each cell with the freehand selection tool and using the Analyze > Measure function. Mean and standard error were then calculated.

Senescence associated beta-galactosidase stain

Staining for senescence associated beta-galactosidase (SA-β-Gal) was performed on 10cm dishes of the U87 cells transformed with empty vector control or the MARCKS over-expression

lentivirus after selection with 100μg/mL Zeocin as described above. The Senescence β-Galactosidase Staining Kit (Catalog #9860, Cell Signaling) was used according to manufacturer's protocol. Images were captured at 20x magnification with an AxioCam MRc5 on a Zeiss Observer.A1 microscope. Senescence was quantified by counting the percentage of SA-β-Gal positive cells in 15 fields at 10x magnification. Mean and standard error were then calculated.

Mitotic catastrophe

$4x10^5$ cells were plated in 6-well plates and allowed to adhere overnight. The cells were then treated with 8 Gy radiation and fixed at 24, 48 and 72 hours with 4% paraformaldehyde. The cells were then stained with 300ng/mL DAPI for 10 minutes and washed 3 times with PBS. Images were captured at 40x magnification on an EVOS fl digital inverted microscope (AMG). Cells with the presence of more than one nucleus within the same cell were designated as undergoing mitotic catastrophe (see Supplementary Fig. S4). 15 fields were counted and mean and standard error were then calculated.

Apoptosis

$4x10^5$ cells were plated in 6-well plates and allowed to adhere overnight. The cells were then treated with 8 Gy radiation and fixed at 24, 48 and 72 hours with 4% paraformaldehyde. The cells were then stained with 300ng/mL DAPI for 10 minutes and washed 3 times with PBS. Images were captured at 40x magnification on an EVOS fl digital inverted microscope (AMG). Cells with condensed, pyknotic, and fragmented nuclei were counted as apoptotic (see Supplementary Fig. S4A). These were distinguished from mitotic figures by the presence of condensed chromosomes (Supplementary Fig. S4B). 15 fields were counted and mean and standard error were then calculated.

Cell Cycle Analysis

$1x10^5$ U251 control and MARCKS knockdown cells were plated in 6-well dishes in triplicate and allowed to adhere overnight. The following day the cells were irradiated with 8 Gy. The cells were harvested by trypsinization at the indicated time points, the trypsin was neutralized with media and the cells were collected by centrifugation at 1000rpm for 10 minutes. The cells were re-suspended in 1mL of cold PBS and were then fixed with the addition of 4ml of -20°C ethanol at 4°C overnight. The next day, the cells were again collected by centrifugation and the fixative was aspirated. The cells were then re-suspended in 300µL PBS + 1µL RNAse (25mg/mL) and incubated at 37 degrees Celsius for 30 minutes. 30µL propidium iodide (in PBS 100µg/mL final concentration) was added to each sample. Flow cytometry was performed at the UAB Flow Cytometry Center for Aids Research (CFAR) Core.

DNA Damage Quantification

U251 MARCKS knockdown and control cells were cultured and seeded on sterile cover slips, allowed to attach overnight, and subsequently treated with mock or 8 Gy using a 320 kV X-ray irradiator (Kimtron Inc., Woodbury, CT). At the appropriate time points, cells were rinsed in PBS and incubated for 5 minutes at 4°C in ice-cold cytoskeleton buffer (10mM Hepes/KOH, pH 7.4, 300mM sucrose, 100mM NaCl, 3mM $MgCl_2$) supplemented with 1mM PMSF, 0.5mM sodium vanadate and proteasome inhibitor (Sigma, 1:100 dilution) followed by fixation in 70% ethanol for 15 minutes. The cells were blocked and incubated with primary antibodies (1:500 dilution, phospho-H2AX Ser139, Millipore, catalog # MI-07-164). The secondary antibody was anti-rabbit Alexa Fluor 594–conjugated antibody (1:2000 dilution; Invitrogen). DAPI (Invitrogen, catalog # D21490) was used for nuclear staining. The cover slips were subsequently mounted onto slides with mounting media (Aqua poly mount, Polysciences, Inc. catalog # 18606) and analyzed via fluorescence microscopy (Carl Zeiss, Thornwood, NY). Positive and negative controls were

included on all experiments. A total of 500 cells were assessed. For foci quantification, cells with greater than 10 foci were counted as positive according to the standard procedure (1-3).

Survival analysis

For the REMBRANDT data, gene expression for MARCKS was calculated as the average over the normalized intensity values for five MARCKS probes (201668_x_at, 201669_s_at, 201670_s_at, 213002_at, and 225897_at) and dichotomized at the median expression level. Survival in months was modeled with a Cox proportional hazards model using the coxph routine in the Survival package (version 2.35-7) in R (version 2.10.0). Kaplan-Meier curves for dichotomized MARCKS expression were generated using predicted survival times from the Cox model using the survfit function from the survival package. For the TCGA data, gene expression data that has been summarized by gene and across three platforms was obtained as previously described (4). Survival data was obtained using the "days_to_death" calculated field from the TCGA data portal after filtering out patients with prior glioma (http://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp). MGMT methylation status was kindly provided by Neil Hayes at UNC, and GBM subtype was assigned as previously described (4). Cox proportional hazards models were fit using the coxph routine in the Survival package (version 2.35-7) in R (version 2.10.0). We summarized the model fit using estimates of the hazard ratio (HR) for each term along with 95% confidence intervals for the effect estimates, as well as p-values for testing the null hypothesis of no effect. Model R^2 and adjusted R^2 given age were calculated to account for censoring using a formula given in (van Wieringen et al., 2009) (5),

$$R^2 = 1-\exp(-(2/n)\,(l(\beta) - l(0)))$$

where n is the sample size, $l(\beta)$ is the likelihood of the full model, and $l(0)$ is the likelihood of the reduced model (either null model or model with just age). Kaplan-Meier curves for MARCKS quartiles or other expression categories were generated by predicting the survival times from

the Cox model at the median age value. This was implemented using the survfit function from the survival package. The proportional hazards assumption was evaluated graphically using the Kaplan-Meier curves and tested using the cox.zph function (6). We found no evidence of proportional hazards violation for MARCKS quartiles or other expression categories. Median survivals were determined from the Kaplan-Meier curves using the program UN-SCAN-IT.

SUPPLEMENTARY TABLES

Supplementary Table 1

Tumor ID	EGFR Amplification	PTEN Status	TP53 Status	MGMT Methylation	IC Tumor Growth (Days)	Radiation Sensitive
GBM 6	Yes (vIII)	WT	R273C	No	52	YES
GBM 10	No	Null	WT	No	55	NO
GBM 12	Yes (WT)	WT	Exon 2 splice	Yes	28	NO
GBM 14	No	Exon 1 (2bp)	WT	Yes	33	YES
GBM 15	Yes (WT)	WT	WT	Yes	63	YES
GBM 22	No	WT	R273C	Yes	43	NO
GBM 39	Yes (vIII)	WT	WT	Yes	31	YES
GBM 59	Yes (vIII)	Null	WT	Yes	46	YES

Supplementary Table 1. Human glioblastoma xenoline characteristics. EGFR=Epidermal Growth Factor Receptor; PTEN=Phosphatase and tensin homolog; TP53=tumor protein 53; MGMT=O^6 methylguanine-DNA methyltransferase; IC= intracranial; WT=wild type; vIII= EGFR vIII variant; bp= base pair. Radiation sensitivity testing for these lines has already been published (7) but is summarized in the table.

Supplementary Table 2

Model	Sample	R^2	R^2|age	Term	HR	*L*	*U*	*P*
MARCKS continuous	All	17.96%	2.00%	Age	1.03	1.02	1.04	<.0001
				MARCKS	0.77	0.59	0.99	0.04
	Proneural	35.19%	14.06%	Age	1.05	1.02	1.06	<.0001
				MARCKS	0.46	0.26	0.77	0.003
	Classical	46.57%	44.83%	Age	1.01	0.99	1.03	0.23
				MARCKS	0.84	0.47	1.47	0.54
	Neural	16.12%	3.17%	Age	1.04	0.99	1.08	0.07
				MARCKS	0.86	0.20	3.57	0.83
	Mesenchymal	37.89%	15.71%	Age	1.04	1.01	1.07	0.003
				MARCKS	0.65	0.32	1.28	0.21
MARCKS quartiles	Proneural	46.07%	28.49%	Age	1.06	1.03	1.07	<.0001
				MARCKS Q2	1.80	0.81	3.98	0.15
				MARCKS Q3	1.39	0.64	3.00	0.40
				MARCKS Q4	0.28	0.11	0.70	0.01
MARCKS quartiles ***(Q4 vs.Q1-Q3)***	Proneural	43.90%	25.61%	Age	1.06	1.03	1.08	<.0001
				MARCKS>Q4	0.21	0.09	0.48	0.0002
MARCKS quartiles (Q4 vs.Q1-Q3) ***Add MGMT***	Proneural	63.17%	51.16%	Age	1.06	1.03	1.08	<.0001
				MGMT	1.48	0.77	2.86	0.24
				MARCKS>Q4	0.18	0.07	0.45	<.0001
MARCKS (Q4 vs. Q1-Q3) ***Unmethlyated***	Proneural	47.38%	32.82%	Age	1.07	1.03	1.10	<.0001
				MARCKS>Q4	0.11	0.03	0.39	<.0001
MARCKS (Q4 vs. Q1-Q3) ***Methlylated***	Proneural	44.97%	12.48%	Age	1.06	1.01	1.11	0.01
				MARCKS>Q4	0.39	0.09	1.56	0.19

Supplementary Table 2. Various Cox Proportional Hazards models for survival outcome as a function of MARCKS expression (continuous or dichotomized using quartiles), age, MGMT status (as a covariate or a stratifying factor), and GBM subtype (as a stratifying factor). Model statistics include total explained variance (R^2), additional explained variance after correcting for age (R^2|age), and hazard ratio with upper and lower confidence bounds and p-value for each term in the model (HR, L, U, and p, respectively).

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. GBM proliferation rates. Proliferation was determined for each cell line using a WST-1 cell proliferation assay as described in the Methods.

Supplementary Figure 2. MARCKS knockdown and radiation sensitivity. Immunoblotting for activation of Akt in the U251 MARCKS knockdown cell line treated with 10μM of LY294002 for 1 hour (A). Proliferation was measured with the MARCKS knockdown cells treated with LY294002 (B). Representative photos of colonies formed for control and knockdown U251 cells with radiation dose and number of cells plated indicated below each photo (C). (*, $p = 0.0287$)

Supplementary Figure 3. DNA repair and cell cycle. Immunoblotting of the U251 MARCKS cell line treated with 10μM LY294002 and 8 Gy radiation (A). A table of the distribution of the cell cycle phases in our control and knockdown cell line in the basal state and after 8 Gy radiation (B). A graph of the percent of the total population versus the phase of cell cycle with significance noted (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$)(C).

Supplementary Figure 4. Mitotic catastrophe and apoptosis. Representative photos of the U251 MARCKS knockdown and control cell lines at 24, 48, and 72 hours after 8 Gy radiation. The arrow heads indicate apoptotic nuclei. The block arrows denote cells undergoing mitotic catastrophe (A). Representative photos of a normal nucleus, a nucleus undergoing mitotic catastrophe, a pyknotic nucleus, and nuclei undergoing several phases of mitotis (B).

Supplemental References:

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