

5-15-2013

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Recommended Citation

Quann, Kevin; Gonzales, Donna M; Mercier, Isabelle; Wang, Chenguang; Sotgia, Federica; Pestell, Richard G; Lisanti, Michael P; and Jasmin, Jean-François, "Caveolin-1 is a negative regulator of tumor growth in glioblastoma and modulates chemosensitivity to temozolomide." (2013).
Department of Stem Cell Biology and Regenerative Medicine Faculty Papers & Presentations.
Paper 6.
https://jdc.jefferson.edu/stem_regenerativefp/6

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Caveolin-1 is a negative regulator of tumor growth in glioblastoma and modulates chemosensitivity to temozolomide

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Keywords: Caveolin-1, glioma, brain cancer, tumor progression, tumor suppressor, microarray, mouse model, chemotherapy, temozolomide

Abbreviations: Cav-1, Caveolin-1; GBM, glioblastoma multiforme; MAPK, mitogen activated protein kinase; PI3K, phosphatidylinositol 3-kinase; RPS6, ribosomal protein S6; mTOR, mammalian target of rapamycin; TMZ, temozolomide; TGFβ, transforming growth factor beta; TGFβRI, transforming growth factor beta receptor I; TP53, tumor protein 53; PTEN, phosphatase and tensin homolog; p-gp, permeability glycoprotein

Caveolin-1 (Cav-1) is a critical regulator of tumor progression in a variety of cancers where it has been shown to act as either a tumor suppressor or tumor promoter. In glioblastoma multiforme, it has been previously demonstrated to function as a putative tumor suppressor. Our studies here, using the human glioblastoma-derived cell line U-87MG, further support the role of Cav-1 as a negative regulator of tumor growth. Using a lentiviral transduction approach, we were able to stably overexpress Cav-1 in U-87MG cells. Gene expression microarray analyses demonstrated significant enrichment in gene signatures corresponding to downregulation of MAPK, PI3K/AKT and mTOR signaling, as well as activation of apoptotic pathways in Cav-1-overexpressing U-87MG cells. These same gene signatures were later confirmed at the protein level in vitro. To explore the ability of Cav-1 to regulate tumor growth in vivo, we further show that Cav-1-overexpressing U-87MG cells display reduced tumorigenicity in an ectopic xenograft mouse model, with marked hypoactivation of MAPK and PI3K/mTOR pathways. Finally, we demonstrate that Cav-1 overexpression confers sensitivity to the most commonly used chemotherapy for glioblastoma, temozolomide. In conclusion, Cav-1 negatively regulates key cell growth and survival pathways and may be an effective biomarker for predicting response to chemotherapy in glioblastoma.

Glioblastoma multiforme (GBM) is the most common and most deadly primary brain tumor affecting adults. Despite advancements made in surgical, radiological and chemo-therapies for this grade IV astrocytoma, prognoses have remained very poor: median survival time from diagnosis remains at 9–15 mo, with less than 10% of patients surviving beyond 5 y.^{1,2}

Caveolin-1 (Cav-1) is the principle structural protein responsible for the formation of caveolae, or invaginating microdomains, in the cell membrane. The capacity for Cav-1 to associate with a wide variety of proteins has implicated it in a number of processes, ranging from vesicular transport and cholesterol homeostasis to nitric oxide production and cell migration, among others.^{3–7} Its ability to regulate cell cycle progression and intracellular signal transduction have resulted in the substantial characterization of Cav-1 in many cancers, where it has been shown to act as both a tumor suppressor and tumor promoter depending on the tissue

type.^{8–11} In gliomas, expression of Cav-1 appears to increase proportionally to tumor grade, with most GBM lesions exhibiting more intense Cav-1 immunoreactivity than their grade II and III counterparts.^{12–14} However, little is currently known as to the role of Cav-1 as it relates to GBM in vivo. Recent in vitro studies conducted using the GBM-derived cell line U-87MG have demonstrated that Cav-1 acts as a putative tumor suppressor in GBM by downregulating α5β1 integrin expression and subsequent TGFβ/SMAD pathway activity.^{15,16} Consistent with these findings, we here show that U-87MG cells stably overexpressing Cav-1 exhibit diminished mitogenic signaling, upregulated activation of apoptotic pathways and a significantly decreased ability to form tumors in vivo. Additionally, we show that expression of Cav-1 confers sensitivity to the alkylating agent temozolomide (TMZ), the most commonly used chemotherapy for GBM. These studies further support the role of Cav-1 as a putative tumor suppressor in

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Submitted: 03/15/13; Accepted: 03/28/13
<http://dx.doi.org/10.4161/cc.24497>

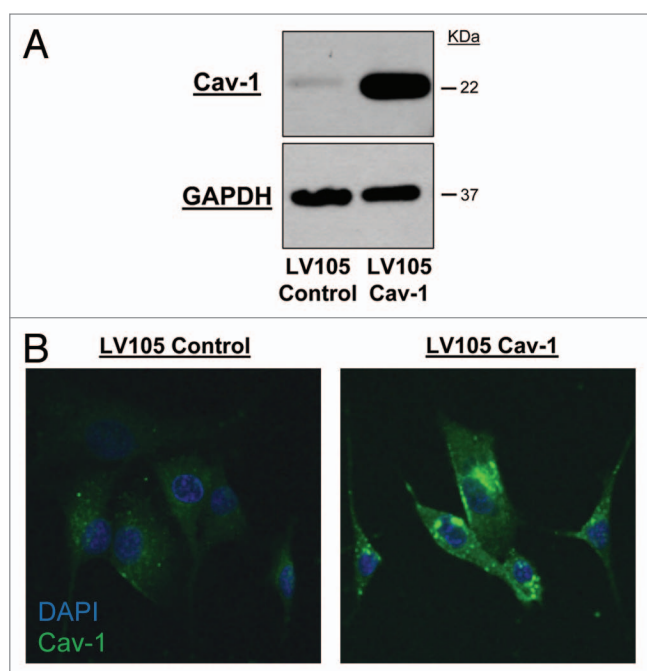


Figure 1. Stable expression of Cav-1 in U-87MG cells. (A) Expression levels of Cav-1 measured by immunoblot analyses of U-87MG cells transduced with either LV105 control or LV105 Cav-1 lentivirus. (B) Immunofluorescent staining of Cav-1 in transduced U-87MG cells (magnification = 40 \times).

gliomas and serve to underscore the potential of Cav-1 to serve as a favorable prognostic factor in GBM.

Results

Stable expression of Cav-1 in U-87MG cells. In order to establish durable expression of Cav-1 over time in a cell line model, we chose to use a lentiviral transduction approach over the transient transfection methods used in previous *in vitro* studies.^{15,16} After selection with puromycin, U-87MG cells transduced with lentiviral constructs stably expressing full length Cav-1 cDNA (LV105 Cav-1) were shown to effectively upregulate Cav-1 compared with an empty control lentivirus (LV105 Control) as demonstrated by western immunoblot (Fig. 1A). Changes in Cav-1 protein expression were also confirmed by immunofluorescence, where overexpressing cells demonstrated increased cytoplasmic and membrane localization of Cav-1 following lentiviral transduction (Fig. 1B).

Cav-1 regulates cancer-associated gene expression. Using a microarray consisting of > 20,000 transcript probes, we were able to identify 2,001 genes (~10%) significantly modulated by Cav-1 overexpression (Tables 1 and 2; Tables S1–3). Gene set enrichment analyses performed on microarray expression data obtained from LV105 control and LV105 Cav-1 U-87MG cells indicates that Cav-1 expression corresponds to changes in a variety of cancer-associated gene signatures. Specifically, by comparing expression data to biological process gene ontology sets, it was found that Cav-1-overexpressing U-87MG cells demonstrated significant ($p < 0.001$) enrichment among gene sets related to negative

regulation of signal transduction, MAP-kinase activity, cell proliferation and transcription (Table 2; Table S1). Signatures related to caspase activation, apoptosis and the transforming growth factor β pathway were also highly enriched (Table 2; Table S1). When expression data was compared with a curated canonical pathway database, gene sets related to PI3K/AKT, mTOR and ERK signaling, as well as cell death and extracellular matrix signaling, were found to be significantly enriched (Table 2; Table S1).

Cav-1 mediates major proliferative and cell-survival pathways. To validate the results obtained from our microarray analyses, we next sought to confirm Cav-1 mediated modulation of intracellular signaling pathways at the protein level. Overexpression of Cav-1 in U-87MG cells results in abrogated activity of proliferative pathways as shown by reduced phosphorylation of ERK1/2 and decreased expression of the cell cycle driver cyclin D1 when compared with control as shown by western immunoblot (Fig. 2A). Overexpression of Cav-1 further reduced the activity of protein synthesis pathways as shown by reduced activation of the AKT/mTOR/ribosomal protein S6 (RPS6) signaling pathways (Fig. 2B). Additionally, U-87MG cells overexpressing Cav-1 demonstrated increased presence of the apoptosis activator cleaved caspase 3 (Fig. 2C).

U-87MG cells stably overexpressing Cav-1 exhibit decreased tumor growth *in vivo*. To evaluate the ability of Cav-1 to regulate tumorigenicity *in vivo*, U-87MG cells infected with either Cav-1-expressing or control lentivirus were injected subcutaneously into the flanks of athymic nu/nu male mice. After 4 wk, mice were sacrificed and tumors were collected, weighed and measured. Importantly, mice harboring Cav-1 overexpressing tumors demonstrated markedly reduced (~7-fold) tumor weights and volumes as compared with their control counterparts ($p < 0.001$, Fig. 3A and B).

Cav-1 overexpressing tumors show reduced signaling activity *in vivo*. Similar to results obtained *in vitro*, our immunohistochemical analyses show that explanted xenograft tumors overexpressing Cav-1 demonstrate fewer cells staining positive for phospho-ERK1/2 and cyclin D1 (Fig. 4). Additionally, RPS6 and MTOR pathways were shown to be silenced in LV105 Cav-1 tumors, as shown by the absence of their active, phosphorylated isoforms (Fig. 4).

Cav-1 confers chemosensitivity in U-87MG cells. To further examine the effect of Cav-1 on chemotherapeutic-induced apoptosis, U-87MG cells were stained for the cell death marker Annexin V and measured by flow cytometry. Cav-1-overexpressing U-87MG cells cultured for 72 h in the presence of 500 μ M of temozolomide (TMZ), the most commonly used chemotherapeutic for GBM, showed significant reductions in cell viability when compared with TMZ-treated LV105 control cells (5.5%, $p < 0.01$, Fig. 5A). This effect was found to be most pronounced among cells initiating apoptosis, as Cav-1-overexpressing U-87MG cells treated with TMZ demonstrated > 400% increase in early apoptotic cells compared with TMZ-treated LV105 control cells ($p < 0.01$, Fig. 5B). Interestingly, although not statistically significant, overexpression of Cav-1 yielded expanded late-apoptotic and dead cells after 72 h of TMZ treatment (Fig. 5C and D).

Table 1. Differential gene expression of Cav-1 overexpressing U-87MG cells

Probe	Fold change	p-value	Description
CCDC3	94.19	0.00E+00	coiled-coil domain containing 3
COL3A1	75.99	0.00E+00	collagen, type III, α 1
MTUS1	46.14	2.77E-10	microtubule associated tumor suppressor 1
NUP210	34.18	5.08E-24	nucleoporin 210 kDa
COL4A1	30.33	1.38E-22	collagen, type IV, α 1
APOE	24.59	0.00E+00	apolipoprotein E
LRRC17	21.54	0.00E+00	leucine rich repeat containing 17
PRL	16.66	1.07E-22	prolactin
SULF1	16.35	5.88E-20	sulfatase 1
UBE2QL1	15.93	0.00E+00	ubiquitin-conjugating enzyme E2Q family-like 1
FAT3	14.40	0.00E+00	FAT tumor suppressor homolog 3 (Drosophila)
CSAG1	13.78	0.00E+00	chondrosarcoma associated gene 1
SOX4	12.86	1.89E-26	SRY (sex determining region Y)-box 4
CTNNA2	12.75	0.00E+00	catenin (cadherin-associated protein), α 2
GPM6B	11.94	1.51E-14	glycoprotein M6B
CSAG1 CSAG2 CSAG3	11.88	1.42E-15	chondrosarcoma associated gene 1 CSAG family, member 2 CSAG family, member 3
COL1A2	11.26	0.00E+00	collagen, type I, α 2
RCAN2	11.10	7.01E-45	regulator of calcineurin 2
MAF	10.52	2.69E-11	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)
CGNL1	10.41	5.06E-26	cingulin-like 1
ARHGAP28	10.03	4.15E-29	Rho GTPase activating protein 28
MGAT4A	9.69	7.01E-45	mannosyl (α -1,3-)-glycoprotein β -1,4-N-acetylglucosaminyltransferase, isozyme A
WISP2	9.27	4.48E-44	WNT1 inducible signaling pathway protein 2
SULF2	9.21	4.22E-15	sulfatase 2
CBLN2	8.73	1.58E-39	cerebellin 2 precursor
CELF2	8.36	0.00E+00	CUGBP, Elav-like family member 2
QPRT	8.31	3.44E-39	quinolinate phosphoribosyltransferase
CALCA	7.63	3.89E-12	calcitonin-related polypeptide α
ALX4	7.62	8.08E-32	ALX homeobox 4
EPHA3	7.53	0.00E+00	EPH receptor A3
CD33	7.53	1.37E-40	CD33 molecule
TNFRSF9	7.12	0.00E+00	tumor necrosis factor receptor superfamily, member 9
KIT	6.96	7.30E-37	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
FOXO1	6.82	1.16E-14	forkhead box O1
F2RL2	6.59	4.88E-33	coagulation factor II (thrombin) receptor-like 2
ZNF229	6.58	0.000241	zinc finger protein 229
CCDC69	6.55	2.06E-28	coiled-coil domain containing 69
MEX3A	6.51	3.28E-21	mex-3 homolog A (C. elegans)
CKB	6.47	4.76E-42	creatine kinase, brain
THY1	6.30	8.38E-18	Thy-1 cell surface antigen
ABLIM1	6.12	1.62E-30	actin binding LIM protein 1
PCDH7	6.04	1.54E-29	protocadherin 7
SLC9A3R1	6.02	0.00E+00	solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1
BIRC7	5.98	2.51E-17	baculoviral IAP repeat-containing 7
MEF2C	5.86	6.40E-43	myocyte enhancer factor 2C

Top 100 microarray hits demonstrating the most significantly up- and downregulated genes in Cav-1 overexpressing U-87MG cells. For a complete list see **Table S2**. (n = 3 samples from each group).

Table 1. Differential gene expression of Cav-1 overexpressing U-87MG cells (continued)

Probe	Fold change	p-value	Description
PIK3R3	5.80	2.36E-18	phosphoinositide-3-kinase, regulatory subunit 3 (gamma)
CD24	5.61	4.33E-22	CD24 molecule
CALCB	5.58	0.00E+00	calcitonin-related polypeptide β
UGT2B4	5.56	9.90E-13	UDP glucuronosyltransferase 2 family, polypeptide B4
SGCD	5.55	7.22E-35	sarcoglycan, delta (35 kDa dystrophin-associated glycoprotein)
DLX4	5.50	0.00E+00	distal-less homeobox 4
TP53	5.49	1.54E-28	tumor protein p53
BCL2L11	5.33	1.26E-27	BCL2-like 11 (apoptosis facilitator)
MDK	5.33	2.69E-32	midkine (neurite growth-promoting factor 2)
COL14A1	5.30	1.96E-19	collagen, type XIV, α 1
DPP4	5.25	8.24E-31	dipeptidyl-peptidase 4
FRMPD4	5.12	1.69E-10	FERM and PDZ domain containing 4
SORL1	5.06	5.16E-10	sortilin-related receptor, L(DLR class) A repeats-containing
RCOR2	5.02	1.79E-18	REST corepressor 2
LCP1	-4.95	1.89E-14	lymphocyte cytosolic protein 1 (L-plastin)
EPHB2	-4.99	3.43E-18	EPH receptor B2
LOC100509788...	-5.04	1.15E-14	hypothetical LOC100509788 hypothetical LOC100507248
ID1	-5.06	4.56E-35	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein
IL13RA2	-5.25	0.00E+00	interleukin 13 receptor, α 2
NAV2	-5.25	0.00E+00	neuron navigator 2
TOX2	-5.28	1.60E-37	TOX high mobility group box family member 2
HLA-DRB1...	-5.30	9.97E-19	major histocompatibility complex, class II, DR β 1...
FST	-5.32	5.01E-29	folliculin
KRT15	-5.33	3.11E-35	keratin 15
CRYM	-5.40	6.12E-17	crystallin, mu
AFF3	-5.52	7.41E-14	AF4/FMR2 family, member 3
NAMPT	-5.54	0.00E+00	nicotinamide phosphoribosyltransferase
COL4A6	-5.56	1.74E-21	collagen, type IV, α 6
HS3ST2	-5.63	8.54E-29	heparan sulfate (glucosamine) 3-O-sulfotransferase 2
DNER	-5.92	9.79E-12	delta/notch-like EGF repeat containing
C3orf14	-5.94	4.38E-22	chromosome 3 open reading frame 14
TXNIP	-6.02	5.66E-32	thioredoxin interacting protein
IL8	-6.44	1.49E-36	interleukin 8
GALNT12	-6.73	4.86E-30	UDP-N-acetyl- α -D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 12 (GalNAc-T12)
FAM133A	-6.93	4.20E-45	family with sequence similarity 133, member A
ACPP	-7.14	5.48E-32	acid phosphatase, prostate
PTX3	-7.23	1.76E-35	pentraxin 3, long
DCC	-7.24	6.16E-23	deleted in colorectal carcinoma
FARP1	-7.26	1.40E-24	FERM, RhoGEF (ARHGEF) and pleckstrin domain protein 1 (chondrocyte-derived)
BDKRB1	-7.27	0.00E+00	bradykinin receptor B1
TFPI2	-7.56	0.00E+00	tissue factor pathway inhibitor 2
IL1RN	-8.00	5.74E-19	interleukin 1 receptor antagonist
FOXF1	-8.20	0.00E+00	forkhead box F1
DDIT4L	-8.25	2.01E-37	DNA-damage-inducible transcript 4-like

Top 100 microarray hits demonstrating the most significantly up- and downregulated genes in Cav-1 overexpressing U-87MG cells. For a complete list see **Table S2**. (n = 3 samples from each group).

Table 1. Differential gene expression of Cav-1 overexpressing U-87MG cells (continued)

Probe	Fold change	p-value	Description
COL13A1	−8.38	0.00E+00	collagen, type XIII, α 1
VAT1L	−8.62	2.06E−29	vesicle amine transport protein 1 homolog (T. californica)-like
PLAU	−9.17	4.46E−25	plasminogen activator, urokinase
BEX1	−9.73	4.20E−45	brain expressed, X-linked 1
MGC87042	−13.51	2.14E−20	STEAP family protein MGC87042
TFAP2C	−13.77	0.00E+00	transcription factor AP-2 gamma (activating enhancer binding protein 2 gamma)
STC1	−15.24	2.80E−45	stanniocalcin 1
SBSN	−16.74	0.00E+00	suprabasin
MMP3	−19.01	0.00E+00	matrix metalloproteinase 3 (stromelysin 1, progelatinase)
IL1B	−25.20	0.00E+00	interleukin 1, β
MMP1	−37.04	0.00E+00	matrix metalloproteinase 1 (interstitial collagenase)

Top 100 microarray hits demonstrating the most significantly up- and downregulated genes in Cav-1 overexpressing U-87MG cells. For a complete list see **Table S2**. (n = 3 samples from each group).

Table 2. Cav-1 regulates cancer-associated gene expression

A. Gene ontology: Biological process				
Gene Set	Enrichment Score (ES)	Normalized ES	Nominal p-value	FDR q-value
NEGATIVE_REGULATION_OF_SIGNAL_TRANSDUCTION	0.500	2.023	< 0.001	0.092
REGULATION_OF_TRANSFORMING_GROWTH_FACTOR_BETA_RECEPTOR_PATHWAY	0.619	1.846	< 0.001	0.071
CASPASE_ACTIVATION	0.566	1.748	< 0.001	0.067
NEGATIVE_REGULATION_OF_MAP_KINASE_ACTIVITY	0.521	1.537	< 0.001	0.160
NEGATIVE_REGULATION_OF_CELL_PROLIFERATION	0.331	1.460	< 0.001	0.190
NEGATIVE_REGULATION_OF_TRANSCRIPTION	0.340	1.367	< 0.001	0.211
APOPTOSIS_GO	0.313	1.338	< 0.001	0.227
POSITIVE_REGULATION_OF_CELL_ADHESION	0.501	1.308	< 0.001	0.243
B. Canonical pathways				
Gene set	Enrichment score (ES)	Normalized ES	Nominal p-value	FDR q-value
BIOCARTA_AKT_PATHWAY	0.647	1.965	< 0.001	0.137
SA_PROGRAMMED_CELL_DEATH	0.739	1.923	< 0.001	0.142
KEGG_ECM_RECEPTOR_INTERACTION	0.496	1.857	< 0.001	0.158
KEGG_MTOR_SIGNALING_PATHWAY	0.487	1.831	< 0.001	0.125
REACTOME_PI3K_AKT_SIGNALING	0.479	1.682	< 0.001	0.144
REACTOME_INTEGRIN_CELL_SURFACE_INTERACTIONS	0.423	1.592	< 0.001	0.142
BIOCARTA_ERK_PATHWAY	0.493	1.583	< 0.001	0.151
KEGG_APOPTOSIS	0.360	1.521	< 0.001	0.169

ES, enrichment score; NES, normalized enrichment score; FDR, false discovery rate. q-value for selected gene sets enriched in LV105 Cav-1 cells vs. control using (A) gene ontology: biological process and (B) canonical pathway molecular signature databases (n = 3 samples from each group). For a detailed list of genes see **Table S1**.

Discussion

Although it has been demonstrated that Cav-1 expression in glioma increases variably in accordance with grade, little is currently known about its biological effects on tumor onset and progression.^{12–14,17–19} Previous in vitro studies using transient transfection techniques have shown that loss of Cav-1 in U-87MG cells resulted in the adoption of a more proliferative and invasive phenotype,

whereas its forced overexpression conferred the opposite effects.¹⁵ In line with previous studies, we here show that Cav-1 functions as a putative tumor suppressor in glioblastoma. Using a novel lentivirus transduction system we created a stable Cav-1 overexpressing cell line based on the U-87MG background.

By subjecting transiently transfected U-87MG cells to a panel of reverse transcription-PCR primers, Martin et al. identified genes pertaining to cell invasion, metastasis and cell adhesion as

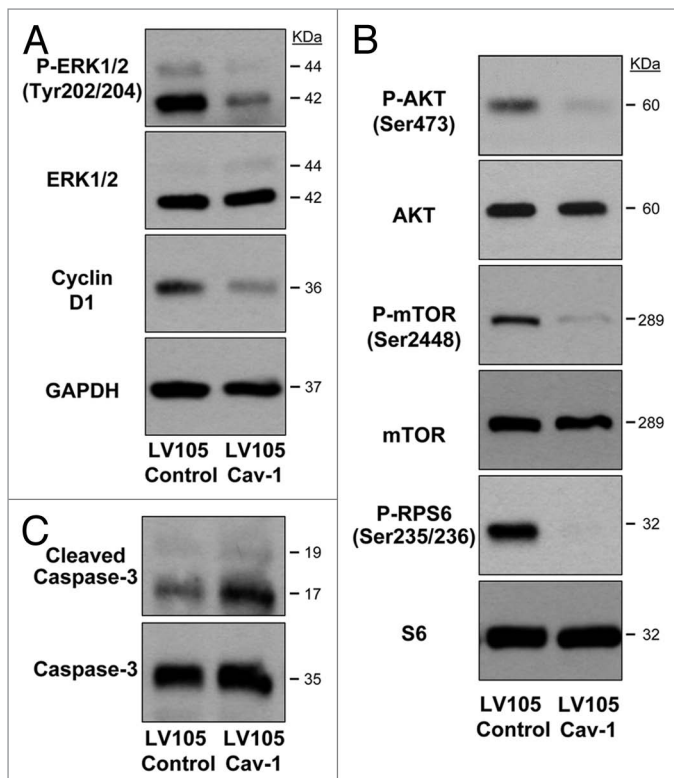


Figure 2. Cav-1 mediates major proliferative and cell survival pathways. Western immunoblot analysis of LV105 control and LV105 Cav-1 U-87MG cells showing cyclin D1 and cleaved caspase-3 expression as well as phosphorylation status of ERK1/2, AKT, mTOR and RPS6 pathways, with respective total protein levels for loading controls. Total caspase-3 and GAPDH serve as loading controls for cleaved caspase-3 and cyclin D1, respectively.

those being the most differentially regulated by Cav-1 expression. Particularly, they showed that the integrin genes ITGA1, ITGA3, ITGA5, ITGAV, ITGB1 and ITGB5 were significantly downregulated in Cav-1-overexpressing cells, with cells treated with Cav-1-specific siRNA demonstrating marked upregulation of these same genes. Matrix metalloproteinase genes MMP1 and MMP2 as well as transforming growth factor β receptor I (TGFRBI) were also shown to be significantly modulated by Cav-1.¹⁵ A follow-up study using Cav-1-silenced U-87MG cells further clarified a mechanism in which Cav-1 acts as a negative regulator of integrin signaling by inhibiting the expression of these integrins themselves as well as sequestering downstream TGF β /TGF β RI/SMAD2 and ERK pathways.¹⁶ Here, we implemented a similar, albeit much more expansive, microarray-based approach to study gene perturbations as a result of Cav-1 overexpression. Using gene set enrichment analyses, we indeed show similar expression profiles to those found previously, with gene sets related to integrin interactions, as well as regulation of TGF β receptor/SMAD pathways showing significant enrichment. In our study, however, we detected a multitude of other significantly enriched gene sets that have not been demonstrated previously in Cav-1-expressing GBM cells. For instance, U-87MG cells overexpressing Cav-1 demonstrated significant upregulation of genes responsible for

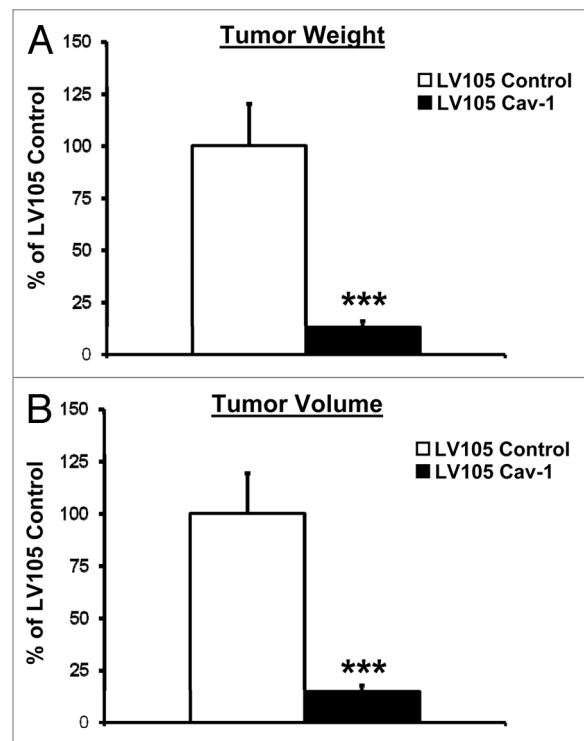


Figure 3. U-87MG cells stably overexpressing Cav-1 exhibit decreased tumor growth in vivo. (A) Tumor weight and (B) tumor volume of U-87MG xenografts grown in athymic nu/nu male mice after 4 wk (n = 30 per group, ***p < 0.001).

negative regulation of signal transduction, particularly within ERK, PI3K/AKT and mTOR pathways. Although it has long been known that Cav-1 serves to negatively regulate the activity of p42/p44 (ERK1/2) signaling proteins of the MAPK pathway, our evidence also suggests it has the capability to sequester PI3K and mTOR activity.²⁰⁻²⁶ This is notable due to the fact that ERK, PI3K and mTOR signaling axes are frequently upregulated in GBM, suggesting that loss of Cav-1 could lead to unchecked activation of these pathways.²⁷⁻³¹ Two of the most commonly silenced genes in GBM are the tumor suppressor proteins PTEN and TP53, which serve to antagonize the PI3K/AKT/mTOR pathway and regulate cell cycle response to DNA damage and cell death, respectively.³² Of note is that these two genes were among the most upregulated in cells overexpressing Cav-1, which would likely explain the gene signatures corresponding to downregulation of signaling pathways and reduced invasiveness.

A major hallmark of GBM is the ability of tumor cells to invariably metastasize to distant sites in the CNS despite aggressive treatment. This is often attributed to the excessive release of matrix metalloproteinases and urokinase plasminogen activator.³³ Here we show that the genes MMP1, MMP3 and PLAU (urokinase plasminogen activator) are highly downregulated in our Cav-1-overexpressing U-87MG cells, which is consistent with reports that Cav-1 negatively regulates tumor invasiveness.^{15,34-36} These genes have been shown to be regulated by Erk and TP53, therefore, their reduction may be secondary to Cav-1 modulation of these pathways.³⁷⁻⁴⁰ Of note, we also found that genes

responsible for sequestering cell cycle progression and transcription were overexpressed in LV105 Cav-1 cells (FOXN3, HDAC5, VHL, CDKN1C, among others). Conversely, genes responsible for progression through cell cycle, such as CCND1 (cyclin D1), were found to be significantly downregulated in Cav-1-overexpressing cells, consistent with previous reports that Cav-1 transcriptionally represses cyclin D1.⁸ Perhaps our most notable finding, however, is that a substantial number of genes involved in the activation of apoptotic and cell death pathways are increased as a result of Cav-1 overexpression (TP53, MOAP1, CASP3, CASP9, BCL2L11, BAK1, BID among others). Although the role of Cav-1 in apoptosis is contentious, with reports indicating both pro- and anti-cell death roles, it may be possible that expression of Cav-1 promotes apoptotic activity in U-87MG cells by inhibiting the BIRC5 gene product, survivin, as is suggested here and in previous reports.⁴¹⁻⁴⁵ In support of these microarray data, we were able to demonstrate, at the protein level, silencing of ERK, AKT, mTOR, RPS6 and cyclin D1 pathways with corresponding activation and cleavage of the key apoptosis initiator caspase 3.

Importantly, we here show for the first time that forced expression of Cav-1 in vivo results in a dramatic reduction of tumor burden in U-87MG xenografts. Although Cosset et al. have demonstrated that explanted human glioma tissue lacking Cav-1 expression results in increased expression of $\alpha 5\beta 1$ integrin subunits, we were able to demonstrate a direct inverse relationship with Cav-1 expression and cell proliferation in an animal model.¹⁶ In line with our in vitro data, these xenograft tumors displayed reduced activity of ERK, RPS6 and mTOR pathways. As these pathways have been previously shown to play major roles in glioma progression, it is likely that Cav-1 could act as a critical regulator of tumor growth and protein synthesis in a clinical setting. As examples of this, studies have shown that exogenous administration of cavtratin, or a soluble peptide consisting of the Cav-1 scaffolding domain fused to an internalization domain, results in reduced MAPK activity in oligodendroglial cells in vivo, as well as reduced tumor volumes in a xenograft model of Lewis lung carcinoma.^{23,46} A separate study demonstrated that in vitro administration of full-length Cav-1 prevented invasion of three different GBM-derived cell lines using a Boyden-chamber assay.⁴⁷ In this regard, it may be suggested that Cav-1 be explored as a therapeutic agent in GBM.

Lastly, we have also demonstrated that expression of Cav-1 confers sensitivity to the most commonly used chemotherapeutic in GBM, temozolomide. This could be due in part to the action of the permeability glycoprotein (P-gp) transporter, a multidrug exporter that normally prevents the influx of drugs across the blood brain barrier. However, cancerous cells can also express this protein, rendering treatment of GBM with conventional chemotherapeutics less effective.⁴⁸ Cav-1 has been shown to

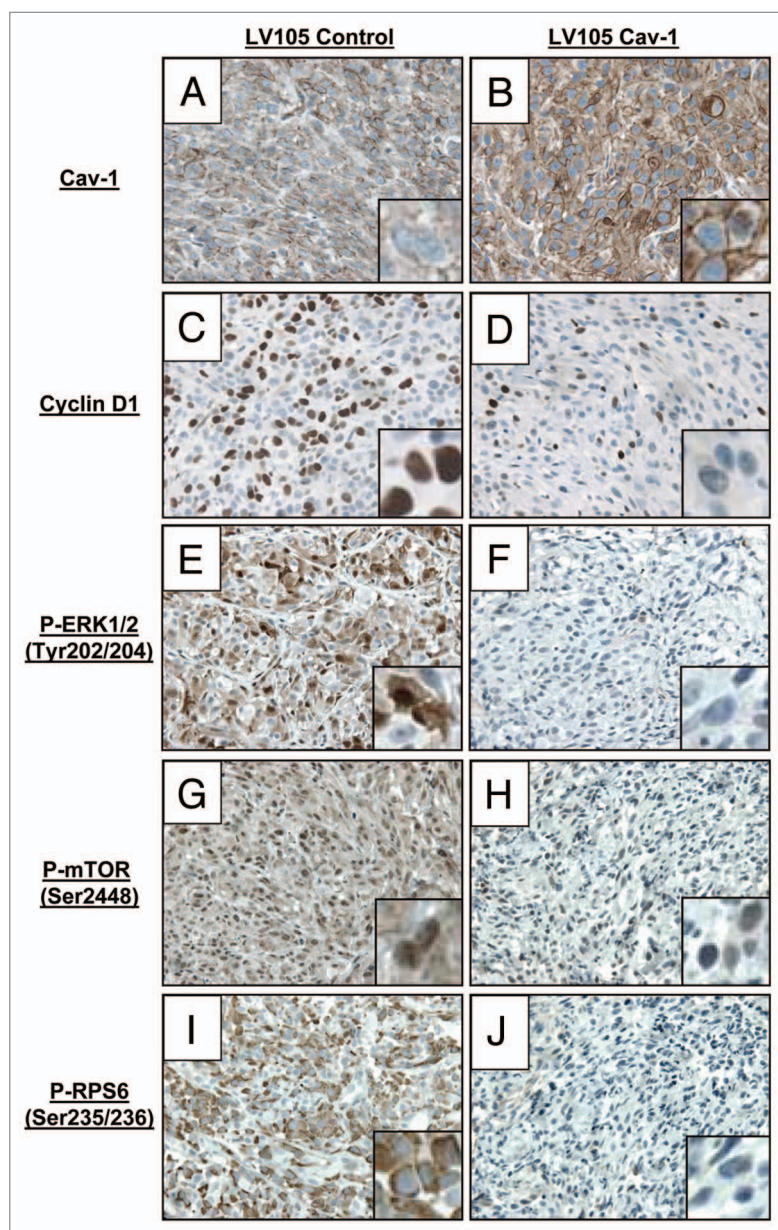


Figure 4. Cav-1-overexpressing tumors show reduced signaling activity in vivo. Immunohistochemical staining of explanted tumors for Cav-1, cyclin D1 and phosphorylated ERK1/2, mTOR and RPS6 (magnification = 60 \times).

associate with P-gp and negatively regulate its activity; therefore, overexpression of Cav-1 most likely results in improved access of TMZ to the intracellular compartment of U-87MG cells in our model.^{49,50} Interestingly, a separate study showed that treatment with TMZ resulted in upregulation of Cav-1 expression in vivo using orthotopic GBM xenograft models.⁴⁷ In light of our data, this could suggest a positive feedback loop exists in which treatment with TMZ serves to auto-sensitize GBM cells through a Cav-1 dependent mechanism. This finding implicates Cav-1 as a potential biomarker predicting response to chemotherapies for GBM, as it has been shown for other cancers such as breast, lung and oral squamous cell carcinomas.⁵¹⁻⁵³

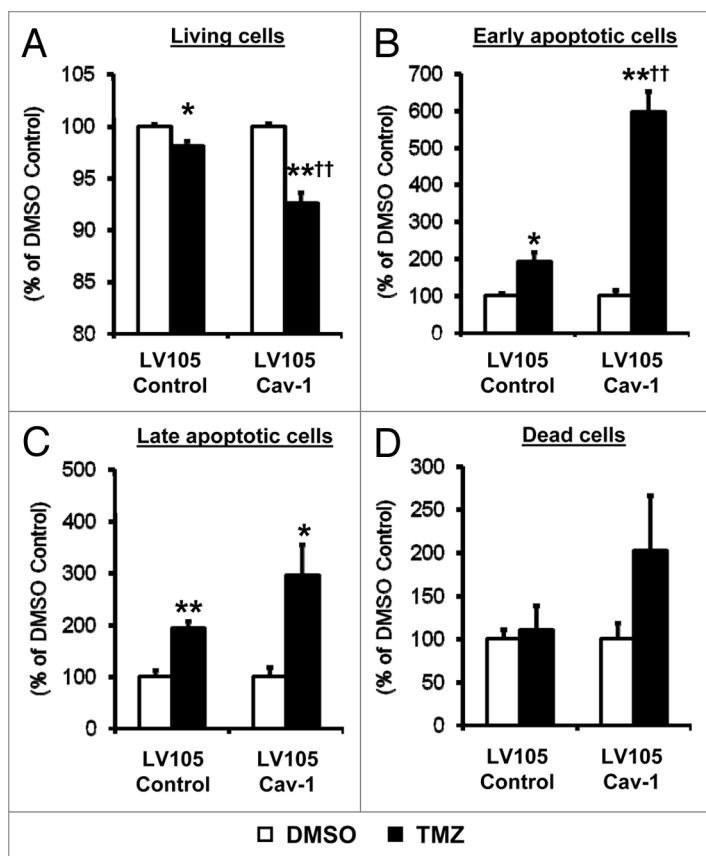


Figure 5. Cav-1 confers chemosensitivity in U-87MG cells. Annexin V and propidium iodide staining of U-87MG cells treated with either DMSO control or 500 μ M TMZ reveals percentages of (A) live cells, (B) early apoptotic cells, (C) late apoptotic cells and (D) dead cells as measured by flow cytometry. Each group is normalized to its own DMSO control (* $p < 0.05$ and ** $p < 0.01$ vs. internal DMSO control, † $p < 0.01$ vs. TMZ treated LV105 control, $n = 3$ per group).

Taken together, these studies confirm and expand upon previous work identifying Cav-1 as a putative tumor suppressor in GBM. We here show that stable overexpression of Cav-1 in a widely used model of GBM results in silencing of key proliferative and cell survival pathways in vitro as well as in vivo (Fig. 6). Additionally, we have demonstrated its ability to modulate sensitivity to commonplace chemotherapeutics for GBM. These findings highlight the potential of Cav-1 to serve as a novel biomarker indicating potential response to therapy and also a candidate therapy for treatment of GBM.

Materials and Methods

Cell lines and reagents. The human glioblastoma-derived cell line U-87MG was obtained from American Type Culture Collection (ATCC) and cultured in Eagle's modified essential medium (EMEM, ATCC) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies). Cells were cultured in the presence of 5% CO₂ at 37°C. Temozolomide (TMZ) was obtained from Sigma-Aldrich and dissolved in DMSO to a concentration of 100 mM. The following antibodies

were used: mouse anti-Caveolin-1 (2297, BD Bioscience), rabbit anti-Caveolin-1 (N-20, Santa Cruz Biotechnology), mouse anti-cyclin D1 (DCS-6, Santa Cruz Biotechnology), rabbit anti-phospho mTOR (Ser2448, D9C2, Cell Signaling), rabbit anti-phospho-ERK1/2 (Thr202/Tyr204, Cell Signaling), rabbit anti-phospho-AKT (Ser473, D9E, Cell Signaling), rabbit anti-phospho-ribosomal S6 (Ser235/236, 91B2, Cell Signaling), rabbit anti-ERK1/2 (Cell Signaling), rabbit anti-AKT (Cell Signaling), rabbit anti-ribosomal S6 (5G10, Cell Signaling), rabbit anti-cleaved caspase 3 (Asp175, Cell Signaling), rabbit anti-caspase 3 (Cell Signaling) and mouse anti-GAPDH (6C5, Fitzgerald Industries).

Stable lentiviral transduction of U-87MG cells. Plasmids Ex-Neg-LV105 (empty control vector) and Ex-D0159-LV105 (Cav-1 cDNA vector) were obtained from Genecopoeia and transfected into the packaging cell line Genecopoeia 293Ta using the Lenti-Pac HIV Expression Packaging Kit as per manufacturer's instructions. Forty-eight h post-transfection, lentivirus containing supernatants were collected and centrifuged at 500 \times g for 10 min to clear cellular debris. U-87MG cells were cultured in viral supernatants supplemented with 5 μ g/ml polybrene (Santa Cruz) for 24 h prior to changing back into complete medium containing 2.5 μ g/ml puromycin hydrochloride (Santa Cruz) to select for lentiviral-transduced cells. After 1 wk of selection, cells were allowed to grow in complete medium without puromycin.

Western immunoblot. Cells at 70% confluence were collected, pelleted at 300 \times g, washed twice with Dulbecco's PBS (DPBS) and resuspended in RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, pH 7.5) including Complete Protease Inhibitor Cocktail (Roche Diagnostics) and Halt Phosphatase Inhibitor Cocktail (Thermo-Scientific). Lysates were sonicated and centrifuged at 10,000 \times g for 10 min to clear cellular debris prior to protein quantification by BCA assay (Thermo-Scientific) as per manufacturer's instructions. Proteins were separated by sodium dodecyl sulfate PAGE (SDS-PAGE; 8–12% acrylamide), transferred to nitrocellulose membranes (Whatman) and blocked for 1 h in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 8.0) with 5% bovine serum albumin (BSA). Membranes were incubated with primary antibodies diluted in TBST + 1% BSA overnight at 4°C followed by incubation in either horseradish peroxidase (HRP) conjugated anti-mouse (Thermo-Scientific) or anti-rabbit (BD Biosciences) antibodies. Detection of bound antibodies was accomplished with the use of Supersignal chemiluminescent substrates (Thermo-Scientific).

Immunofluorescence analysis. U-87MG cells grown on glass coverslips in 6-well plates were fixed in ice-cold methanol for 20 min, washed with PBS and incubated with anti-Cav-1 primary antibody (BD Bioscience) in immunofluorescence (IF) buffer (PBS + 5% BSA, 0.5% NP40) for 30 min at 37°C before incubation with secondary fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody (Jackson Labs) in IF buffer. Cells were counterstained with Hoechst nuclear dye (Life Technologies) prior to coverslipping and visualization with a Zeiss LSM 510 confocal microscope (Carl Zeiss Microscopy).

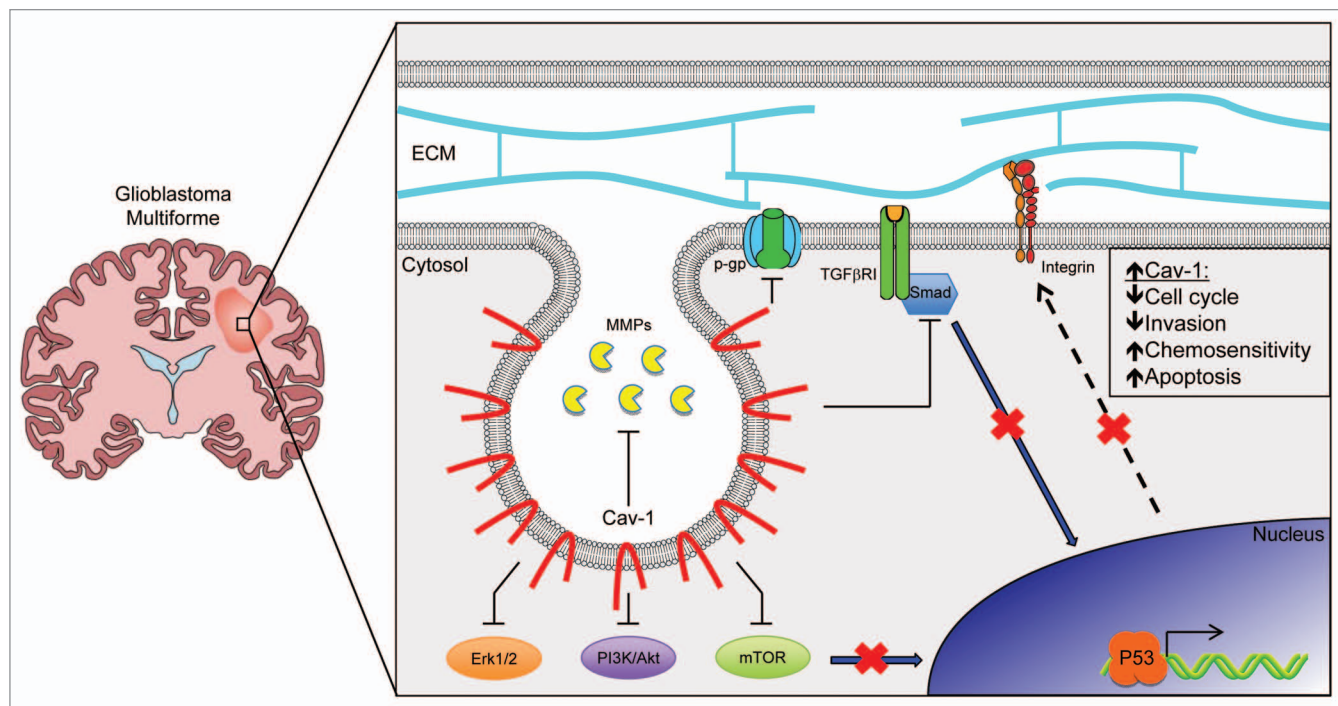


Figure 6. Schematic representation of the role of Cav-1 in glioblastoma. Gliomas are highly heterogeneous tumors that have been demonstrated to contain populations of cells with varied levels of Cav-1 expression. In this case, enhanced Cav-1 expression among a tumor cell prevents the activation of the TGFβRI/SMAD pathway, which, in turn, suppresses expression of integrin subunits at the transcriptional level and their subsequent signaling activity. Increased Cav-1 expression is also correlated with a decrease in the availability of matrix metalloproteinases, downregulated activity of the Erk1/2, PI3K/Akt and mTOR signaling pathways as well as the inhibition of the trans-membrane drug exporter p-gp. Together, this may implicate that tumors with increased Cav-1 levels are less likely to progress through the cell cycle or invade into surrounding tissues and are primed to undergo P53 mediated apoptosis, making these cells more easily targeted by standard chemotherapy regimens.

Gene array. DNA microarray analysis was performed using the Human Whole Genome OneArray v2 (Phalanx Biotech). RNA quality and integrity were determined utilizing an Agilent 2100 Bioanalyzer (Agilent Technologies) and absorbance at A260/A280. Only high quality RNA, having a RIN of > 7.0 and an A260/280 absorbance ratio of > 1.8, was utilized for further experimentation. RNA was synthesized to double-stranded cDNA and amplified using in vitro transcription that included amino-allyl UTP, and the aRNA product was subsequently conjugated with Cy5 NHS ester (GE Healthcare Lifesciences). Fragmented aRNA was hybridized at 42°C overnight using the HybBag mixing system with 1× OneArray Hybridization Buffer (Phalanx Biotech), 0.01 mg/ml sheared salmon sperm DNA (Promega), at a concentration of 0.025 mg/ml labeled target. After hybridization, the arrays were washed according to the OneArray protocol. Raw intensity signals for each microarray were captured using a Molecular Dynamics™ Axon 4100A scanner, measured using GenePixPro™ Software. Significantly up or downregulated genes in LV105 Cav-1 cells were identified as having normalized intensities above background (> 50), a fold change of ± 1.5 compared with control and $p < 0.05$.

Gene set enrichment analyses. Pre-processed expression data was subjected to Gene Set Enrichment Analysis using C5.BP.V3.0 (gene ontology: biological processes) and C2.CP.V3.0 (canonical pathways) MSigDB gene sets.^{54,55} Genes expression data were ordered based on a Signal2Noise metric

and compared statistically to existing gene sets at a resolution of 1,000 permutations. Statistical significance of gene set enrichment was assumed at nominal $p < 0.05$, with a false discovery rate (FDR) $q < 0.25$.

Tumor xenografts. All animal studies were conducted in accordance with the guidelines set forth by the National Institutes of Health and the Thomas Jefferson University Institutional Animal Care and Use Committee (IACUC). Briefly, U-87MG cells were washed with DPBS, trypsinized, counted and resuspended in a volume of complete medium yielding 1×10^6 cells/50 μ l, which was subsequently injected subcutaneously into the flanks of 6–8-wk-old male athymic nu/nu mice (NCI). After 4 wk, mice were sacrificed and tumors were excised, weighed and measured prior to further processing.

Immunohistochemistry. Explanted xenograft tumors were fixed in 10% phosphate buffered formalin solution for 24 h prior to dehydration in 70% ethanol, paraffin embedding and sectioning onto slides. Following xylene deparaffinization and rehydration, slides were subjected to 10 min of heat antigen retrieval in 10 mM sodium citrate buffer pH 6.0 and endogenous peroxide quenching in 3% hydrogen peroxide for 20 min. Tissues were blocked in 10% normal goat serum (NGS, Vector Labs) for 1 h at room temperature and incubated overnight with primary antibody in 10% NGS at 4°C. Slides were then washed in PBS and blocked with Biotin-Blocking System (Dako) before incubating with the appropriate secondary antibody in PBS and developing

with 3,3-diaminobenzidine (DAB) substrate (Dako). Slides were counterstained with hematoxylin (Sigma), dehydrated and coverslipped prior to imaging with an Olympus BX51 light microscope equipped with a Micropublisher 5.0 CCD camera (QImaging).

Flow cytometry. U-87MG cells (50,000/well) were plated in 12-well tissue culture dishes and allowed to attach overnight prior to changing their medium into complete EMEM containing either 500 μ M TMZ or DMSO control and culturing them for an additional 72 h. Cells were then trypsinized, centrifuged at 300 \times g for 5 min and resuspended in binding buffer with APC-conjugated anti-Annexin V antibody (BD Biosciences) and 0.33 μ g/ml Propidium Iodide (PI, KPL). All samples were run on a BD FACSCalibur flow cytometer (BD Biosciences). Cells were quantified according to staining as follows: viable (Annexin V-negative, PI-negative), early apoptotic (Annexin V-positive, PI-negative), late apoptotic (Annexin V-positive, PI-positive) and dead (Annexin V-negative, PI-positive).

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Statistical analysis. All data were expressed as mean \pm SEM. Differences between groups were evaluated by either unpaired Student's t-test or one-way ANOVA followed by Tukey's multiple-group comparisons test, where appropriate. Statistical significance was assumed at $p < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Jean-Francois Jasmin was supported by a Susan G. Komen Career Catalyst Research Grant. Michael P. Lisanti and his laboratory were supported via the resources of Thomas Jefferson University.

Supplemental Materials

Supplemental materials may be found here:
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