Supporting Information

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SI Materials and Methods

Plasmids. Small hairpin (sh)RNA for mda-9/syntenin (sh*mda-9*/syntenin) was created with pSilencer hygro expression vectors according to the manufacturer's protocol (Ambion). The specific hairpin siRNA oligonucleotides sequences are: sense 5′GATCC-GCGGATGGCACCAAGCATTTTCAAGAGAAATGCTTGG-TGCCATCCGCTTTT TTGGAAA-3′ and antisense 5′AGCTTT-TCCAAAAAAGCGGATGGCACCAAGCATTTCTCTTGAAA-ATGCTTGGTGCC ATCCGCG-3′

The oligo nucleotides were annealed and ligated to pSilencer vector by T4 DNA ligase. Alternate siRNA sequences were obtained through Qiagen with the following sequences: 5′-TTGACTCTTAAGATTATGTAA-3′ (si*mda-9* #3). sh*mda-9* resistant *mda-9*/*syntenin* plasmid was created using the following primer sequences: forward 5′-GCCTGCTTTTATCTTTGAAC-ATATTATTAAGCGAATGAAGCCTAGTATAATGAAAA-GCCTAATGGACCACACCATTCCTGAG-3′ and reverse 3′-CGGACGAAAATAGAAACTTGTATAATAATTCGCTTA-CTTCGGATCATATTACTTTT CGGATTACCTGGTGTGG-TAAGGACTC-5′.

These plasmids were cleaved and transfected into HEK-293 cells to obtain the corresponding Ad.5/3-based vectors. The viruses were then expanded using HEK-293 cells and purified by cesium chloride double ultracentrifugation (Beckman SW28 rotor) using standard protocols (OD260 Inc.). The infectious viral particles were titered by plaque assay, as described previously (1).

Cell Lines, Cell Culture, and Chemicals. The human glioma cell line U1242-luc-GFP was kindly provided by Kristofer Valerie, Virginia Commonwealth University, Richmond, VA. U1242/luc-GFP (U1242-Luc), U87 cells (ATCC), and U251 were cultured in DMEM supplemented with 10% FBS and antibiotics. VG2, VG9, VG10, VG11, U87vIII cells were grown in media supplemented additionally with nonessential amino acids (Thermofisher). The cumulative culture length of the cells was less than 6 mo after resuscitation. Early passage cells were used for all experiments and authenticated. All of the cell lines were frequently tested for mycoplasma contamination using a mycoplasma detection kit from Sigma. All primary GBM cells were authenticated by IDEXX Bioresearch (Columbia, MO).

Virus Infections. Viral infections were performed as described previously (2).

Chemicals and Treatment. Erlotinib, rapamycin, CQ diphosphate, 3MA, and FAKi were obtained from Sigma. The GSCs were treated with the drugs for 24 h at 10-μM concentration before assessing their effect on autophagy.

Antibodies. Antibodies against EGFR, pEGFR, PKCα, pPKCα, LC3, BCL2, were obtained from Cell Signaling. pBCL2, β-actin, and α-tubulin were obtained from Abcam, and MDA-9/Syntenin was from Abnova. Lamp1 (CD 107a) was obtained from Miltenyi Biotech. ATG5 antibody was procured from R&D Systems.

Intracellular Flow Cytometry. ATG5, LC3B, Lamp1, and EGFR were stained according to the manufacturer's instructions, followed by flow cytometry analysis using BD DIVA. pEGFR, pBCL2 proteins were assessed by intracellular flow cytometry, as described previously (3). Cells were fixed, permeabilized and stained with antibodies according to the manufacturer's instructions. Flow cytometry was performed after 24 h of incubation before any cell death was observed. This was followed by flow cytometry analysis with BD DIVA.

Preparation of Whole-Cell Lysates and Western Blotting Analysis. Cell lysates were prepared after 24 h of incubation before any cell death was observed. X-ray films from Western blots were scanned and analyzed with ImageJ software (NIH) for densitometry evaluation.

Live/Dead Cell Assay. Live/dead cell staining was performed as directed by the manufacturer (Invitrogen), followed by imaging by laser confocal microscopy (Leica). The GSCs were imaged after 48 h of incubation. The resulting images were analyzed by Zen software.

Intracranial Implant of Cells in Nude Mice. Athymic female NCr-nu/nu mice were purchased from the National Cancer Institute, Frederick, MD. Mice that weighed about 25 g were selected for study. Mice were maintained under pathogen-free conditions as approved by the American Association for Accreditation of Laboratory Animal Care, as well as in agreement with present regulations and standards of the US Department of Agriculture, US Department of Health and Human Services, and NIH. At least five mice were designated per group, and the experiment was replicated at least twice for a minimum total of 10 mice per group. Mice were anesthetized through intraperitoneal administration of ketamine (40 mg/kg) and xylazine (3 mg/kg) and then the fully unconscious mice were placed in a stereotactic frame (Stoelting). A 24-gauge needle attached to a Hamilton syringe was inserted into the right basal ganglia to a depth of 3.5 mm. The skull entry point was 2-mm lateral and 1-mm dorsal to the bregma. The inserted needle was then withdrawn 0.5 mm to accommodate intracerebral injected tumor cells, and 1.5×10^4 cells in 2 μL per mouse were injected over 10 min using an automated injector (Stoelting). The skull opening was sealed with sterile bone wax, treated with antibiotic ointment, and the incised skin flaps were surgically stapled under sterile conditions. The mice were killed according to an approved protocol, and the resultant tumors were resected, weighed, preserved and stained for immunohistochemical analyses.

Immunohistochemistry. VG2-luc primary glioma GSCs were infected with Ad.5/3.sh*con* or Ad.5/3.sh*mda-9* virus at a multiplicity of infection of 1,000 vp per cell. These cells were injected into the brain of athymic nude mice, within 24 h of virus infection, as described previously (3, 4). The mice were monitored and killed according to an approved protocol and the brains were dissected. The brain samples from both the groups of animals were fixed in phosphate-buffered formalin and paraffin sections were prepared using standard histology protocols. Paraffin-embedded sections were dewaxed and rehydrated through incubations in xylene and a gradient series of alcohol. Antigen retrieval was performed in 10 mM citric acid (pH 6.0) with microwave treatment for 20 min. Endogenous hydrogen peroxidase was quenched by 3% (vol/vol) H_2O_2 treatment for 20 min. Nonspecific binding sites were blocked with a solution of 5% (vol/vol) normal sera, and the sections were incubated with antibody overnight. The sections were incubated with biotinylated secondary antibodies and subsequently with avidin–biotin complex peroxidase (Vector Elite; Vector Laboratories). Colorimetric reactions were developed by DAB substrate [0.02% DAB (3,3′-diaminobenzidine), 0.005% hydrogen peroxide] treatment followed by 10% (vol/vol) Harris hematoxylin counterstaining. H&E staining was also conducted

following a standard protocol as described previously (2). The images were analyzed under an Olympus BX41 microscope system equipped with DP25 digital camera and software.

RT-PCR. GSCs were pelleted by centrifugation and total RNA was extracted by TRIzol extraction (Invitrogen) and the RNeasy kit (Qiagen). cDNA was synthesized with cDNA synthesis kit (Applied biosystems). Quantitative PCR studies were carried out by using TaqMan Gene expression assays (Invitrogen), using a ViiA 7 fast real-time PCR system and the data were normalized to 18S expression (Invitrogen) according to the manufacturer's protocol (Applied Biosystems).

1. Mittereder N, March KL, Trapnell BC (1996) Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. *J Virol* 70:7498–7509.
2. Boukerche H, et al. (2005) mda-9/syntenin: A positive regulator of melanoma metastasis. *Cancer Res* 65:10901–10911.
3. Talukdar S, et al. (2016) Novel function of MDA-9/syntenin (SDCBP) as a regulator of survival and stemness in glioma stem cells. *Oncotarget* 7:54102–54119.
4. Kegelman TP, et al. (2017) Inhibition of radiation-induced glioblastoma invasion by genetic and pharmacological targeting of MDA-9/syntenin. *Proc Natl Acad Sci USA* 114:370–375.

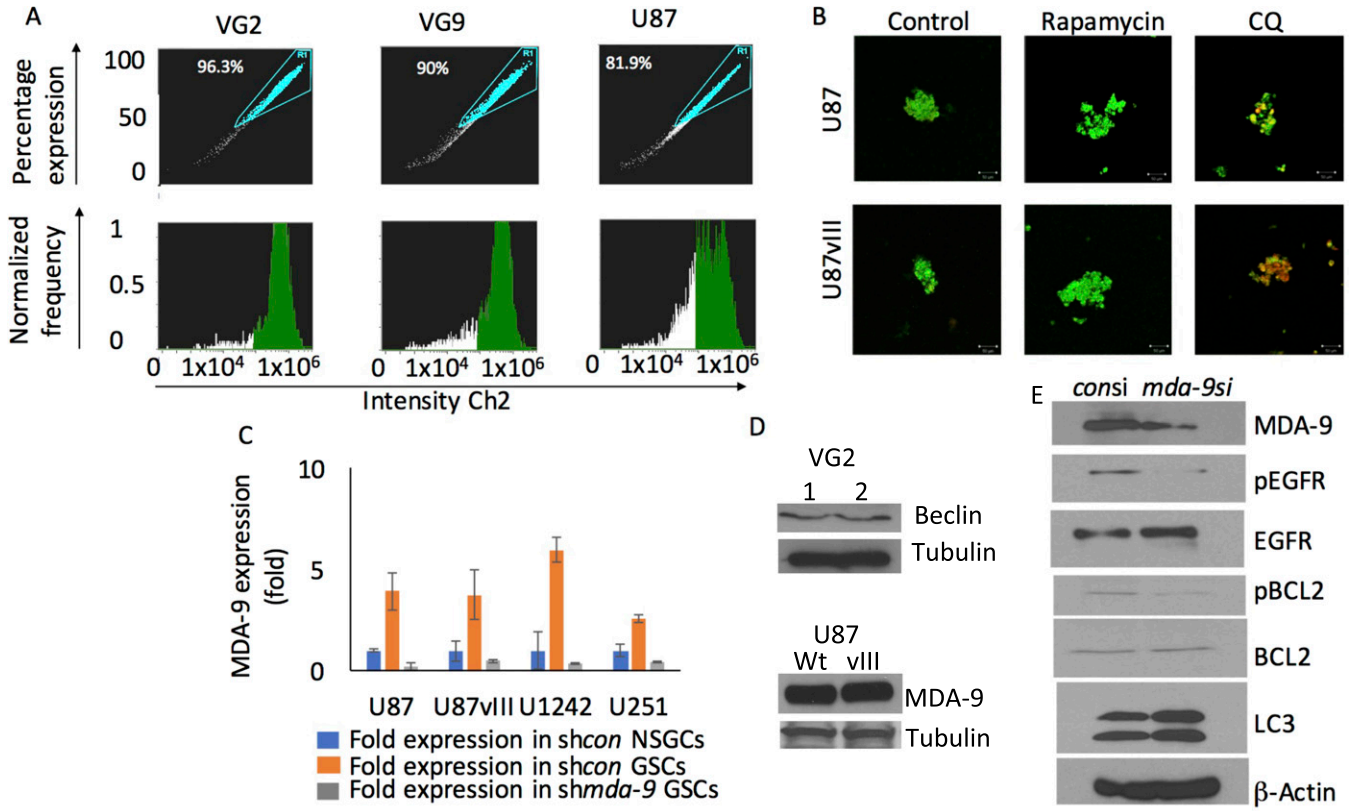

Fig. S1. Autophagy is important in maintaining survival of anoikis resistant GSCs, and this process correlates with *mda-9* expression. (*A*) Anoikis-resistant GSCs show autophagy. (*B*) Effect of autophagy induction (rapamycin treatment) or inhibition (CQ treatment) on GSC viability. (Magnification: 100×.) (*C*) Expression of *mda-9* in NSGCs, sh*con*, and sh*mda-9* GSCs. (*D*, *Upper*) Beclin-1 expression in VG2 GSCs. 1, sh*con*; 2, sh*mda-9*. (*Lower*) MDA-9 expression in U87 and U87vIII GSCs. (*E*) Effect of MDA-9 suppression (alternate siRNA) on pEGFR, EGFR, BCL2, pBCL2, and LC3B as shown by Western blot.

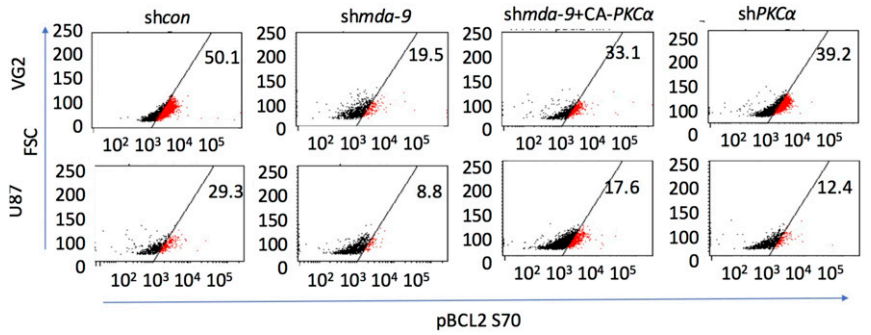

Fig. S2. PKC regulates BCL2 phosphorylation at S70. Effect of PKC signaling on pBCL2 is shown by percentage of cells expressing pBCL2 by flow cytometry.

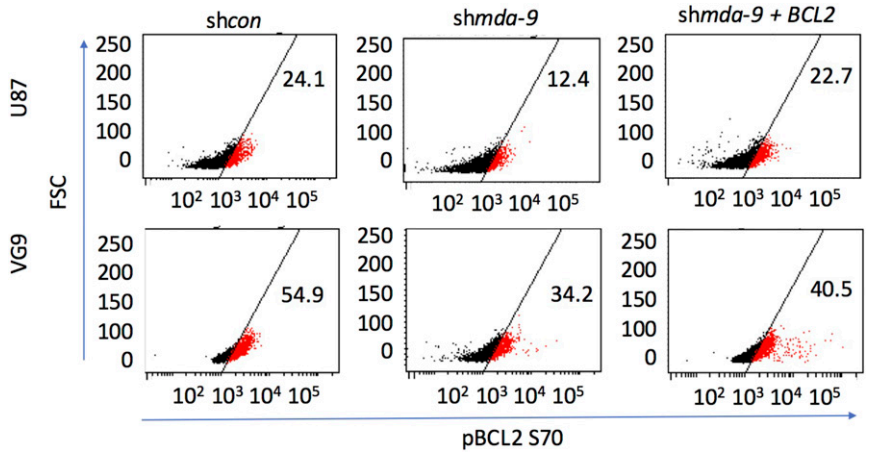

Fig. S3. Effect of *BCL2* overexpression on pBCL2 is shown by percentage of cells expressing pBCL2 by flow cytometry.

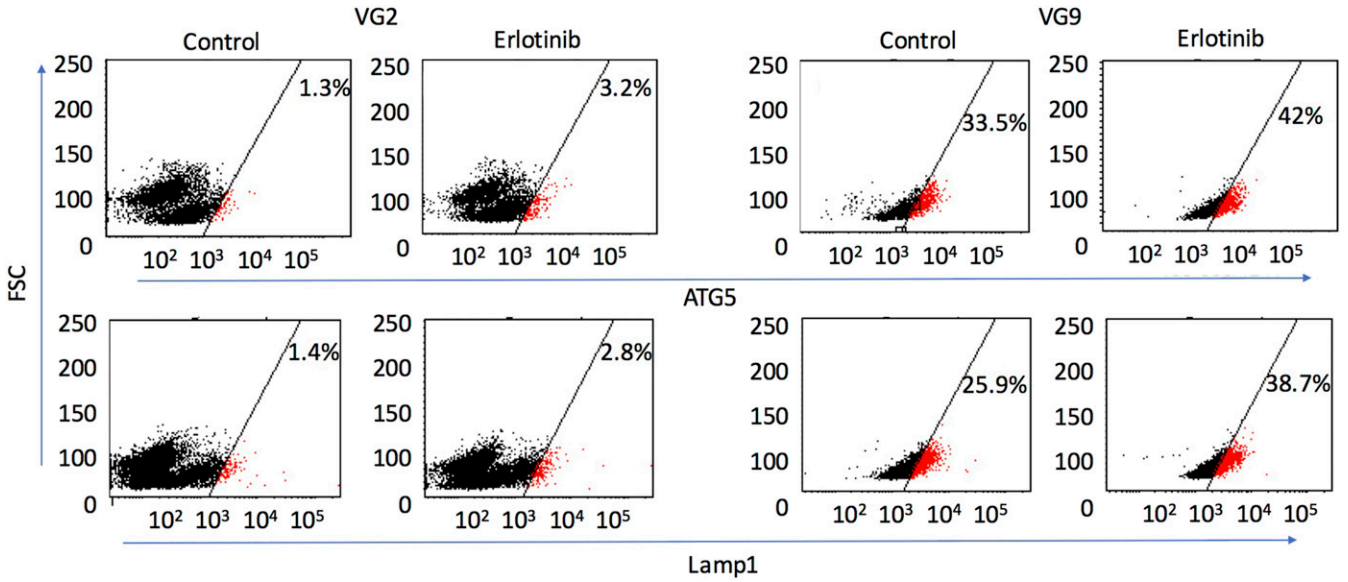

Fig. S4. Erlotinib causes up-regulation of autophagy in nonadherent GSCs. Effect of EGFR inhibition by erlotinib on autophagy markers ATG5 and Lamp1 as determined by flow cytometry.

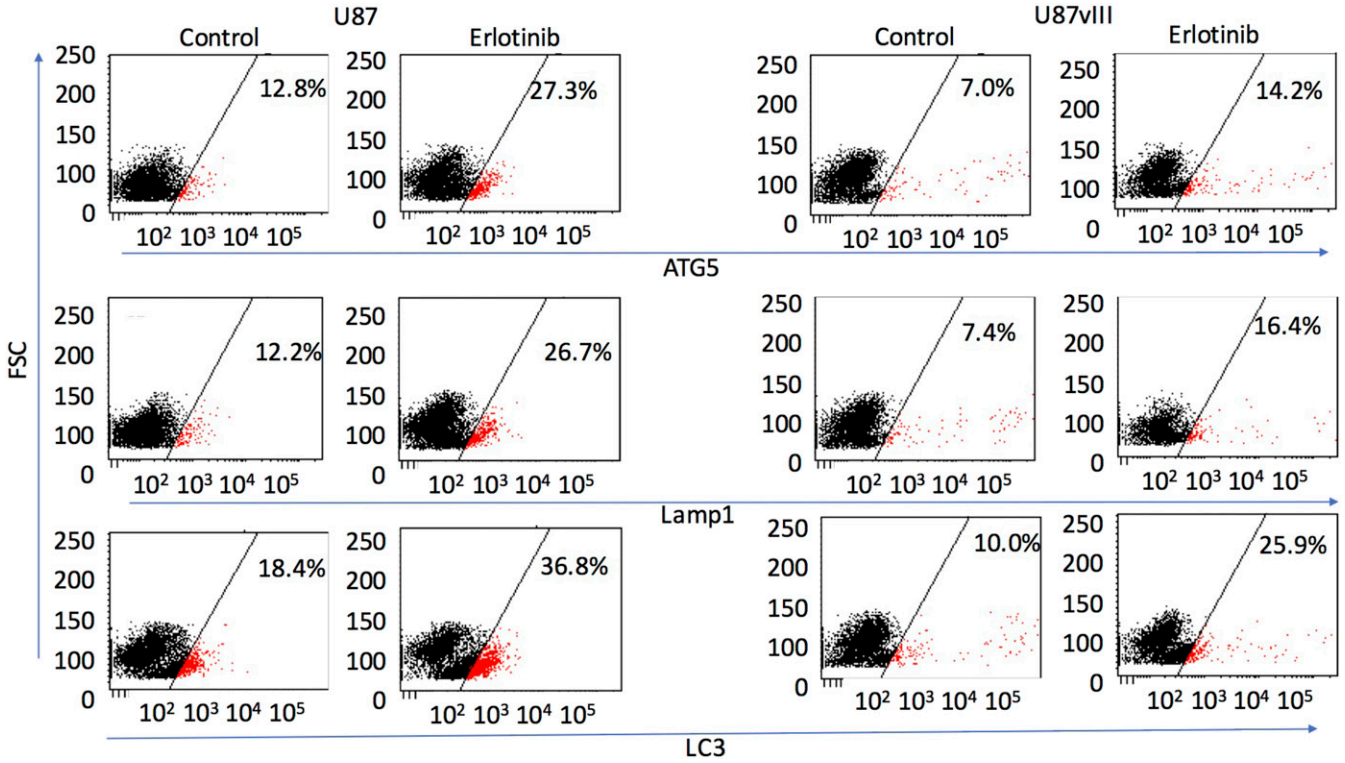

Fig. S5. EGFR activation causes down-regulation of autophagy in nonadherent GSCs. Effect of EGFRvIII overexpression in DMSO and erlotinib-treated U87 GSCs on autophagy markers ATG5, Lamp1, and LC3B as shown by flow cytometry.

Table S1. Expression of EGFR, pEGFR, autophagy markers (ATG5, Lamp1, LC3), and pBCL2 in sh*con* and sh*mda*-9 GSCs

Cell line (protein tested)	% Expression in sh*con* GSCs	% Expression in sh*mda-9* GSCs
U1242		
EGFR	46.9 ± 5.02	38.7 ± 0.35
pEGFR	45.5 ± 2.29	10.9 ± 2.18
ATG5	13.9 ± 1.76	27.5 ± 3.47
Lamp1	17.1 ± 1.14	32.3 ± 4.08
LC3	23.6 ± 0.81	56.2 ± 4.79
pBCL2	10.3 ± 0.19	2.1 ± 0.01
VG9		
EGFR	50.6 ± 3.84	49.8 ± 1.28
pEGFR	44.6 ± 4.13	15.7 ± 1.07
ATG5	59.5 ± 1.38	72.3 ± 2.36
Lamp1	55.2 ± 1.04	73.1 ± 6.51
LC3	64.8 ± 3.25	86.5 ± 5.54
pBCL2	26.5 ± 2.55	0.6 ± 0.02
VG10		
EGFR	93.4 ± 8.23	90.1 ± 9.71
pEGFR	90.1 ± 4.09	23.6 ± 5.69
ATG5	23.6 ± 3.22	43.2 ± 2.45
Lamp1	38.3 ± 2.40	48.5 ± 1.09
LC3	50.9 ± 1.47	62.6 ± 6.83
pBCL2	16.9 ± 2.08	1.3 ± 0.04