Supplemental Information

LY6K Promotes Glioblastoma Tumorigenicity via CAV-1-Mediated ERK1/2 Signaling Enhancement

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Supplementary Methods:

Brain Tumor Xenografts, *In Vivo* Bioluminescent Imaging (BLI), and Immunohistochemistry (IHC) Studies

All experiments using animals were conducted under an Institutional Animal Care and Use Committee (IACUC)-approved protocol at Northwestern University in accordance with NIH and institutional guidelines. Athymic (Ncr nu/nu) mice at six weeks of age (Taconic Farms) were employed for all animal experiments as described previously.[1,2] Patient-derived glioma stem-like cells (GSCs) in 2-5 μl culture media were stereotactically implanted into the left striatum of nude mice, with three to five mice per group. 5 x 10^4 cells were used for hematoxylin and eosin (H&E) staining and 1 x 10^4 cells were used for survival analysis. All mice were monitored regularly for the development of neurological symptoms due to tumor growth. Mice were maintained until the development of neurological symptoms, including hunched back, loss of body weight, reduced food consumption, and inactivity. After development of aforementioned symptoms, mice were humanely sacrificed.

For in vivo bioluminescent imaging (BLI) of mice, tumor-bearing mice were injected 200 mg/kg of D-luciferin (potassium salt, Gold Biotechnology) before isoflurane anesthesia. Radiance (photons/s/cm^2/steradian) was measured 10 min after substrate injection using Living Image 4.3.1 software (Caliper Life Sciences) or Aura software (Spectral Imaging).

For H&E brain sections, mice were humanely euthanized two to four weeks after implantation, and brains were harvested as previously described.[1,3] Each mouse brain was removed and embedded in O.C.T compound (Thermo Fisher) and stored at -80°C. Brains bearing xenografted tumors were sectioned on a cryostat (Leica) at 10 μm thickness. The whole brain was sectioned from most anterior to posterior. Every fifth tumor-bearing brain section of each brain was subjected to H&E staining to determine the locations from beginning to the end of each tumor. After careful comparison, the section with the largest tumor area in each tumor-bearing brain was used for the measurement. Tumor volume was estimated by using the formula $V = \frac{ab^2}{2}$, where a and b are the length and width of the tumor, respectively.

Bioinformatic Analyses

RNA-seq data of expression levels of LY6K and CAV-1 gene expression levels in GBM and low-grade glioma (LGG) of TCGA datasets were downloaded from FireBrowse (http://firebrowse.org/). This includes 530 LGG and 166 GBM samples. A total of 47 classical (CL), 63 mesenchymal (MES), and 39 proneural (PN) subtype tumors were included in GBM samples. The Mann-Whitney U-test was performed to determine whether *LY6K* and/or *CAV-1* were differentially expressed in GBM and LGG. One-way ANOVA with the Student-Newman-Keuls post-hoc test was used for the comparison of means among groups. Kaplan-Meier survival analyses were used to assess the correlation between *LY6K* and/or *CAV-1* and overall survival time in patients with gliomas. In the analyses of the TCGA dataset, the median or quartile expression of *LY6K* and/or *CAV-1* in all glioma samples were selected as a cutoff to divide samples

as high-and low- expression groups. In addition, a second dataset from GSE4271 was used to confirm the results of TCGA analyses.[4] The numerical data were presented as mean ± standard deviation (SD) of at least three determinations.

For amplification/copy number gain and expression data for *LY6K* (see Supplementary Figure 1), data were analyzed and downloaded from cBioPortal.[5,6] Amplification, as defined by cBioPortal, indicates high-level amplification/copy number gain. Values are generated from the GISTIC or RAE algorithms. The threshold for amplification/copy number gain for individual cancers is defined by the specific study from which the alteration frequency was generated.[5,6]

Lentiviral Plasmids

TRC lentiviral control and target-specific shRNA vectors were purchased from Dharmacon (*LY6K*: TRCN0000117952 and TRCN0000117956; *CAV-1*: TRCN0000007999 and TRCN0000011218). TRCN0000117952 (referred to as shL1 in the figures) targets the 5'-UTR of *LY6K* and TRCN0000117956 (referred to as shL2 in the figures) targets the body of the gene. For *LY6K* overexpression, the *LY6K* open reading frame (ORF) was inserted into the *pCMV6-Entry* vector to generate a construct containing *LY6K* including *Myc* and *DDK* tags. The resulting cDNA fragment was then subcloned into the *pCDH-EF1-MCS-IRES-RFP* vector to generate *pCDH-LY6K* lentiviral construct.

For rescue experiments, GSC83 or GSC30 cells stably expressing a lentiviral shRNA vector that targets the 3'-UTR of *LY6K* (TRCN0000117952; shL1) were infected

with the *pCDH-LY6K*. For cells expressing a lentiviral shRNA vector that targets the body of *LY6K* (TRCN0000117956; shL2), a mutated version of the *LY6K* gene was used. Specifically, the 3rd base of three codons were mutated in the shRNA target gene sequence. This ensured that all mutations were silent, resulting in no change in the amino acid sequence, but rendering the construct resistant to the shRNA. Site-directed mutagenesis was performed with a QuikChange Site-Directed Mutagenesis Kit (Agilent), according to the manufacturer's instructions.

For the *LY6K-ΔGPI* domain deletion mutant, cloning was performed as described for *LY6K-WT* above. The ORF for *LY6K-ΔGPI* excluded the region of the *LY6K* gene starting at the most likely ω site (GPI-attachment site),[7] as predicted by UnitProt (https://www.uniprot.org/uniprot/Q17RY6).

Lentiviral Infection

HEK293T cells were seeded on the evening prior to the transfection in 10 cm dishes. The morning of the transfection, 6 µg of *psPAX2*, 6 µg of *VSV-G*, and 12 µg of the target vector (shRNA against *LY6K* or *CAV-1*, or full-length *LY6K* or *LY6KΔGPI* mutant) were transfected into cells using PEI as the lipophilic agent. Serum-containing media was changed 4-6 hours after transfection and cells were incubated for 48 hours at 37^0C. To establish stable cell lines, the supernatants containing lentivirus were harvested with polybrene (10 mg/ml, Sigma) and used to infect target cells. 72 hours after transduction, infected cells were selected with FACS.

Cell Proliferation Assays

In vitro cell proliferation assays were performed as previously described[3]. Briefly, GSC spheres or U87 glioma cells were dissociated into single cells, and cell density was quantified by counting viable (Trypan Blue-negative) cells using a hematocytometer. Cells were seeded into 24-well or 48-well plates (three wells averaged per time point and three or four time points). Each well contained a density of 6,000 (GSC83 & GSC30), 9,000 (GSC528), or 15,000 (U87) cells per well. Cells were counted at two, four, six, and/or eight days using a hemocytometer. All proliferation assays were repeated three times.

Limiting Dilution Assays

Limiting dilution/sphere forming frequency assays were conducted as described previously[1]. Briefly, dissociated cells from glioma spheres seeded in 96-well plates at density of 1, 10, 50, or 100 for all assays. For MES-like and PN-like GSCs, seven and 14 days after seeding respectively, each well was examined for formation of tumor spheres, and sphere forming frequencies were calculated and plotted as described online (http://bioinf.wehi.edu.au/software/elda/).[8]

In Vitro Signaling Assays

All signaling assays were conducted with starvation media. For all assays with U87 cells, cells were trypsinized and counted using a hemocytometer and seeded in fresh media with 10% FBS. Six hours after seeding, media was removed and replaced with fresh serum-free media overnight. For all assays with GSCs, cells were counted

using a hemocytometer and seeded in fresh stem cell media. Six hours after seeding, media was removed and replaced with fresh growth factor-free stem cell media overnight. All treatments below were conducted at 37°C.

For EGF stimulation, EGF (20 ng/ml) was added to cells and incubated for 10 min. For SB590885 (BRAF inhibitor) experiments, 0.1 nM SB590885 was added to cells for 1 hour. For PD98059 (MEK inhibitor) experiments, 50 μM PD98059 was added to cells for 1 hour. For latrunculin A experiments, 10 μM latrunculin A was added to cells for 20 min. For mannosamine hydrochloride experiments, 5 mg/ml D-mannosamine hydrochloride was added to cells for 1 h at 37°C. For MG132 experiments, 10 μM MG132 was added to cells and allowed to incubate overnight at 37°C. For PI-PLC experiments, cells were counted using a hemocytometer and suspended in Opti-MEM with 2 U/ml PI-PLC for 2 hours.

Co-Immunoprecipitation

Cells were lysed in RIPA lysis buffer (ProteinTech) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Lysates were pre-cleared by with 50 μl Protein A agarose bead slurry per 1 mg lysate for 1 hour at 4°C on a rotator. They were subsequently centrifuged at 1,000 rpm for 3 min at 4°C. After preclearing, lysates were subjected to immunoprecipitation with either 4 μg LY6K for 12 hours (ProteinTech, Cat 12026-1-AP) or 5 μg CAV-1 overnight (Thermo Fisher, Cat PA1-064) at 4°C.

Following antibody incubation, the immunocomplex was captured with 50 µl Protein A agarose bead slurry and incubated at 4°C (LY6K 12 hours; CAV-1 5 hours). The pull-down complex was then washed and eluted twice with a glycine elution buffer. Eluents were pooled, neutralized, and subjected to immunoblotting (IB) analysis with 4X sodium dodecyl sulfate (SDS) sample buffer. Lysates were heated for 10 min, run on a 12% SDS gel, subjected to electrotransfer, and subsequently probed using appropriate antibodies.

Immunoblotting Analyses

Cells that were lysed directly with 2X SDS buffer or immunocomplex in 4X SDS buffers described above were supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Protein samples were subjected to SDS-PAGE (12% polyacrylamide) gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes. Blocked membranes were incubated with indicated antibodies overnight at 4°C (1:500 or 1:1000). Antibodies used include anti-LY6K (ProteinTech, Cat 12026-1-AP), anti-GAPDH (Santa Cruz, Cat sc-47724), anti-p-ERK1/2 (Cell Signaling, Cat 9101S), anti-ERK1/2 (Cell Signaling, Cat 9102S), anti-p-AKT (Cell Signaling, Cat 4060S), anti-AKT (Cell Signaling, Cat 9272S), anti-p-EGFR (Cell Signaling, Cat 3777S), anti-EGFR (BD, Cat 610016), anti-CAV-1 (Thermo Fisher, Cat PA1-064), anti-p-GSK3β (Cell Signaling, Cat 9331S), anti-GSK3β (Cell Signaling, Cat 5676S), anti-p-SMAD2 (Cell Signaling, Cat 8828S), anti-SMAD2 (Cell Signaling, Cat 3102S), anti-p-SRC (Cell Signaling, Cat 2101S), anti-SRC (Santa Cruz, Cat sc-19), anti-p-STAT3 (Cell Signaling, Cat 9131S), anti-STAT3 (Cell Signaling, Cat 9132S), anti-p-PDGFRα (Santa Cruz, Cat sc-12910-R), anti-PDGFRα (Cell Signaling, Cat 3164), anti-p-AXL (R&D Systems, Cat

AF2228), anti-AXL (R&D Systems, Cat AF154), anti-p-MET (Upstate Cell Signaling Solutions, Cat 07-211), and anti-MET (Santa Cruz, Cat sc-10) antibodies. Following washing with 0.1% TBS-T, membranes were incubated with corresponding peroxidase-labeled secondary antibodies (1:1000). Blots were developed with enhanced chemiluminescence (ECL, Amersham Bioscience) reaction according to manufacturer's instructions.

Immunofluorescent (IF) Staining

Cells were cultured in chamber slides and fixed with 4% formaldehyde (Fisher) for 30 min. Cells requiring permeabilization were subsequently treated with 0.2% Triton X-100 for two min. Cells were blocked with AquaBlock (East Coast Bio, North Berwick, ME) for 60 min and probed with an anti-LY6K antibody (Abnova Cat PAB21148, 1:100) overnight at 4°C. After being washed three times with PBS, cells were incubated with Alexa 488 labelled secondary antibodies (1:200) and DAPI-containing mounting solution Vectashield (Vector Laboratories), and then visualized by using a Nikon A1R (A) Spectral laser scanning confocal microscope.

Methylation Analyses & Ionizing Radiation Treatment

Genomic DNA (gDNA) extraction was performed using QIAamp DNA Mini Kit (Qiagen) and used for Illumina 450K array profiling that interrogates 485,577 CpG loci at the NUSeq Core at Northwestern University. Chip processing was carried out according to manufacturer's instructions. The signal intensities obtained from the Illumina GenomeStudio was converted to β-values and normalization was carried out to

remove biases between the Infinium I and II probes. Probes on X and Y-chromosomes were also removed. As we previously described, in order to preserve the biological variations on methylation profiles among different subtypes, no further normalization was performed.[9] In addition to removal of X and Y chromosomes (~12,000 probes), we removed probes that had unusual distribution of β-values (high variance) on the same sample subtypes. Data were deposited to GEO with a deposit number of GSE90498.

Methylation analyses of *LY6K* CpG island promoter region was carried out using combined bisulfite and restriction analyses (CoBRA) and direct bisulfite sequencing and confirmed with clone sequencing as previously described.[1,10] Bisulfite conversion of gDNA was carried out prior to CoBRA analyses using Epitect Bisulfite kit (Qiagen), according to manufacturer's instructions. Methylated positive controls were also generated by incubating unconverted samples with S-Adenosyl methionine (SAM) and DNA methyltransferase (New England Biolabs) at 37°C for 2 hours. Nested CoBRA primers were designed using previously published standard primers designing criteria for bisulfite converted gDNA.[10] 10 µl PCR product was treated for 1 hour with Bsh1236I (Thermo Scientific) at 37°C. After digestion, samples were resolved by agarose gels. Digested (methylated) samples showed multiple bands on the gel, while undigested (unmethylated) samples showed one band matching the uncut samples.

Direct bisulfite sequencing was carried out using agarose gel-purified bisulfite PCR products with the QIAquick Gel Extraction Kit (Qiagen). Samples were then directly submitted to the NUSeq Core. The sequencing chromatograms were compared

to the genomic *LY6K* sequence to determine which CpG sites had been subjected to bisulfite conversion and thus were methylated. For each sample, 10 ng of DNA and 10 picomoles/μl primer was used for sequencing. To confirm the results, gel-purified bisulfite PCR products were then subcloned into pGEM-T (Promega, Cat#: A3600). Successful clones were extracted and sequenced by using T7 promoter sequencing primer at the NUSeq Core at Northwestern University Feinberg School of Medicine and subjected to the chromatogram analysis described above. Each sequencing reaction was carried out with samples in triplicates.

For ionizing radiation treatment, GSCs were dissociated into single cells and placed in 6-well plates. They were subsequently subjected to 2 Gy IR using an X-Ray Irradiator (RS-2000 Series by Rad Source Technologies, Inc), followed by incubation at 37°C. Cells were collected at 24, 72, 120 hours, and 10 days after IR and subjected to bisulfite sequencing analyses. For cell proliferation assays following IR, cells were seeded into 48-well plates and subjected to 2 Gy IR. They were then monitored for proliferation over the next eight days.

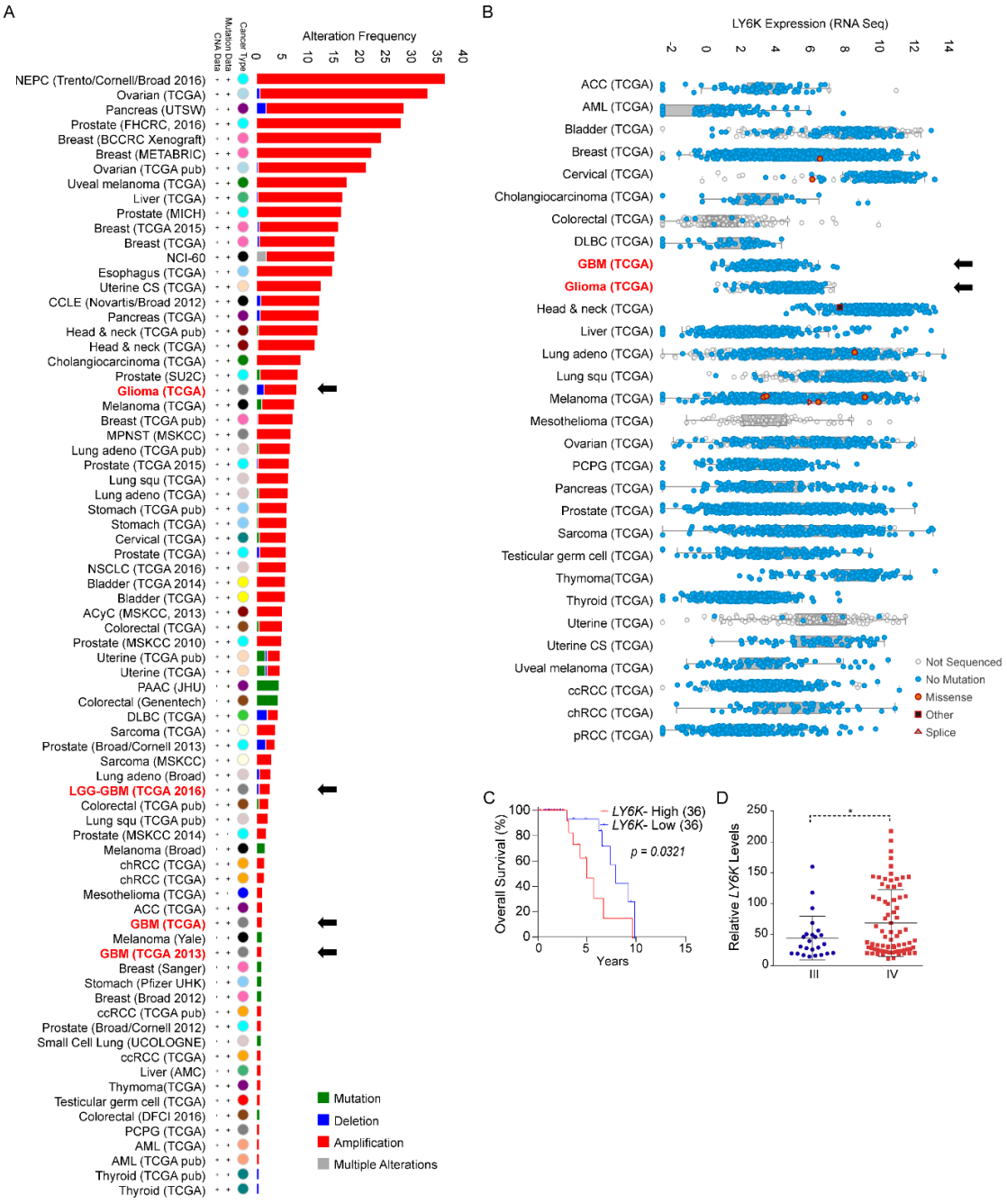


Figure S1 (Accompanying Fig 1)

LY6K is frequently amplified or has copy number gain in clinical cancers. (A-B) RNA Seq analysis of TCGA data. *LY6K* is amplified or has copy number gain across various cancer types. Only cancers with alteration frequencies above greater than 0% are shown (A). Few mutations and splice variants of *LY6K* have been discovered, but none have been observed in gliomas (B). For A & B: Arrows and corresponding text in red indicate data from glioma or GBM. (C-D) Analyses of dataset GSE4271. Expression of *LY6K* is associated with GBM prognosis (C) and tumor progression (D). *p < 0.05.

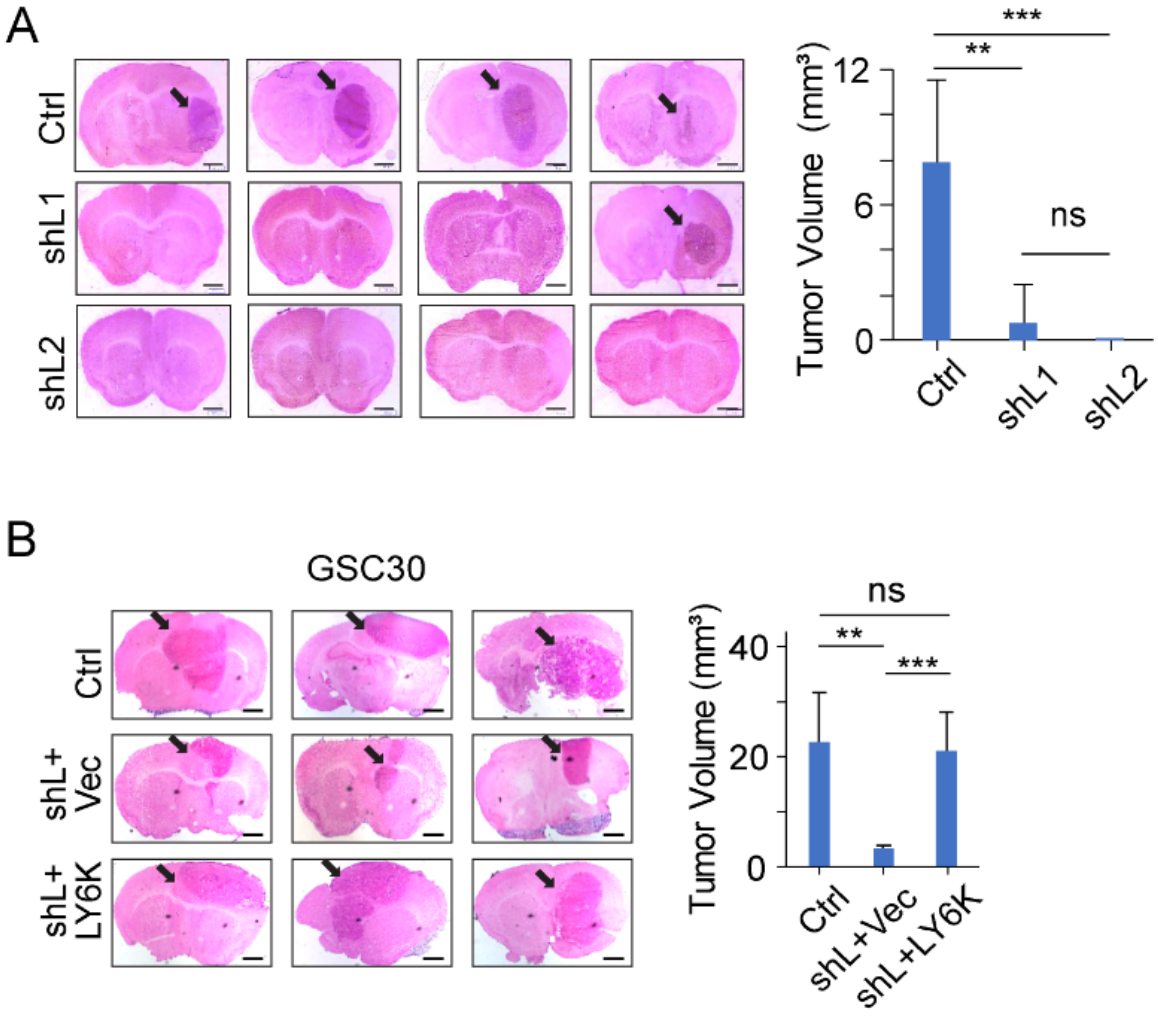


Figure S2 (Accompanying Fig 2)

LY6K promotes GBM tumorigenicity *in vivo*. (A) H&E staining analysis of mouse brain sections with GSC83 tumor xenografts with indicated modifications. Graph on right shows quantification of tumor volume in indicated GSC83 xenograft tumors. (B) H&E staining analysis of mouse brain sections with GSC30 tumor xenografts with indicated modifications. Graph on right shows quantification of tumor volume in indicated GSC30 xenograft tumors. Scale bar in (A) and (B) is 200 μm. Data are representative from two to three independent experiments with similar results. **p < 0.03, ***p < 0.01.

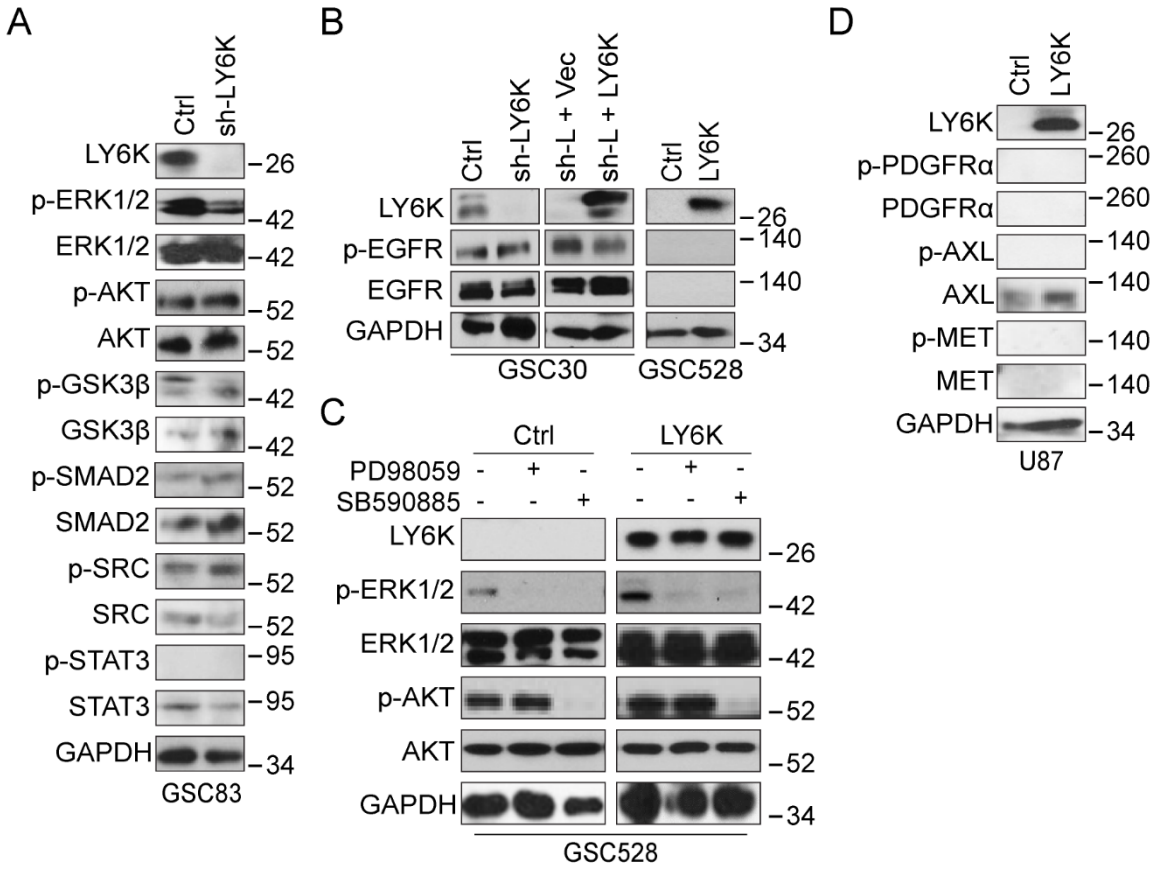


Figure S3 (Accompanying Fig 3)

LY6K selectively activates p-ERK1/2 but not other signaling pathways, and functions independent of EGFR signaling. (A-D) IB. (A) Of all signaling mediators tested, LY6K expression enhanced only p-ERK1/2 expression faithfully and reproducibly. (B) No changes in p-EGFR or EGFR were detected commiserate to the changes seen in p-ERK1/2 in GSC30 (left) cells. No p-EGFR or EGFR was detectable in GSC528. (C) MEK inhibitor PD98059 or B-Raf inhibitor SB590885 inhibited LY6K-enhanced p-ERK1/2 in GSC528 cells, despite these cells lacking EGFR expression as observed in (B). (D) Activation of PDGFRα, AXL, or c-MET was not affected by the expression of LY6K.

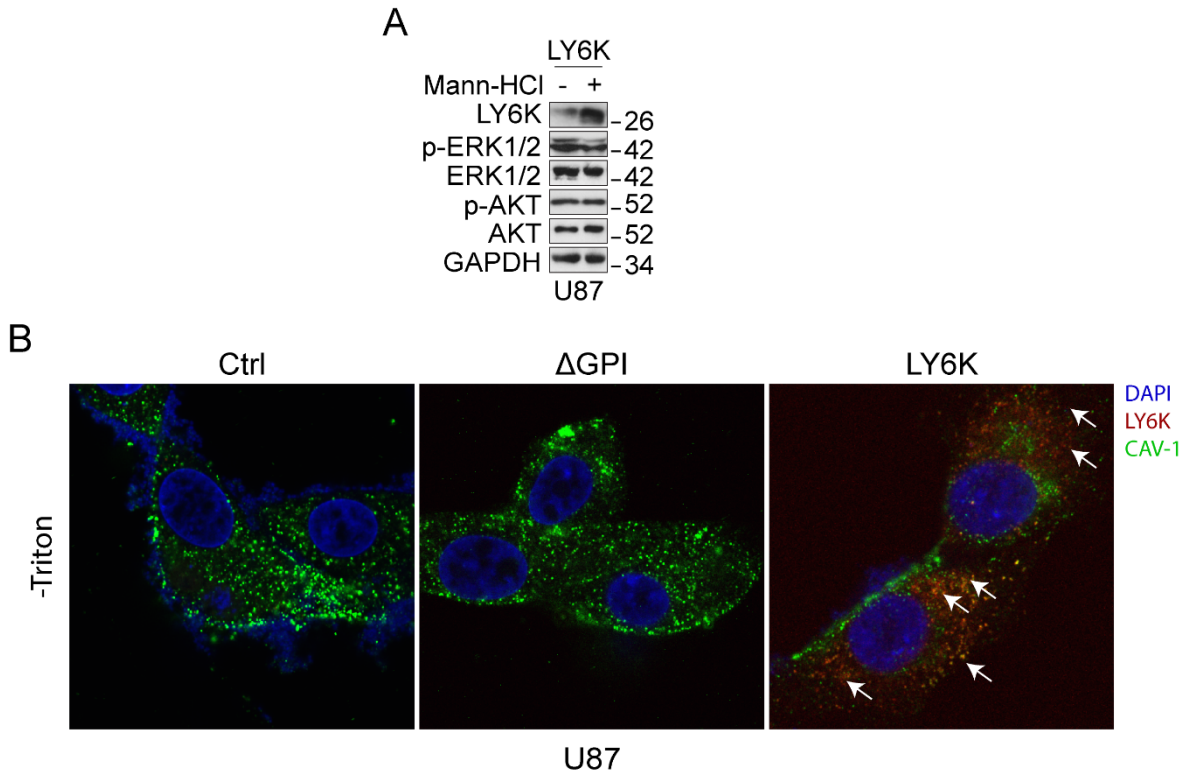
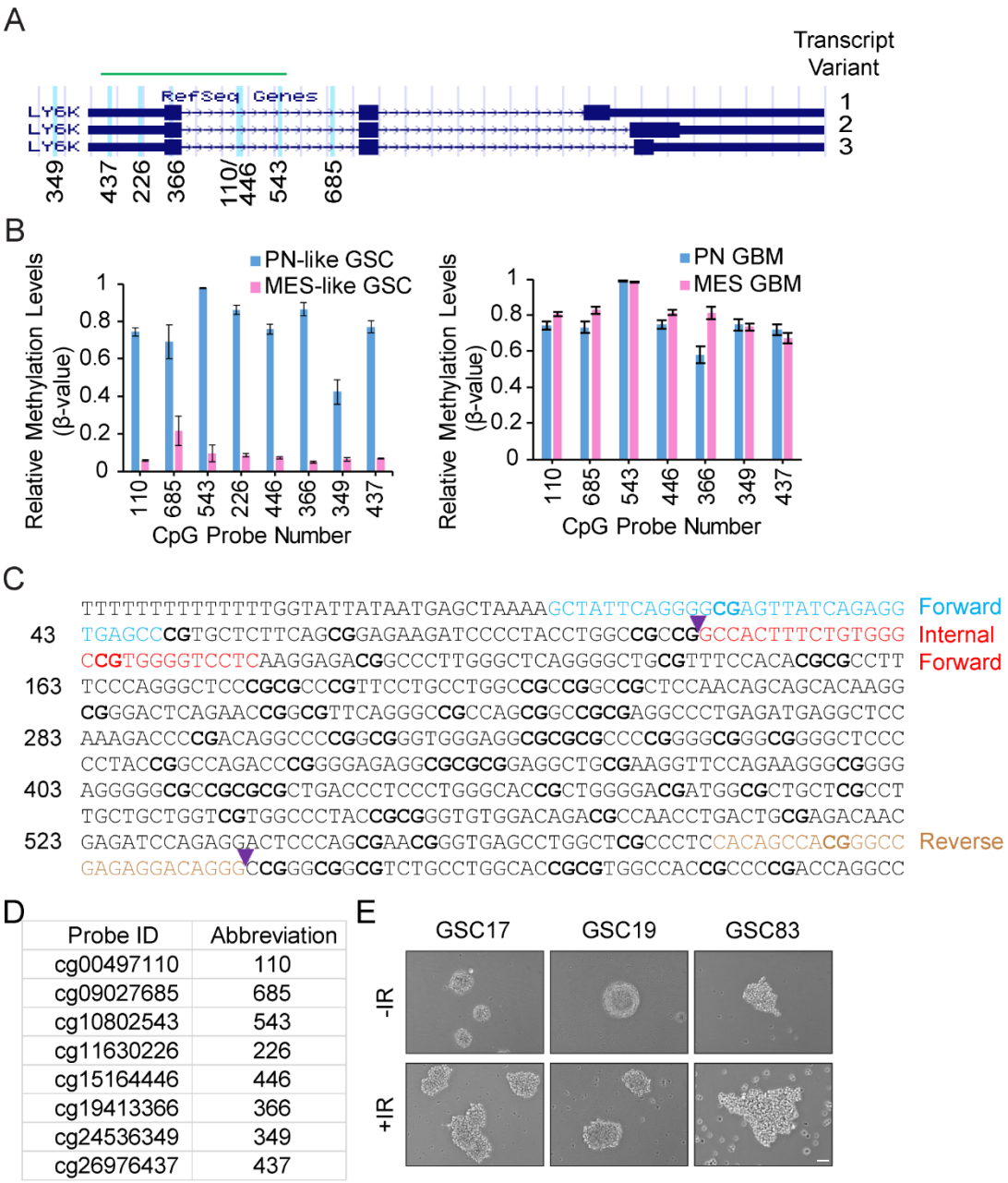


Figure S4 (Accompanying Fig 5)

The GPI-anchor domain of LY6K is required for its activity. (A) IB. Treatment with mannosamine hydrochloride (a compound that inhibits GPI anchor incorporation) reduced LY6K-enhanced p-ERK1/2 levels. (B) Immunofluorescent staining. Unlike control cells and the LY6K-ΔGPI mutant, LY6K-WT can co-localize with CAV-1 on the cell membrane. White arrows indicate the co-localization between LY6K and CAV-1. Scale bar in B is 10 μm.



Probe ID	Abbreviation
cg00497110	110
cg09027685	685
cg10802543	543
cg11630226	226
cg15164446	446
cg19413366	366
cg24536349	349
cg26976437	437

Figure S5 (Accompanying Fig 6)

DNA methylation of *LY6K* gene promoter regulates its expression and GSC response to irradiation. (A) Schematic showing the location of various CpG sites in the promoter region of *LY6K.* Light blue lines indicate CpG sites. Green bar indicates predicted CpG island. (B) Relative methylation levels for CpG sites in the *LY6K* promoter in PN-like or MES-like GSCs (left) or PN or MES GBM (right). (C) Illustration of nested primers used for CoBRA PCR, showing forward (blue), internal forward (red), and reverse (brown) primers, with CpG sites bolded. Sequence between the purple arrowheads is the PCR-amplified sequence. The base pair location is indicated on the left. (D) Table showing the CpG probe IDs and abbreviations used in (A) and (B). (E) Visual representation showing how PN-like GSC17 and GSC19 change from having tight spheres typical of PN-like GSCs to having loose spheres, similar to MES-like GSC83 in the presence of IR. Scale bar in E is 200 μm.

References:

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