Supplementary Information

SEC61G Assists *EGFR*-Amplified Glioblastoma to Evade Immune Elimination

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The Supporting Information contains Supplementary Materials and Methods,
7 Supplementary Figures and 3 tables.

SI Materials and Methods

Cell culture, transfection and treatment

Normal human astrocytes (NHA), human glioblastoma cell lines LN229, U251, U87MG and U87-EGFR*VIII,* mouse GBM cell line GL-26 and CT2A, and embryonic kidney 293T cells were cultured in DMEM medium (Gibco) supplemented with 10% bovine calf serum (Gibco). The GBM patient-derived GBM0603, GBM1226, GBM0108, GBM0109, GBM0919 and GBM0709 cells were primarily cultured in the lab. Only early-passage primary GBM cells were used for the study. No cell lines used in this study were found in the database of commonly misidentified cell lines that is maintained by NCBI Biosample. Cell lines were authenticated by short tandem repeat profiling and were routinely tested for mycoplasma contamination.

Transient transfection of plasmids and siRNAs was performed using lipofectamine 2000 (Invitrogen) or X-tremeGENE siRNA transfection reagents (Roche), respectively. For cell treatment, the standard concentrations of the following reagent are: Tunicamycin, 10μg/ml; cycloheximide (CHX), 50 μg/ml; MG132, 20 μM; EGF, 100 ng/ml; erlotinib, 2 μM; lapatinib, 1μM; Eeyarestatin I (ES I), 5 μM; unless otherwise specifically indicated.

Plasmid construction

Plasmids Flag-PD-L1, Flag-PVR and Flag-PD-L2 were purchased from Beijing Yiqiao Shenzhou Technology. Human *SEC61G* cDNA was amplified from GBM0108 cells and cloned into pcDNA3-HA vector. To construct plasmids expressing human or

mouse *SEC61G* and *PD-L1* shRNAs, the sense and antisense oligonucleotides of the target sequences were annealed and then cloned into pLKO.1-puro vector. All plasmids were confirmed by DNA sequencing. The sequences of the oligonucleotides were shown in Table S2.

Quantitative PCR

Total RNA was extracted using Trizol reagent (Invitrogen, USA) and then reverse-transcribed using the HiScrip II Q RT SuperMix (Vazyme, Nanjing, China), according to the manufacturer's instructions. The reverse-transcribed cDNA products were used for quantitative PCR (qPCR) analysis using UltraSYBR Mixture (Cwbiotech, Beijing, China). For quantification of genomic DNA copy number, the genomic DNA of different cells were extracted using the TIANamp Genomic DNA kit (Tiangen, Beijing, China). All quantitative PCR results were expressed as mean ± SEM from three independent experiments. The sequences of the oligonucleotides used for qPCR were shown in Table S2.

Lentiviral stable cell lines

To generate lentiviral stable cells, the lentiviral expression plasmids, packaging plasmid psPAX2, and envelope plasmid pMD2.G (4:3:1) were co-transfected into 293T cells using Lipofectamine 2000 reagent (Invitrogen, USA). The cell culture supernatants containing lentiviral particles were collected 48 hours after transfection,

and then added onto GBM cells in six-well plates. The infected GBM cells were selected by puromycin (2 μg/ml) or neomycin (200 μg/ml) for 1 week.

T Cell-mediated tumor cell killing assays

In brief, human peripheral blood mononuclear cells (PBMC) (LDEBIO, Guangzhou, China) were cultured in ImmunoCult™-XF T cell expansion medium (10981, StemCell Technologies) with ImmunoCult™ Human CD3/CD28/CD2 T cell activator (10970; STEMCELL Technologies) and IL-2 (1000U/mL, R&D) for one week according to the manufacturer's protocol. $CD8^+$ T cells were then purified from PBMCs using CD8 Dynabeads (#11147D, Invitrogen, USA) following the manufacturer's instructions. GBM cells were planted on 24-well plates. After 24h, $CD8^+$ T cells were added and co-culture with GBM cells for 48h. The ratios between GBM cells and activated T cells (target-effector ratio) were modified according to the purpose of each experiment. After co-culture with activated T cells, GBM cells were washed with PBS for 3 times, and the surviving GBM cells were then visualized by violet staining, and spectrophotometric absorbance were quantified by a spectrometer at OD 570 nm.

Immunofluorescence and immunohistochemical analysis

For immunofluorescence (IF) assays, cells in 6-well plates were treated with 4% formaldehyde for 15 min and then treated with 0.5% Triton X-100 for 15 min. The slides were incubated with antibodies against CD8α, granzyme B, PD-L1, PVR or

GRP94, and then incubated with a fluorescent-conjugated second antibody (Alexa Fluor, Life Technologies). Nuclei were co-stained with DAPI. Images were taken using a confocal microscope (Carl Zeiss).

Anonymous archived human GBM specimens were obtained from the Department of Neurosurgery of Nanfang Hospital of Southern Medical University under a protocol approved by the institutional review board. All tissue samples were collected in compliance with the institution's informed consent policy. For immunohistochemical (IHC) staining, tissue slides were deparaffinized, rehydrated through an alcohol series, and then stained with primary antibodies against EGFR, SEC61G, CD8α, granzyme B, PD-L1, PVR, PD-L2, PCNA or cleaved Caspase-3. Staining was scored according to the percentage of cells with positive staining and to the staining intensity, as we described previously (2). We assigned the percentage score as follows: 1 if 0-25% of cells had staining, 2 if 25-50%, 3 if 50-75%, 4 if more than 75% of cells had staining. We scored the staining intensity as 1 for weak, 2 for moderate, and 3 for strong. The total score was obtained by multiplying the percentage score by the intensity score. Three individuals who were blinded to the slides examined and scored each sample. The final score was the median value of the scores provided by the individuals. Table S3 contains the detail information of antibodies used in the study.

Flow cytometry

For mouse brain tissues, single cell suspension of GL-26 GBM tumors were obtained by rapid and gentle stripping, physical grinding and filter filtration. Cells were stained with PE/Cyanine7-CD45, PerCP/Cyanine5.5-CD3, and APC-CD8 for 30 min. After fixation and permeabilization by Fixation Buffer (#420801, Biolegend) and Intracellular Staining Perm Wash Buffer (#421002, BioLegend), intracellular GZMB was stained using FITC-GZMB antibody. The stained cells were analyzed using a DxP Athena flow cytometry system (Cytek Biosciences, USA), and data were analyzed by CytExpert and Flow Jo 10.0 software.

Cell viability assays

For cell viability analysis, human GBM0108 and GBM0709 cells and mouse GL-26 cells (1000 cells/well) in 96-well plates were treated with erlotinib (2nM) and lapatinib (1nM), and cultured in 5% CO_2 incubator at 37 °C for 10 days. The viability of cells was assessed by CCK8 assays (Vazyme Biotech, Nanjing, China), according to the manufacturer's instructions.

Cellular ubiquitination assays

Cells transfected with the indicated plasmids were treated with the MG132 (20 μM) for 6 h, and then lysed using RIPA lysis buffer (50 mM Tris-base pH 6.8, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 10 mM NaF, 10 mM dithiothreitol (DTT), 0.2 mM Na_3VO_4, 1% cocktail protease inhibitors, 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysates were immunoprecipitated using

the indicated antibodies, and the resultant precipitates were separated by SDS page and then analyzed by immunoblotting.

Bioinformatic analysis

TCGA-GBM patients in the UCSC Xena database and CPTAC-GBM patients in the LinkedOmics database (http://www.linkedomics.org/login.php) were used to analyze the correlation between DNA copy numbers (gene-level) and mRNA expression. The TCGA-GBM simple nucleotide variation (SNV) data and copy number variation (CNV) data obtained from TCGA GDC were used to analyze mutation and copy number alterations through GenePattern (https://www.genepattern.org). Gene Set Enrichment Analysis (GSEA) was performed using the TCGA-GBM datasets downloaded from UCSC Xena (https://xena.ucsc.edu/).

Calcein-AM/propidium iodide (PI) staining

After co-culture with activated T cells, GBM cells were washed with PBS for 3 times to remove the T cells and cell debris, and then stained with 2 μM calcein-AM and 4.5 μM PI (Beyotime, Beijing, China) at 37 °C for 30 min. Images of the cells were obtained using a fluorescence microscope (Carl Zeiss). The percentage of PI positive cells was counted.

***In vivo* intracranial and subcutaneous GBM models**

All animal experiments were approved by the Institutional Animal Care and Use Committee of Southern Medical University. The animals were randomized to different experimental groups. The investigators were blinded to allocation during experiments and outcome assessment. C57BL/6 mice and BALB/c nude (nu/nu) mice (4- to 6-week-old male) were purchased from the Guangdong Medical Laboratory Animal Center. GL-26 cells (5×10^5 cells/mouse) were injected intracranially into C57BL/6 mice or BALB/c nude (nu/nu) mice (5 mice for each group). Tumor growth was quantified by bioluminescence imaging. At the end of the experiments, the mice were humanely killed, and tumor formation was determined by histological analysis of tissue sections stained with hematoxylin and eosin (H&E). Tumor volume was calculated by the formula $V=\pi/6 \times \text{length} \times \text{width}^2$. For *in vivo* therapeutic experiments, 6d after intracranial implantation of GL-26 cells into C57BL/6 mice (5 mice per group), CD8α mAb (Selleck, 100 μg/mouse/d) or IgG2b (Selleck, 100 μg/mouse/d) was injected intraperitoneally every other day for four times. For erlotinib treatment, erlotinib (Selleck, 50 mg/kg/d) in a vehicle of dimethyl sulfoxide/polyethylene glycol 300/Tween 80/ddH_2O (0.5:3:0.5:6 ratio) was injected intraperitoneally every day for 7d and then every other day for three times. In addition, we used mice (5 mice per group) for survival analysis, recorded the survival days when the mice died, and carried out subsequent statistical analysis.

For the subcutaneous mouse model, GL-26 cells (1×10^6 cells/mouse) were injected subcutaneously into C57BL/6 mice. For *in vivo* therapeutic experiments, 6d after implantation of GL-26 cells into C57BL/6 mice (5 mice per group), erlotinib (50

mg/kg/d) in a vehicle of dimethyl sulfoxide/polyethylene glycol 300/tween 80/ddH_2O (Selleck, 0.5:3:0.5:6 ratio) was injected intraperitoneally every day for 7d and then every third day for two times or Eeyarestatin I (2.5 mg/kg/d) in a vehicle of dimethyl sulfoxide/polyethylene glycol 300/tween 80/saline (MedChemExpress, 1:4:0.5:4.5 ratio) was injected intraperitoneally every third day for five times. For combinatorial treatment, mice received injections of erlotinib or Eeyarestatin I on alternating days for 20d. The vehicle alone was used for negative control.

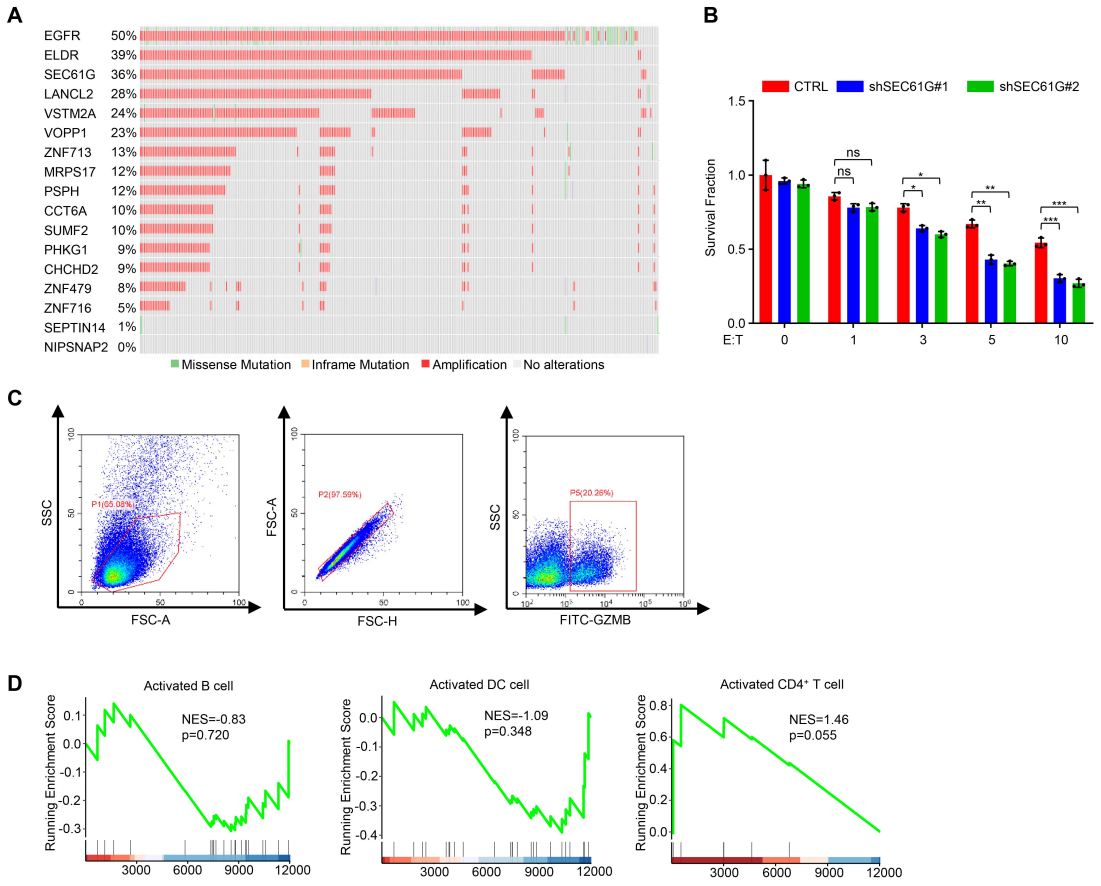

Fig.S1 Screen of genes at 7p11 loci identified SEC61G as a key regulator of cytolytic T cell activity. (A) A schematic view of amplification ratio of different genes on chr7p11 in GBM. (B) GBM0108 cells expressing *SEC61G* shRNAs were co-cultured with activated T cells for 48h with different effect-target (E-T) ratios. The surviving GBM cells were stained with crystal violet, and relative fold ratios of surviving cells were calculated (mean ± SEM, n=3 independent assays). ns, not significant, *P<0.05, **P<0.01, ***P< 0.001. (C) Gating scheme of the isolated $CD8^+$ T cells by flow cytometry. (D) GSEA analysis of the TCGA-GBM datasets demonstrated the correlation between *SEC61G* mRNA levels and the population of activated B cells, DC cells and $CD4^+$ T cells, respectively.

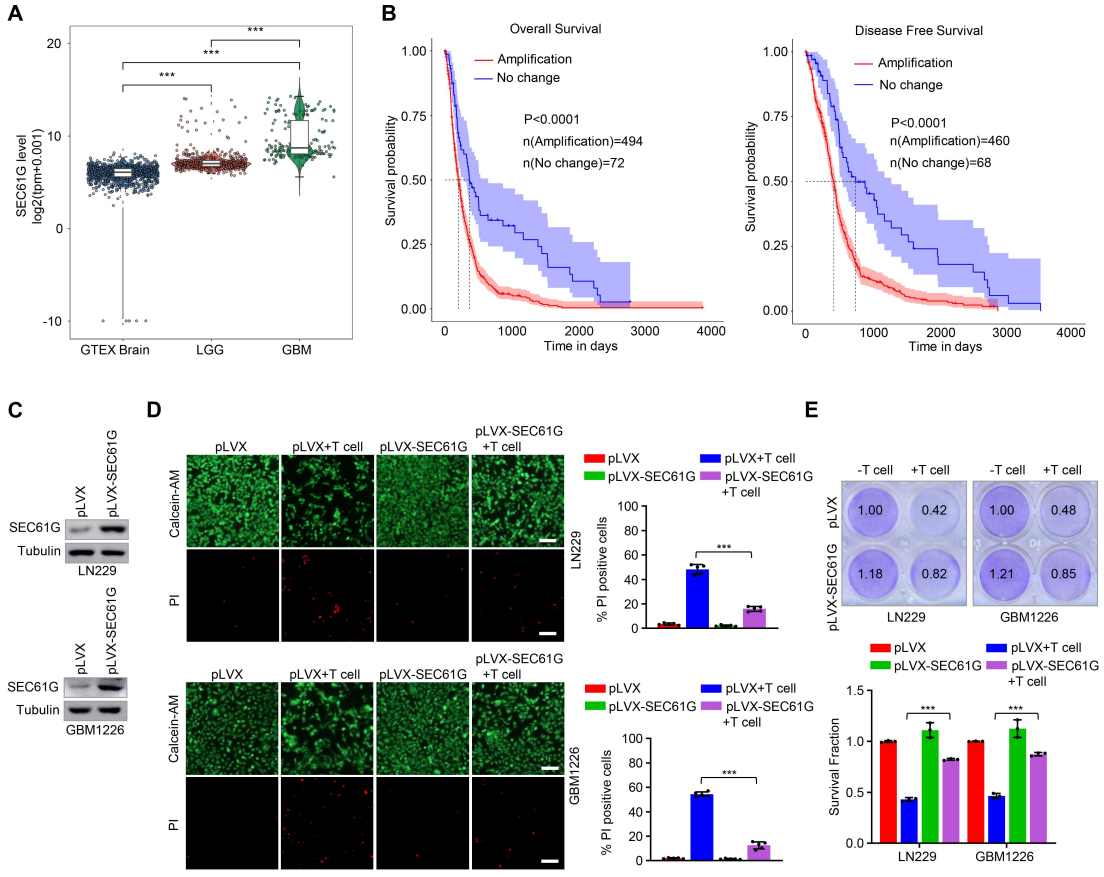

Fig.S2 *SEC61G* amplification predicts poor survival in glioma and inhibits T cell-mediated GBM cell killing. (A) Statistical analysis of SEC61G levels in normal brain, low grade glioma (LGG) and GBM using the TCGA datasets. (B) TCGA datasets demonstrated that amplification of *SEC61G* had poor overall survival and disease-free survival in glioma patients. (C) LN229 and GBM1226 cells stably expressing pLVX-SEC61G were analyzed by immunoblotting. (D) LN229 and GBM1226 cells expressing pLVX-SEC61G were co-cultured with activated T cells for 48 h (E-T ratio, 3:1). The surviving and dead cells were detected by Calcein-AM/PI co-staining. Representative images were shown. Scale bar, 200 μm. The percentage of PI positive cells were counted (mean ± SEM, n=5 randomly selected microscope fields). ***P< 0.001. (E) LN229 and GBM1226 cells expressing

pLVX-SEC61G were co-cultured with activated T cells for 48 h (E-T ratio, 3:1). The surviving cells were stained with crystal violet, and relative fold ratios of surviving cells were calculated (mean ± SEM, n=3 independent assays). ***P<0.001.

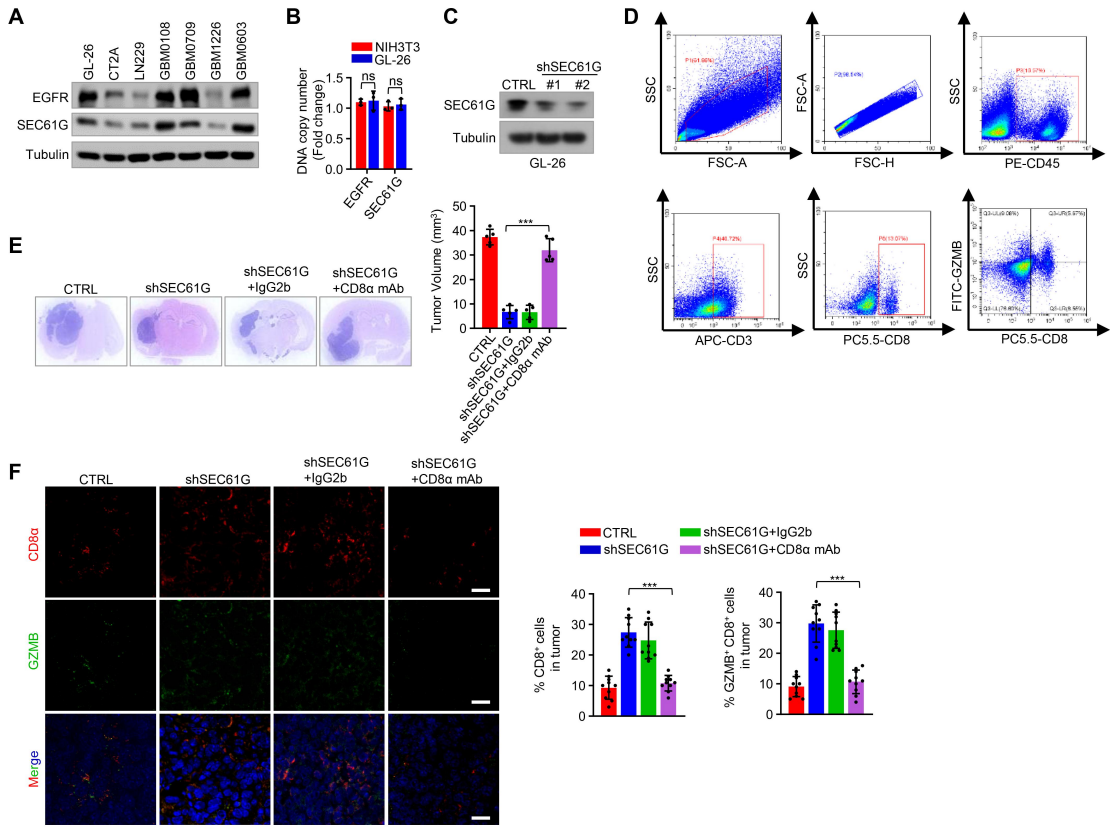

Fig.S3 Depletion of SEC61G inhibits GBM tumorigenesis by enhancing anti-tumor T cell immunity. (A) Immunoblotting analysis of EGFR and SEC61G in mouse GBM cells (GL-26 and CT2A), human GBM cells (LN229) and the patient-derived GBM cells (GBM0108, GBM0709, GBM1226 and GBM0603). (B) DNA copy numbers of mouse *EGFR* and *SEC61G* in GL-26 cells and mouse NIH3T3 cells were quantified by RT-PCR using specific primers. *GAPDH* was used an internal control. Values were normalized to NIH3T3 cells. ns, not significant. (C) GL-26 cells stably expressing *SEC61G* shRNAs were analyzed by immunoblotting. (D) Gating scheme of the isolated $CD8^+$ T cells by flow cytometry in Fig.3E. (E) GL-26 GBM-bearing mice were injected intraperitoneally with CD8α mAb (100 μg/mouse/d) or IgG2b (100 μg/mouse/d). H&E-stained sections show representative tumors. Tumor volumes

were calculated (mean ± SD, n=5 mice for each group, One-way ANOVA test). ***P < 0.001. (F) Mouse brain tissues were double-stained with CD8 and GZMB. Representative images were shown. Scale bar, 50 μm. CD8 and GZMB levels were quantified by ImageJ (mean ± SEM, n=10 randomly selected fields, Student's t-test). ***P< 0.001.

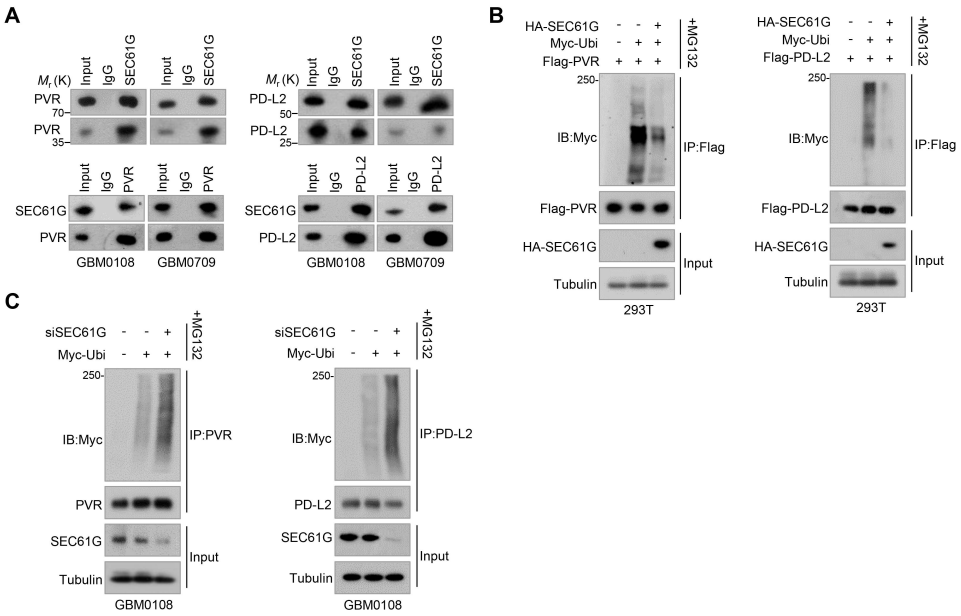

Fig.S4 SEC61G-induced N-glycosylation of the ICLs inhibits their ubiquitination. (A) Reciprocal IP assays demonstrated the interaction of SEC61G with PVR and PD-L2 in GBM0108 and GBM0709 cells. (B) 293T cells were transfected with Myc-Ubi, HA-SEC61G, and Flag-PVR or Flag-PD-L2. Cells were then treated with MG132 for 6 h, and cell lysates were immunoprecipitated using an anti-Flag antibody and then analyzed by immunoblotting. (C) GBM0108 cells were transfected with *SEC61G* siRNA and Myc-Ubi, and then treated with MG132 for 6 h. Cell lysates were immunoprecipitated using an anti-PVR or anti-PD-L2-antibody and then analyzed by immunoblotting.

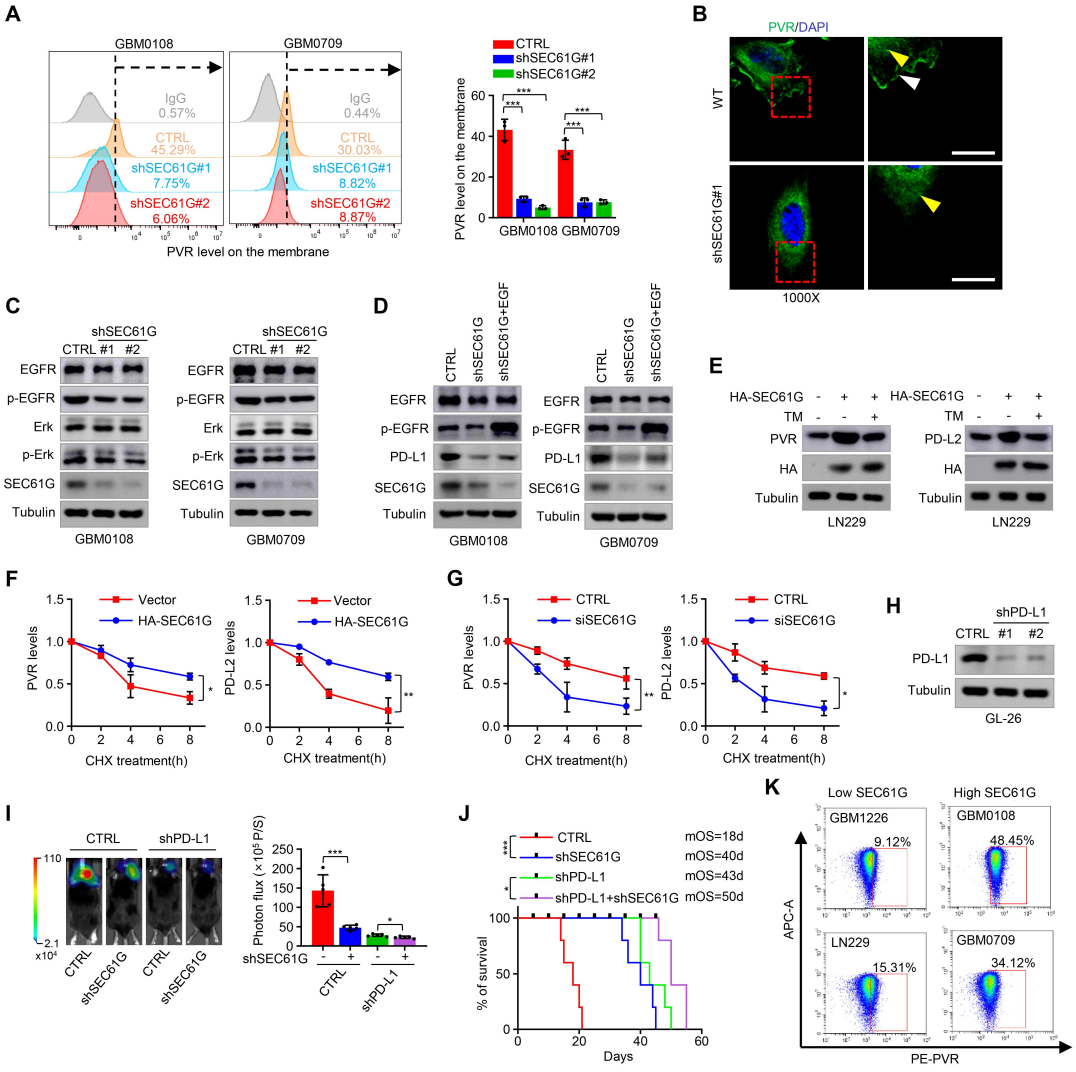

Fig.S5 SEC61G promotes the expression and membrane localization of ICLs. (A) The levels of membrane-bound PVR in GBM0108 and GBM0709 cells expressing *SEC61G* shRNAs were analyzed by FACS. Representative FACS plots were shown. Data are presented as mean ± SEM, n=3 independent assays, ***P<0.001. (B) Immunostaining of PVR in GBM0108 cells expressing control or *SEC61G* shRNA. Scale bar, 20 μm. The white and yellow arrows indicate membrane-bound and cytosol PVR, respectively. (C) GBM0108 and GBM0709 cells expressing *SEC61G* shRNAs were analyzed by immunoblotting using the indicated antibodies. (D) GBM0108 and

GBM0709 cells expressing *SEC61G* shRNAs were treated with EGF (100 ng/ml) for 36 h. Cell lysates were analyzed by immunoblotting using the indicated antibodies. (E) LN229 cells were transfected with HA-SEC61G plasmid and then treated with 5 μg/ml TM for 12 h. Cell lysates were analyzed by immunoblotting. (F-G) Band intensities of PVR and PD-L2 were quantified, and the results are expressed as the levels relative to the control cells (mean ± SD, n=3 independent experiments, paired Student's t-test, right panel). *P< 0.05, **P< 0.01. (H) GL-26 cells stably expressing *PD-L1* shRNAs were lysed and then subjected to immunoblotting. (I) GL-26 cells with SEC61G depletion were engineered to stably express *PD-L1* shRNA, and then were injected intracranially into C57BL/6 mice (5×10^5 cells/mouse). Tumor growth was monitored by bioluminescence imaging. Representative images were shown and tumor bioluminescence was quantified (mean ± SD. n=5 mice for each group, one-way ANOVA test). *P<0.05, ***P<0.001. (J) The survival of mice was evaluated (n=5 mice for each group, Kaplan-Meier model with two-sided log-rank test). *P< 0.05, ***P<0.001. (K) The membrane-bound levels of PVR in the SEC61G-high and SEC61G-low GBM cells were analyzed by FACS.

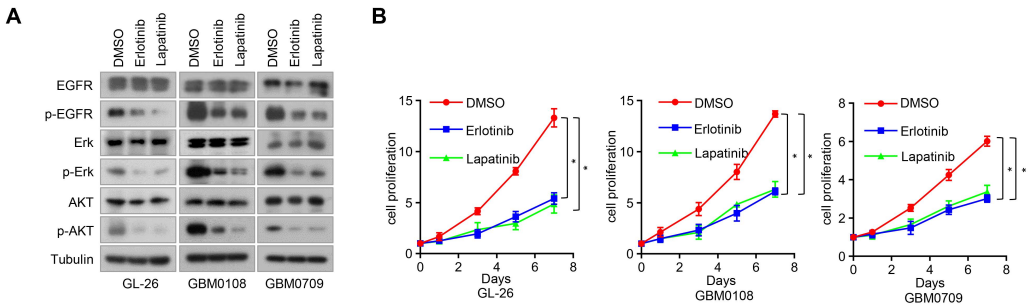

Fig.S6 EGFR inhibition using erlotinib and lapatinib inhibits GBM cell proliferation. (A) Mouse GL-26 cells, and human GBM0108 and GBM0709 cells were treated with erlotinib (2 μM) and lapatinib (1 μM) for 6h. Cell lysates were analyzed by immunoblotting using the indicated antibodies. (B) GBM cells were treated with erlotinib and lapatinib for different time duration, and cell viability was determined by CCK8 assays (mean ± SEM, n=3 independent experiments, paired Student's t-test). *P<0.05.

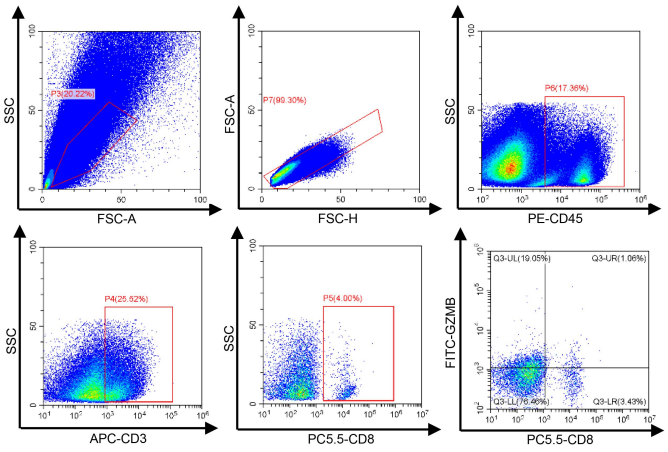

Fig.S7 Flow cytometry gating scheme of tumor-infiltrating lymphocytes in GL-26 tumors.

Supplementary Table S1 Information of patients used for primary GBM cell establishment in the study.

Cell lines	Patient ID number	Sex	Age	Pathological type	Molecular features
GBM0108	001602649	Male	62	Glioblastoma (Grade IV)	P53（+）、EGFR（+++）、VEGF（+++）、IDH1（-）、Ki-67（++）
GBM0109	001204381	Female	23	Glioblastoma (Grade IV)	P53（-）、EGFR（++）、VEGF（++）、IDH1（-）、Ki-67（+）
GBM0919	001132793	Female	14	Glioblastoma (Grade IV)	P53（-）、EGFR（++）、VEGF（++）、IDH1（-）、Ki-67（++）
GBM0709	001852123	Male	48	Glioblastoma (Grade IV)	P53（+）、EGFR（+++）、VEGF（++）、IDH1（-）、Ki-67（+++）
GBM1226	ZA2695227	Male	45	Glioblastoma (Grade IV)	P53（-）、EGFR（+）、VEGF（+）、IDH1（-）、Ki-67（+）
GBM0603	001413801	Male	28	Glioblastoma (Grade IV)	P53（+）、EGFR（++）VEGF（++）、IDH1（-）、Ki-67（++）

Supplementary Table S2 Oligonucleotides used in the study.

Name	Sequence (5’-3’)
SEC61G-Human-sh1	AGCCAAGTCGGCAGTTTGTAA
SEC61G-Human-sh2	ATTCCAGAAGATTGCCATGGC
SEC61G-Mouse-sh1	GCAGTTTGTAAAGGACTCAAT

SEC61G-Mouse-sh2	ACTGATCCATATACCCATTAA
PD-L1-Mouse-sh1	GCGTTGAAGATACAAGCTCAA
PD-L1-Mouse-sh2	GCCACTTCTGAGCATGAACTA
Primer for RT-qPCR	
Human-EGFR	F: 5’-AGGCACGAGTAACAAGCTCAC-3’
	R: 5’-ATGAGGACATAACCAGCCACC-3’
Human-SEC61G	F: 5’-GCAGTTTGTTGAGCCAAGTCG-3’
	R: 5’-CCAGCCGAATGGAGTCCTT-3’
Human-EGFR-Amp	F: 5’-ATCAGTGGCGATCTCCACATC-3’
	R: 5’-TCAGAATATCCAGTTCCTGTGG-3’
Human-SEC61G-Amp	F: 5’-AAGATTGCCATGGCAACAGCA-3’
	R: 5’-AACAGCCTGTCATCTCAAATG-3’
Mouse-EGFR-Amp	F: 5’-TAATGGCATAGGCATTGGTG-3’
	R: 5’-TGGGTCTAGAGGAGGAGTGC-3’
Mouse-SEC61G-Amp	F: 5’-CATGGGATTCATTGGCTTCT-3’
	R: 5’-AACACCACACAGCATTCAGG-3’
siRNA	
SEC61G-siRNA	ATCTTAGAGATTGGTGAACAA

Supplementary Table S3 Information of antibodies used in the study.

Antibody	**Company**	**Cat.no**	**Dilution**

SEC61G	Proteintech	11147-2-AP	IHC: 1:100
SEC61G	Affinity	DF12136	WB: 1:500 IP: 1:500
EGFR	Proteintech	66455-1-Ig	WB: 1:1000 IHC: 1:100
Granzyme B	Proteintech	13588-1-AP	IHC/IF: 1:100
PD-L1	Proteintech	17952-1-AP	WB: 1:1000 IP: 1:500
PD-L1	Abclonal	A1645	IHC/IF: 1:100
PVR	Proteintech	27486-1-AP	WB: 1:1000 IHC/IF: 1:100 IP 1:500
PD-L2	Proteintech	18251-1-AP	WB: 1:1000 IHC: 1:100 IP 1:500
GRP94	Proteintech	60012-2-IG	IF 1:100
CD8α	Santa cruz	sc-7970	IHC/IF: 1:50
p-EGFR	Santa cruz	sc-81489	WB: 1:500
ERK1/ERK2	Abclonal	A16686	WB: 1:1000
p-ERK	Santa cruz	sc-7383	WB: 1:500
p-ERK	Abclonal	AP0974	WB: 1:500
Akt	Proteintech	51077-1-AP	WB: 1:1000
p-Akt	Santa cruz	sc-81433	WB: 1:500
Flag	Proteintech	80010-1-RR	WB: 1:1000 IP 1:500
Myc	Proteintech	16286-1-AP	WB: 1:1000
HA	CST	3724S	WB: 1:1000
PCNA	Proteintech	10205-2-AP	IHC: 1:100
Active caspase3	Immunoway	YM3431	IHC: 1:100
Tubulin	Proteintech	11224-1-AP	WB: 1:10000

FITC-CD274	Biolegend	374509	FC: 0.25 μg per million cells
PE-PVR	Biolegend	337507	FC: 0.25 μg per million cells
PE/Cy7-CD45	Biolegend	103114	FC: 0.5 μg per million cells
PerCP/Cy5.5-CD3	Biolegend	100218	FC: 1 μg per million cells
APC-CD8	Biolegend	100712	FC: 0.25 μg per million cells
FITC-Granzyme B	Biolegend	515403	FC: 0.5 μg per million cells