# USP36 promotes tumorigenesis and drug sensitivity of glioblastoma by deubiquitinating and stabilizing ALKBH5

## Supplemental Data

Supplemental Figure 1

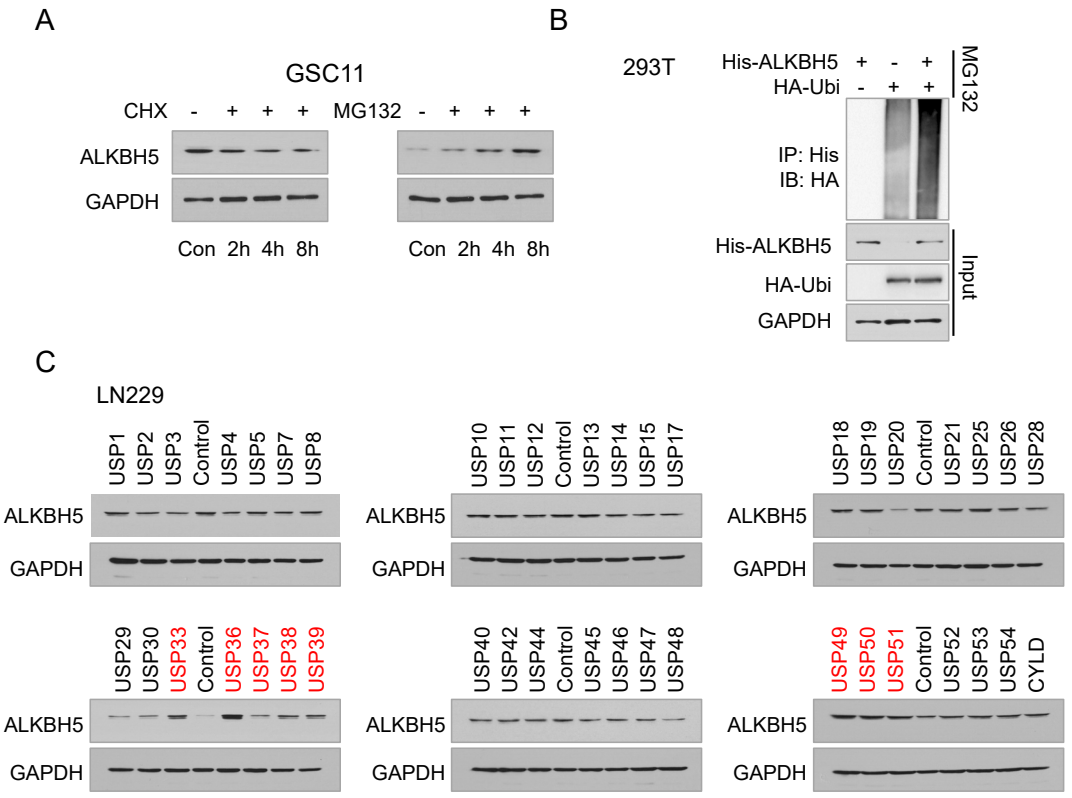



**Figure S1. USP36 is a deubiquitylase of ALKBH5. (A)** GSC11 cells were treated with 100 ng/mL cycloheximide (CHX) or 25 μM MG132 for the indicated time intervals and ALKBH5 expressions were detected by western blot. **(B)** 293T cells were transfected with His-ALKBH5 and HA-Ubi, and then treated with MG132 (25 μmol/L) for 6 hours before harvest. Cell lysates were immunoprecipitated with an anti-His antibody and the ubiquitination of ALKBH5 was analyzed by immunoblotting. **(C)** Total 42 DUBs were transfected into LN229 cells respectively, and cells were treated with CHX (100 ng/mL). The ALKBH5 expressions were detected with western blot and were normalized to NHA transfected with pcDNA3 vectors.

Supplemental Figure 2

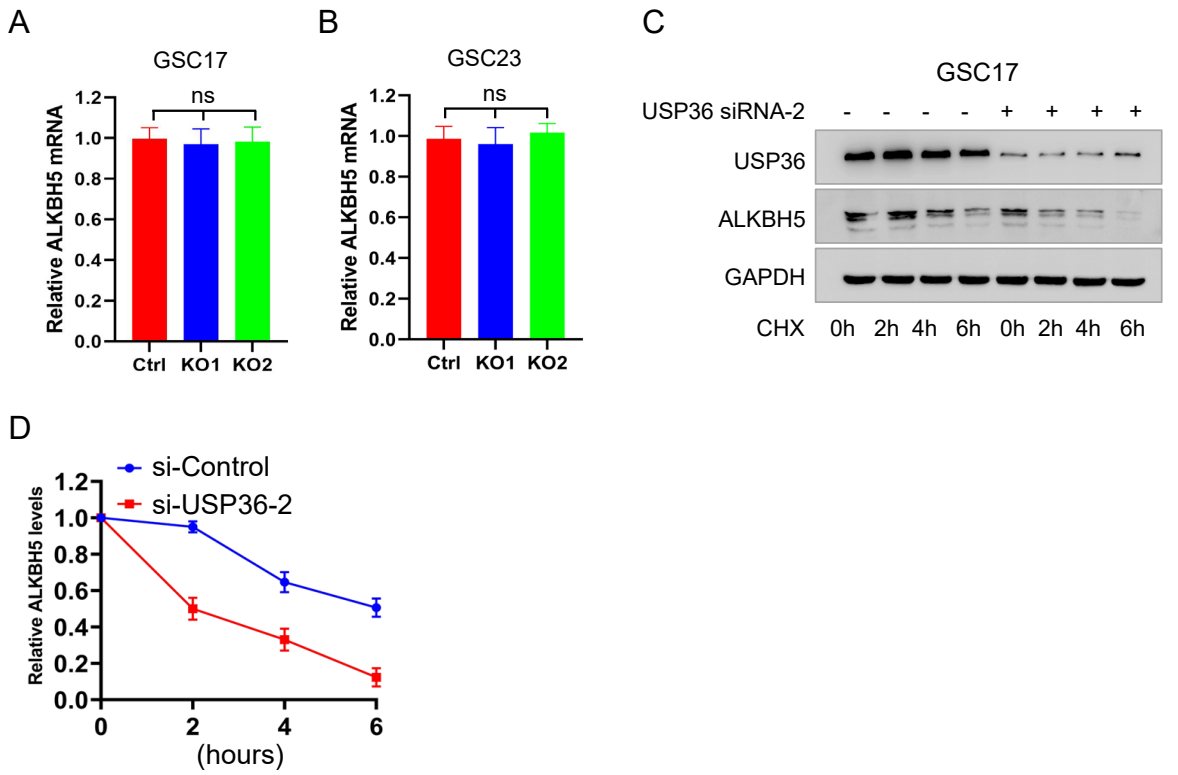



**Figure S2. USP36 stabilizes ALKBH5 but imposes no effects on ALKBH5 mRNA. (A and B)** The ALKBH5 mRNA expressions were not affected by the depletion of USP36 in GSC17 and GSC23 cells. **(C and D)** The ALKBH5 expressions were analyzed by immunoblotting in control- and USP36-siRNA2- GSC17 cells under the treatment with CHX. Western blotting band intensity of ALKBH5 was quantified and normalized to the internal control (D) (Data are mean ± SD for triplicate biological replicates; Student t-test; **, $p < 0.01$).

Supplemental Figure 3

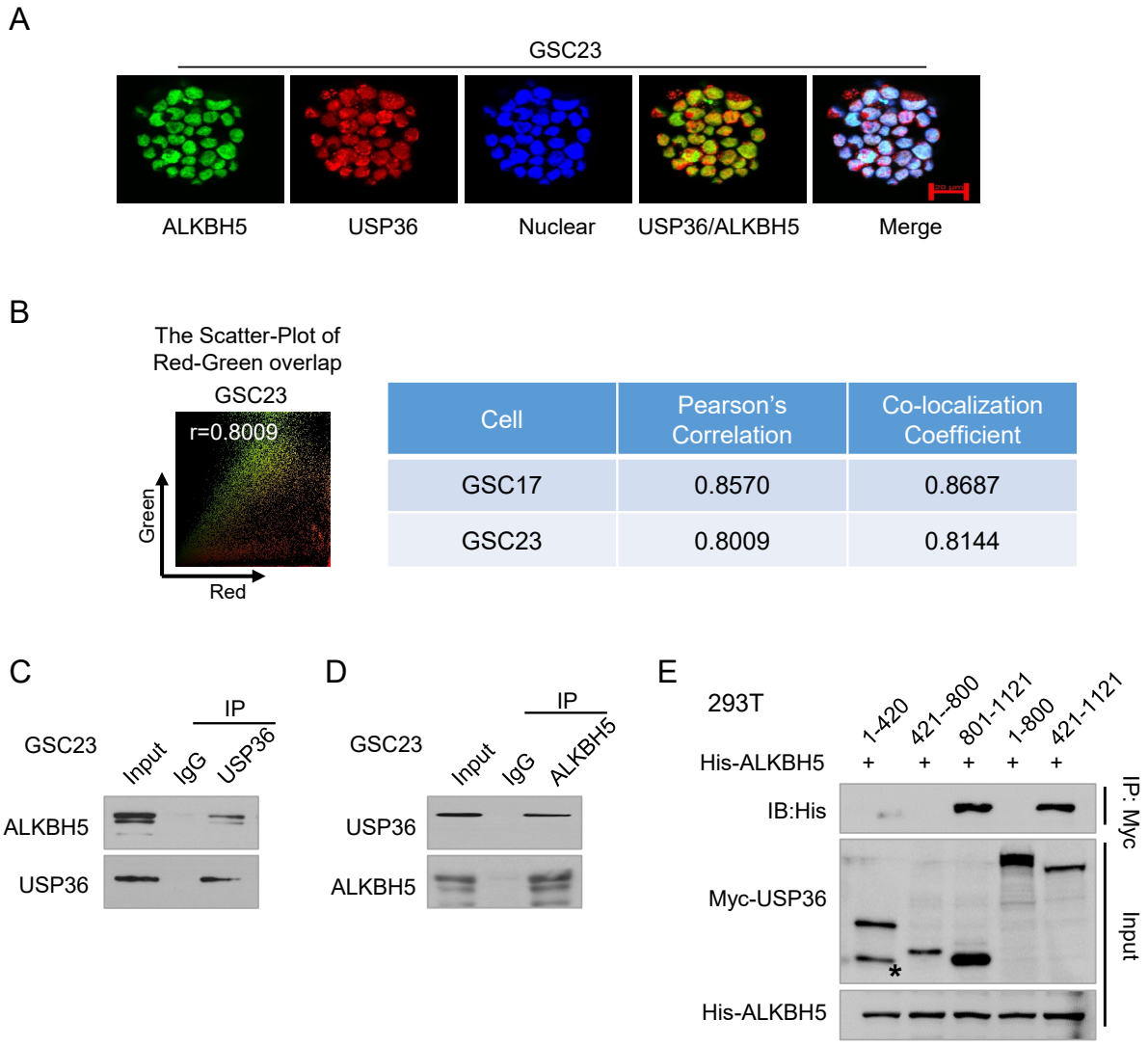



**Figure S3. USP36 binds with ALKBH5 straightly. (A)** Immunofluorescence assays of USP36 and ALKBH5 were performed in GSC23 cells. Cells were co-stained with DAPI (blue). Images were taken using a confocal microscope. Representative images are shown. Scale bar, 20 μm. **(B)** The quantification of overlap between USP36 and ALKBH5 in (A) was determined with the Image Pro Plus software. Left: the representative red-green scatter-plot of USP36 and ALKBH5 images; Right: the Pearson's correlation and co-localization coefficient of USP36 and ALKBH5 proteins. **(Cand D)** GSC23 cell lysates were immunoprecipitated with an anti-USP36 or ALKBH5 antibody and the immunoprecipitates

were analyzed by immunoblotting using the indicated antibodies. **(E)** A series of USP36-deletion constructs were generated and co-transfected with His-ALKBH5 into 293T cells. The cell lysates were immunoprecipitated with an anti-Myc antibody and then subjected to immunoblotting analysis with indicated antibodies. Stars indicated non-specific western bands in this immunoprecipitation result.

Supplemental Figure 4

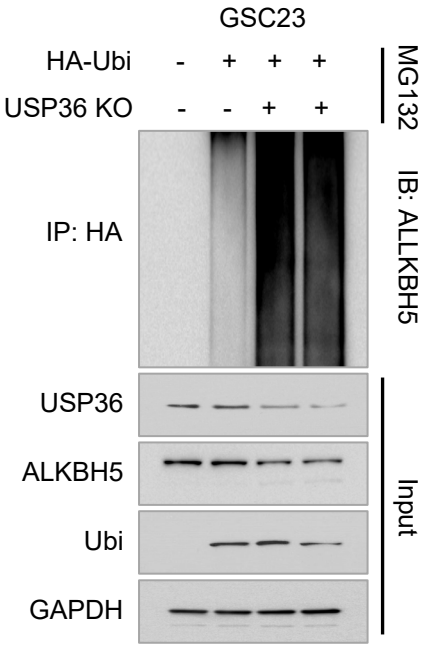
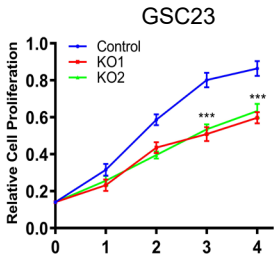


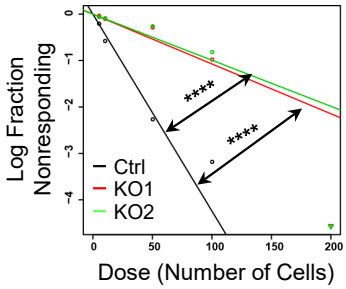

**Figure S4. USP36 regulates ALKBH5 protein level by the deubiquitination process.** The endogenous ubiquitination of ALKBH5 was analyzed in USP36-knockout GSC23 cells after transfected with HA-Ubi. Cell lysates were immunoprecipitated with anti-HA antibody and then analyzed by immunoblotting using the indicated antibodies.

Supplemental Figure 5

A

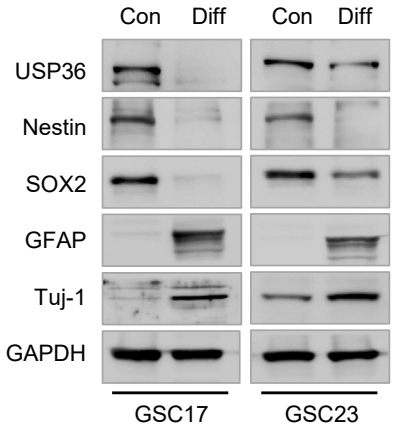



B

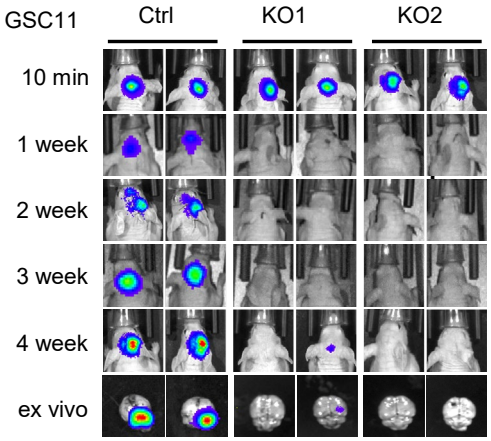



| Stem Cell Frequency | GSC23 | | |
|---|---|---|---|
| | Ctrl | KO1 | KO2 |
| Lower | 29.8 | 115.2 | 124.8 |
| Estimate | 23.6 | 93.2 | 100.8 |
| Upper | 18.7 | 75.5 | 81.4 |

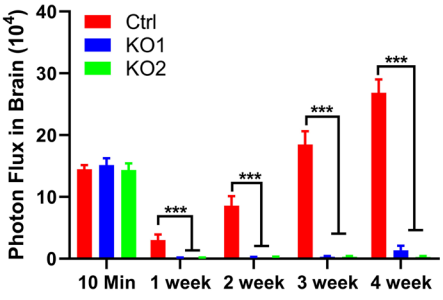
C



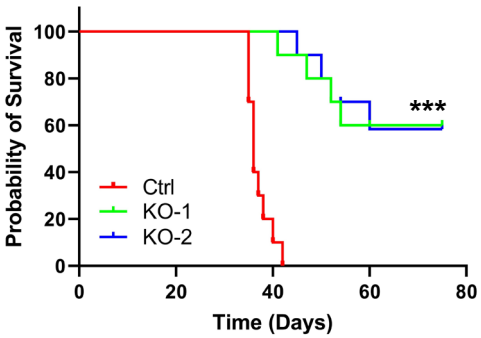

D



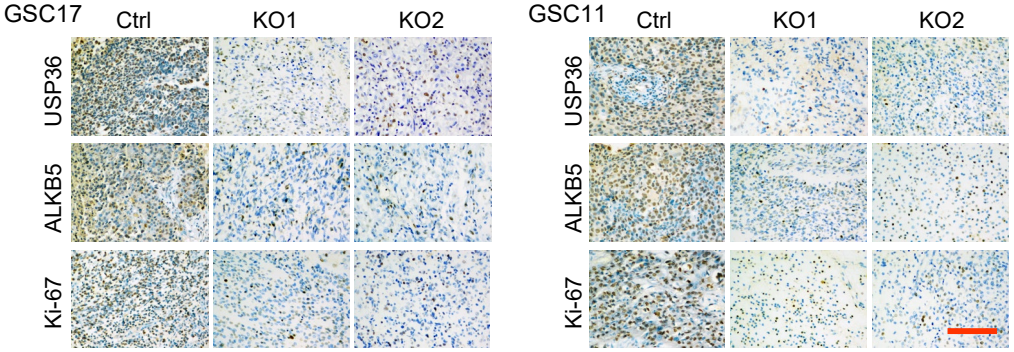

E



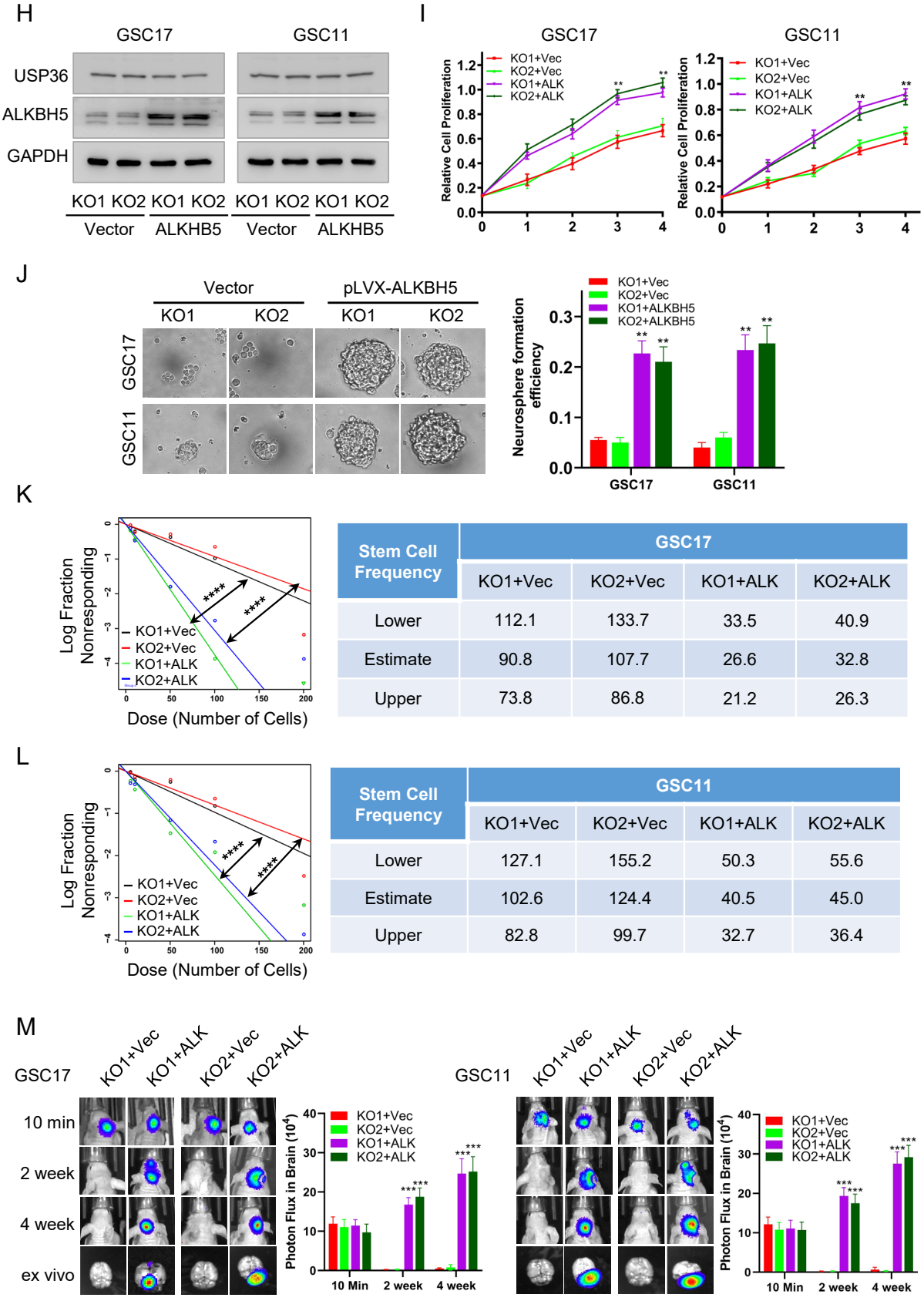

F




G



Supplemental Figure 5




| Stem Cell Frequency | GSC17 | | | |
|---|---|---|---|---|
| | KO1+Vec | KO2+Vec | KO1+ALK | KO2+ALK |
| Lower | 112.1 | 133.7 | 33.5 | 40.9 |
| Estimate | 90.8 | 107.7 | 26.6 | 32.8 |
| Upper | 73.8 | 86.8 | 21.2 | 26.3 |

| Stem Cell Frequency | GSC11 | | | |
|---|---|---|---|---|
| | KO1+Vec | KO2+Vec | KO1+ALK | KO2+ALK |
| Lower | 127.1 | 155.2 | 50.3 | 55.6 |
| Estimate | 102.6 | 124.4 | 40.5 | 45.0 |
| Upper | 82.8 | 99.7 | 32.7 | 36.4 |

**Figure S5. USP36 regulates glioma stem cell proliferation and self-renewal abilities. (A)** The cell proliferations of GSC23 cells after ablation of USP36 was assessed by the CCK8 assay. Data are mean ± SEM, for triplicate biological replicates with 3 technical replicates in each biological one. Student t test; **, $p<0.01$. **(B)** Extreme limiting dilution assay showed the frequencies of neurosphere formation in USP36 depleted GSC23 cells. The significance of the difference between the indicated groups was determined by Chi-square test. ****, $p<0.0001$ (n=3 independent experiments). **(C)** The protein expressions of USP36, SOX2, Nestin, Tuj-1 and GFAP in differentiated (Diff) GSC17 and GSC23 cells was analyzed by western blot analysis. GAPDH served as a loading control. **(D)** Representative BLI images of the GSC11 cell are shown after intracranial injection. (**E)** The value of bioluminescence was quantitated by measuring photon flux. Values are the mean ± SD, n=8, Two-sided Mann-Whitney test, ***$p<0.001$. (**F)** Kaplan-Meier survival analyses for mice injected with control or USP36 sgRNA GSC11 cells (mean ± SD, n = 10; Logrank test, ***, $p < 0.001$). **(G)** The protein levels of USP36, ALKBH5 and Ki-67 in tumor produced by control- or USP36 depleted- GSC17 and GSC11 cells were analyzed by IHC. **(H)** Immunoblotting analysis of ALKBH5 and USP36 protein expressions in USP36 depleted GSC17 and GSC11 cells after rescued ALKBH5 expressions. **(I)** The cell proliferations of USP36 deleted GSC17 and GSC11 cells after rescued ALKBH5 expression were assessed by the CCK8 assay. Student t test; **, $p<0.01$. **(J)** The neurosphere formation efficiency (spheres/cells plated) of USP36 knockout GSC17 and GSC11 cells were detected after re-expressed ALKBH5. The significance was determined by student t-test. Data are mean ± SEM for triplicate samples. **, $p <0.01$; ***, $p<0.001$. **(K and L)** Extreme limiting dilution assay showed the frequencies of neurosphere formation in USP36 depleted GSN17 and GSC11 cells after rescued ALKBH5 expressions. The significance of the difference between the indicated groups was determined by Chi-square test. ****, $p<0.0001$ (n=3 independent

experiments). **(M)** Representative BLI images of the GSC17 and GSC11 cell are shown after intracranial injection and the value of bioluminescence was quantitated by measuring photon flux. Values are the mean ± SD, n=8, Two-sided Mann-Whitney test, ***p<0.001.

Supplemental Figure 6

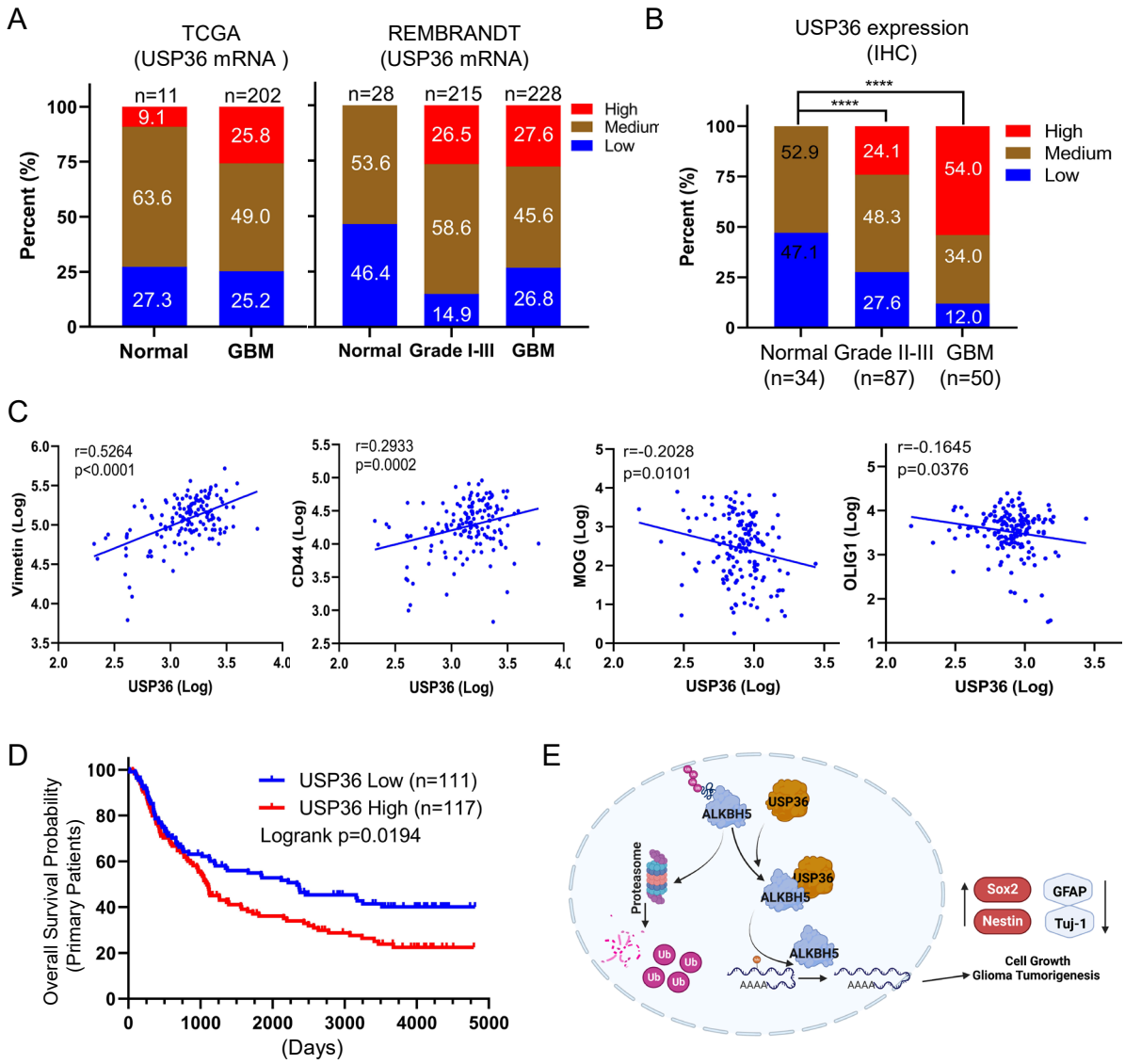



**Figure S6. High expression of USP36 correlated with poor clinical outcome. (A)** Profile of USP36 mRNA expression in normal, GBM, or grade II-III glioma patients in TCGA and REMBRANDT datasets. **(B)** Analysis of USP36 protein expression in 4 TMAs with 34 normal cases, 87 grade II-III astrocytoma cases, and 50 GBM cases, using immunohistochemical analysis. IHC staining score 0-2, low; 3-8, medium; 9-12, high (Chi-square test, ****$p < 0.0001$). **(C)** The correlations among them were statistically analyzed by Pearson correlation test. The data was retrieved from TCGA data (https://www.cbioportal.org/). Note that the scores of some samples overlap. **(D)** Kaplan-Meier survival

curves were used to assess the overall survival of primary glioma patients in the CGGA database. The median was the cutoff value used to classify USP36 mRNA expression as high or low. **(E)** A schematic sketch shows the USP36-ALKBH5 regulatory axis. USP36 interacts with and deubiquitinates ALKBH5, which leads to ALKBH5 stabilization and promotes cell proliferation, self-renewal and tumorigenesis of GSCs.

**Supplemental Table 1. Antibody were used in this study**

| Primary antibody | Company | Catalogue No. | Dilution | Application |
|---|---|---|---|---|
| ALKBH5 | Novus Biologicals | NBP1-82188 | 1:5000 | WB/IHC |
| ALKBH5 | Abnova | H00054890-B01P | 1:1000 | WB/IF |
| ALKBH5 | Sigma | HPA007196 | 1:1000 | WB/IHC |
| USP36 | Proteintech | 14783-1-AP | 1:1000 | WB/IF/IHC |
| USP36 | LifeSpan Bio | LS-C748177-200 | 1:1000 | WB |
| GAPDH | Santa Cruz Biotechnology | sc-365062 | 1:3000 | WB |
| Myc-Tag | Cell Signaling Technology | 2278 | 1:3000 | WB |
| HA-Tag | Cell Signaling Technology | 3724 | 1:3000 | WB |
| HA-Tag | Cell Signaling Technology | 12698 | 1:3000 | WB |
| Sox2 | Cell Signaling Technology | 3579 | 1:4000 | WB |
| GFAP | Cell Signaling Technology | 80788 | 1:2000 | WB |
| Tuj1 | Santa Cruz Biotechnology | sc-58886 | 1:1000 | WB |

| | | | | |
|---|---|---|---|---|
| Nestin | BD Transduction Laboratories | 611658 | 1:1000 | WB |
| Anti-mouse IgG, | Cell Signaling Technology | 7076 | 1:5000 | WB |
| Goat Anti-Rabbit IgG | abcam | ab6721 | 1:5000 | WB |
| Alexa Fluor 488 goat anti-mouse antibody | Molecular Probes | A32723 | 1:1000 | IF |
| Alexa Fluor 594 goat anti-rabbit antibody | Molecular Probes | A32740 | 1:1000 | IF |
| Ki67 Polyclonal antibody | Proteintech | 27309-1-AP | 1:500 | IHC |

**Supplemental Table 2. The primers used in this study**

| Primer | Sequence |
|---|---|
| USP36 | Forward: 5’-CAGATCACGCGTGAAGTGCTCC-3’ |
| | Reverse: 5’-TGGAGGAATGCAGCCGTTCTGG-3’ |
| ALKBH5 | 5’-ATCCTCAGGAAGACAAGATTAG-3’ |
| | 5’-TTCTCTTCCTTGTCCATCTC-3’ |
| GAPDH | Forward: 5’-TGCACCACCAACTGCTTAGC-3’ |
| | Reverse: 5’-GGCATGGACTGTGGTCATGAG-3’ |
| USP36C131A | 5’-ATGGTGGCATTGAGAAAGGCGGTGTTGCCAAGGTTGTG-3’ |
| | 5’-CACAACCTTGGCAACACCGCCTTTCTCAATGCCACCAT-3’ |
| Control gRNA1 | 5’-CGCGATAGCGCGAATATATT-3’ |
| USP36 gRNA1 | 5’-CGCTTTGCCAACTTCAGCG-3’ |
| USP36 gRNA2 | 5’-GAATCACACTCGGGCGGCC-3’ |
| USP36 Fragments 1-420 | 5’-ATGCCAATAGTGGATAAGTTGA-3’ |
| | 5’-ATAGAACAGCACGTAGGCCTGC-3’ |
| USP36 Fragments 421-800 | 5’-CTGCGAATTCCAGGCTCTAAG-3’ |
| | 5’- CTCTGGCAACTGGTGTGGAAG-3’ |
| USP36 Fragments 801-1121 | 5’-GCCAGTGAGCCCCCCCAGAGC-3’ |
| | 5’-GCGGCGATAGCTGAGGCTGGC-3’ |