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Original Article

CDC6 overexpression contributes to the malignant phenotype of glioma via IL6/JAK2/STAT3 signaling

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Abstract: Glioma, a prevalent primary tumor of the central nervous system, is targeted by molecular therapies aiming to intervene in specific genes and signaling pathways to inhibit tumor growth and spread. Our previous bioinformatics study revealed that significant CDC6 overexpression in gliomas was closely correlated with poor patient prognosis. Through qPCR, western blotting, and immunohistochemistry, we will further validate CDC6 expression in clinical glioma specimens, while the effects of silencing and overexpressing CDC6 in the U87 and LN229 glioma cell lines on malignancy will be assessed through MTS, EdU, transwell, and migration assays. Luciferase reporter assays, ChIP, qPCR, and western blotting were used to explore the upstream and downstream molecular mechanisms of CDC6. Our study confirmed the abnormal overexpression of CDC6 in gliomas, particularly in glioblastomas. CDC6 promotes glioma cell activity, proliferation, invasion, and migration by activating the IL6-mediated JAK2/STAT3 signaling pathway. The transcription Factor E2F8 directly regulates CDC6 transcription, playing a crucial role in its abnormal overexpression in gliomas. This research provides vital evidence supporting CDC6 as a molecular target for glioma therapy.

Keywords: Glioma, CDC6, IL6, E2F8

Introduction

Glioma is one of the most common primary brain tumors in neurosurgical clinical practice [1]. The main tumor types include astrocytoma, oligodendroglioma, and ependymoma, classified histologically as low-grade or high-grade tumors. Glioblastoma multiforme (GBM), a grade IV glioma, is the most common primary malignant brain tumor, with an incidence of 3-8 cases per 100,000 person-years and an average age at diagnosis of 64 years [2]. Primary GBM accounts for approximately 80% of all primary malignant tumors in the central nervous system (CNS). Despite standard treatments for newly diagnosed GBM patients, including maximal safe resection and radiation therapy combined with temozolomide (TMZ) followed by maintenance chemotherapy, the median survival period is only 12-18 months [3]. In recent years, with a deeper understanding of glioma mechanisms and treatment research, molecular diagnostic methods have emerged as crucial tools. Molecular diagnostics not only clarify tumor subtypes and molecular characteristics but also guide personalized treatment plans to enhance treatment efficacy [4]. Precision molecular therapies have the potential to reduce unnecessary side effects and improve the quality of life of patients. Therefore, the identification of genes related to glioma development is highly important for therapeutic research.

Cell division cycle protein 6 (CDC6) is a member of the cell division cycle protein family and plays a crucial role in regulating the cell cycle [5]. The human CDC6 gene is located on chromosome 17q21.3 and primarily encodes an AAA+ ATPase. The main function of CDC6 is to regulate DNA replication during the G1/S transition of the cell cycle [6]. The expression of CDC6 exhibits periodic changes throughout the cell cycle, with elevated expression typically occurring during the G1/S transition. This regulation ensures that CDC6 activity is increased

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when cells are preparing for DNA replication [7]. During the G1/S transition, CDC6 forms a complex with CDC18 and interacts with Cyclin-CDK complexes, collectively activating Cyclin-dependent kinases (CDKs) and initiating the commencement of DNA replication [8].

The aberrant expression of CDC6 is closely associated with the occurrence and development of various tumors. For example, the expression levels of CDC6 are typically elevated in breast cancer tissues. The overexpression of CDC6 is linked to abnormal proliferation and an uncontrolled cell cycle in breast cancer cells, potentially inducing excessive DNA replication and proliferation [9]. In ovarian cancer, CDC6 expression is significantly upregulated, potentially leading to excessive DNA replication and enhanced proliferative capacity, thereby promoting the development of ovarian cancer [10]. Aberrant expression of CDC6 is also associated with the onset and progression of colorectal cancer. The overexpression of CDC6 may disrupt the cell cycle, promoting the uncontrolled proliferation of tumor cells [11]. Consequently, CDC6 has emerged as a crucial focus in cancer biology research and potential therapeutic strategies. Inhibiting the function of CDC6 may aid in intervening in the cell cycle of tumor cells, providing novel targets for cancer treatment.

Although our previous bioinformatics research revealed that the upregulation of CDC6 is a poor prognostic signature in glioblastoma multiforme and other studies have reported that CDC6 is a prognostic biomarker correlated with immune infiltrates in glioma, the true expression of CDC6 in gliomas has not been validated in clinical specimens [12, 13]. Moreover, the specific biological roles and regulatory mechanisms of CDC6 in the malignant progression of gliomas remain unexplored. Therefore, on the basis of preliminary research findings, this study confirms the distinct overexpression of CDC6 in glioma tissues. Furthermore, CDC6 promotes the proliferation, invasion, and migration of glioma cells by activating the JAK2/STAT3 signaling pathway, which is mediated by IL6. Additionally, E2F8 transcriptionally regulates CDC6, leading to sustained high expression in glioma cells. Consequently, CDC6 plays a crucial role in the malignant progression of gliomas and holds promise as a significant molecular target for glioma therapy.

Methods

Patient samples and ethical approval

We collected data from 43 glioma patients who were treated at Zibo Central Hospital from January 2018 to December 2023, including 12 patients classified as WHO Grade II, 14 patients classified as Grade III, and 17 patients classified as Grade IV. Concurrently, we assembled a control group consisting of normal brain tissues from 10 patients with brain trauma. This study obtained approval from the Ethics Committee of Zibo Central Hospital (No. 2022H025), and all patients provided signed informed consent agreements.

Cell culture

The glioma cell lines U87, U118 and LN229 were obtained from the Beijing BENA Cell Bank and cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Gibco) at 37°C with 5% CO_2. The IL-6 neutralizing antibody (R&D Systems, Minneapolis, MN, USA) was used at a concentration of 0.5 mg/ml. All analyzed cells were cultured for no more than 20 generations.

RNA extraction and qPCR

The total RNA of glioma tissues and cell lines was extracted using the Mini-BEST Universal RNA Extraction Kit (TaKaRa, Kyoto, Japan) following the manufacturer's instructions. The first-strand cDNA was subsequently synthesized using Prime-Script RT Master Mix (TaKaRa). qPCR detection was carried out using SYBR Green Master Mix (TaKaRa) on a PCR LightCycler 480 (Roche Diagnostics Ltd., Basel, Switzerland). Each sample was run four times, and β-actin was used as an internal control. The qPCR sequences of primers used were as follows: CDC6, forward 5'-CCAGGCACAGGCTACAATCAG-3', reverse 5'-AACAGGTTACGGTTTGGACATT-3'; IL6, forward 5'-CCTGAACCTTCCAAAGATGGC-3', reverse 5'-CCATTTCACCAGGCAAGTCTCCTCA-3'; and β-actin, forward 5'-CATGTAC GTTGCTATCCAGGC-3', reverse 5'-CTCCTTAAT GTCAC GCACGAT-3'.

Western blotting

Total protein was extracted from glioma tissues and cell lines via a Cell Protein Extraction Kit (Beyotime Biotechnology, Beijing, China) following the manufacturer's instructions. A BCA kit (Beyotime Biotechnology) was used to determine protein concentrations. Equal amounts of protein from each sample were subjected to 4-20% SDS-PAGE (GenScript, Nanjing, Jiangsu, China), transferred to a nitrocellulose membrane and blocked with 2% bovine serum albumin (KeyGen Biotechnology). The membrane was then incubated with primary antibodies against CDC6, IL6, p-JAK2, JAK2, p-STAT3, STAT3, and β-actin (Abcam Technology, Cambridge, UK) overnight at 4°C, followed by TBST washing and secondary antibody incubation (Abcam Technology). An enhanced chemiluminescence (ECL) kit (Beyotime) was used to detect bands on each membrane, and quantification was performed using IMAGE J software (National Institutes of Health, Bethesda, MD, USA).

Immunohistochemistry (IHC)

Immunohistochemistry was conducted using an immunohistochemistry kit (Maixin Biotechnology, Fuzhou, Fujian, PR China) following the manufacturer's instructions. Briefly, tissue samples were paraffin-embedded, cut into 4-μm sections, and incubated with primary antibodies against CDC6, IL6, and Ki-67 (Abcam) at 4°C overnight. After DAB staining, the sections were imaged with an optical microscope (Olympus) and the staining intensity was evaluated according to the German immunohistochemical scoring system [14].

Lentiviral vector construction and transfection

Using lentivirus-based vector transfection, CDC6 and E2F8 were overexpressed, and CDC6 was silenced using RNAi, with technical support from Gene-Chem (Shanghai, China). Two siRNA sequences were designed for CDC6 knockdown: forward 5'-GCCAAACUAGAACCAACAATT-3', reverse 5'-UUGUUGGUUCUAGUUUGGCTT-3', and forward 5'-CCUCAAGAAGGAACUGAAATT-3', reverse 5'-UUUCAGUUCCUUCUUGAGGTT-3'. After transfection, all the cells were treated with puromycin (Sigma, Santa Clara, CA, USA) at a concentration of 10 mg/ml for 30 days. The effectiveness of CDC6 and E2F8 overexpression or CDC6 knockdown was verified through qPCR and western blotting.

Cell viability assay

The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI, USA) was used to assess cell viability according to the manufacturer's instructions. Briefly, glioma cells were cultured in 96-well plates at a density of 3×10^3 cells/well for 24, 48, 72, 96, or 120 hours. Subsequently, 20 ml of MTS was added to each well, followed by a 1-hour incubation at 37°C. The absorbance at 495 nm was detected using a UV spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

EdU assay

The 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay (Beyotime) was performed according to the manufacturer's instructions. Glioma cells were seeded into 24-well plates at a density of 1×10^5 cells per well and cultured for 20 hours, followed by a 2-hour treatment with 50 mM EdU at 37°C. The cells were subsequently fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, incubated with 100 ml of Click Additive Solution for 30 minutes, and stained with 100 ml of DAPI. Images were captured using a laser scanning confocal microscope (Olympus), and the percentage of EdU-positive cells was calculated.

Cell migration and Transwell assays

For the cellular migration assay, glioma cells subjected to various treatments were suspended in serum-free medium (HyClone) at a concentration of 2×10^5 cells/ml. Subsequently, 600 μl of DMEM containing 20% FBS was added to the lower chamber while 100 μl of the cellular suspension was placed in the upper chamber (Costar, Corning, NY, USA). After 24 hours of incubation at 37°C, the glioma cells were fixed with 4% paraformaldehyde (Solarbio) for 10 minutes at room temperature and stained with 1% crystal violet solution (Solarbio) for 20 minutes. Finally, cell quantification was performed by determining the mean of 5 randomly selected areas using an inverted microscope (Olympus). For Transwell assays,

an 8 μm pore-size polycarbonate membrane was coated with 100 μl of 50 ng/μl Matrigel solution (BD, Franklin Lakes, NJ, USA). The subsequent procedures were identical to those of the cellular migration assay.

Luciferase activity analysis

The luciferase reporter assay was conducted using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. In brief, the CDC6 reporter plasmids were constructed by GeneChem (Shanghai, PR China). Glioma cells were seeded into 96-well plates at a density of 3×10^3 cells per well and transfected with different plasmids. After 48 hours, the cells were lysed, and luciferase activity was measured using a UV spectrophotometer (Thermo Fisher Scientific). Each experiment was independently repeated three times.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were conducted using the EZ-ChIP™ Immunoprecipitation Kit (Millipore, Billerica, MA, USA) following the manufacturer's instructions. Chromatin complexes were immunoprecipitated with an anti-E2F8 antibody (Abcam). A ChIP Assay Kit (Beyotime) was subsequently used to purify the DNA samples, and the purified DNA was subjected to qPCR analysis. The primer pairs utilized for amplifying the E2F8 binding site in the CDC6 promoter were as follows: forward 5'-TGCAACACTCCCCGGTTATC-3' and reverse 5'-CTTCCACGTATGTGAGCGAGG-3'.

Xenograft experiments

Six-week-old female BALB/c nude mice (Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China) were subjected to orthotopic injection of glioma cells during the exponential growth phase. Each mouse received an injection of 1×10^4 glioma cells. The mice were subsequently monitored daily for neurological symptoms or signs of mortality. The tumor volume was calculated using the following formula: V = (D × d^2)/2, where D represents the longest tumor diameter and d represents the shortest tumor diameter. Survival rates were assessed using the log-rank test and Kaplan-Meier analysis. All animal procedures adhered to the guidelines of the Animal Care Committee of Zibo Central Hospital.

Bioinformatics analysis

The mRNA expression data and clinical information of patients were obtained from the Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov) and the Chinese Glioma Genome Atlas (CGGA, http://www.cgga.org.cn). Gene set enrichment analysis (GSEA, http://www.broadinstitute.org/gsea/index.jsp) was conducted to identify signaling pathway gene sets that exhibited statistically significant differences between groups with higher and lower CDC6 expression. The Jaspar database was used to analyze whether the promoter region of CDC6 contains binding sites for E2F8.

Statistical analysis

Statistical analysis and visualization were performed using GraphPad Prism 9.0 (GraphPad Software, Inc., San Diego, CA, USA). Each experiment was independently replicated at least three times, and the findings are expressed as the mean ± standard error. Group comparisons were conducted using the chi-square test, F test, or t test, as applicable. Statistical significance was determined at a P value < 0.05.

Results

CDC6 is highly expressed in gliomas

Although our previous bioinformatics research revealed significant overexpression of CDC6 in gliomas, the expression of CDC6 has not been detected or validated in clinical specimens. We collected 43 brain glioma patients who visited our hospital in the past 5 years (Grade II: 12 cases; Grade III: 14 cases; Grade IV: 17 cases). Ten normal brain tissue samples were used as a control group. Detection was performed via qPCR (**Figure 1A**), western blotting (**Figure 1B**), and IHC (**Figure 1C**, **1D**). The results indicated that the expression of CDC6 in glioma tissues was significantly greater than that in normal brain tissues. Furthermore, with increasing glioma grade, the expression of CDC6 increased, peaking at Grade IV. These results fully confirm the pronounced overexpression of CDC6 in gliomas.

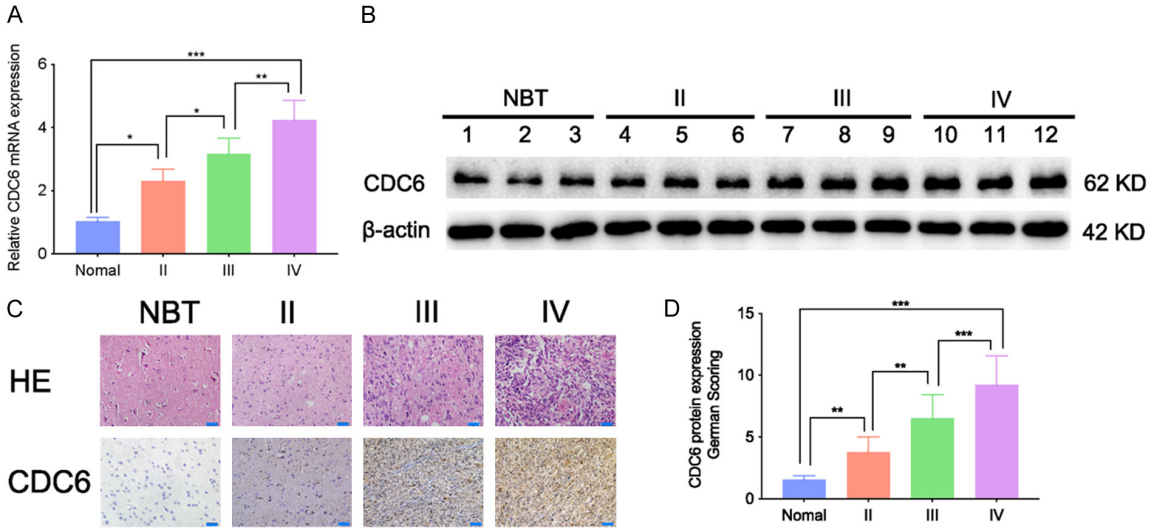

Figure 1. CDC6 is highly expressed in gliomas. A: qPCR assays was performed to assess the expression levels of CDC6 in 10 cases of normal brain tissue and 25 cases of glioma tissues with different grades (II: 8 cases, III: 7 cases, IV: 10 cases). B: Representative western blotting images depicted the differential expression of CDC6 in normal brain tissue and glioma tissues of varying grades. C: Representative images illustrated the immunohistochemical detection of CDC6, demonstrating expression differences in normal brain tissue and glioma tissues of different grades. D: Quantitative analysis of immunohistochemistry was conducted using the German immunohistochemical scoring system to assess the staining intensity of CDC6. All data are presented as the mean ± SD from three independent experiments. Statistical significance was determined as *P < 0.05, **P < 0.01, ***P < 0.001.

CDC6 knockdown inhibited the malignant phenotype of gliomas in vitro

To further investigate whether the expression of CDC6 is involved in regulating malignant phenotypic effects such as the viability, proliferation, and invasion of glioma cells, we silenced and overexpressed CDC6 in the glioma cell lines U87 and U118. First, we utilized qPCR (**Figure 2A**) and western blotting (**Figure 2B**) to accurately and reliably detect the effects of CDC6 silencing and overexpression in U87 and U118 cells. MTS assays revealed a significant decrease in absorbance values after CDC6 silencing, indicating decreased viability of glioma cells in both U87 and U118 cells. Conversely, after CDC6 overexpression the absorbance values significantly increased, increasing the viability of glioma cells (**Figure 2C**, **2D**). EdU assays revealed a marked reduction in the percentage of EdU-positive U87 and U118 cells after CDC6 silencing, while CDC6 overexpression led to a significant increase in the percentage of EdU-positive U87 and U118 cells, confirming that CDC6 promotes the proliferation of glioma cells (**Figure 2E**). Furthermore, transwell and migration assays showed a substantial decrease in the number of invading and migrating U87 and U118 cells after CDC6 silencing. In contrast, CDC6 overexpression resulted in significant increases in invasion and migration, suggesting that CDC6 promotes the invasion and migration of glioma cells (**Figure 2F**, **2G**). These results conclusively demonstrate that silencing CDC6 in glioma cells significantly inhibits their malignant progression.

CDC6 knockdown inhibited IL6/JAK2/STAT3 signaling

To further explore the potential downstream molecular mechanisms of CDC6, we conducted gene set enrichment analysis (GSEA) on the expression of CDC6 in both the TCGA and CGGA databases. The analysis revealed significant enrichment of the IL6/JAK2/STAT3 signaling pathway in patients with high CDC6 expression (**Figure 3A**). Additionally, we examined the correlation between CDC6 and IL6 expression and found a clear positive correlation (**Figure 3B**, **3C**). Using qPCR (**Figure 3D**) and ELISA (**Figure 3E**), we observed a significant decrease in both the expression and secretion levels of IL6 after silencing CDC6, whereas the overexpression of CDC6 led to a

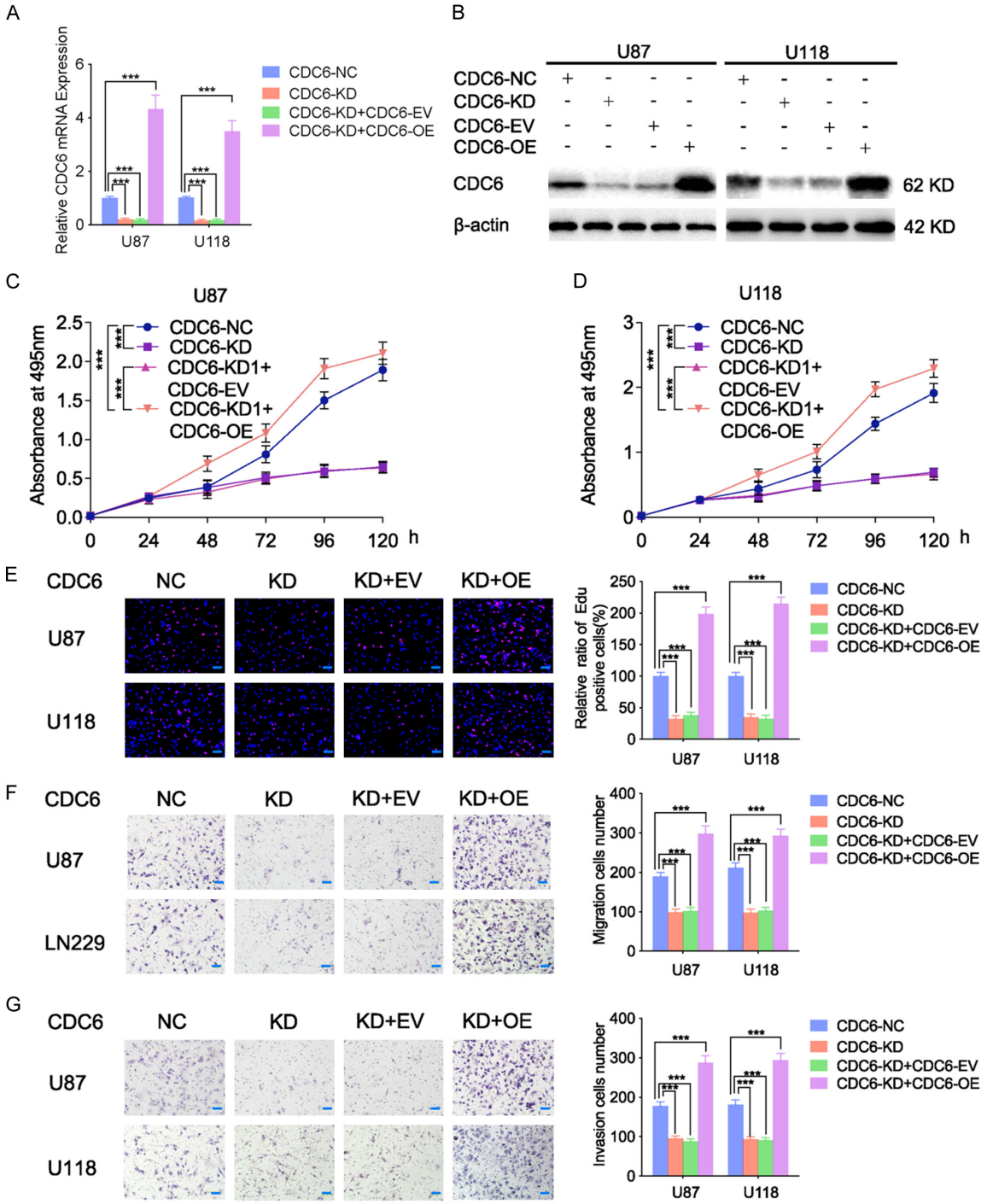

Figure 2. CDC6 knockdown inhibited the malignant pheonotype of gliomas in vitro. A, B: qPCR and western blotting were employed to assess the efficiency of CDC6 silencing or overexpression in glioma cell lines U87 and U118. C, D: MTS assays were conducted to evaluate the cell viability of glioma cell lines U87 and U118 following CDC6 silencing or overexpression. E: EdU assays were performed to examine the cell proliferation activity of glioma cell lines U87 and U118 after CDC6 silencing or overexpression. F: Migration experiments were carried out to measure the number of migrated cells in glioma cell lines U87 and U118 following CDC6 silencing or overexpression. G: Transwell assays were employed to determine the number of invasive cells in glioma cell lines U87 and U118 after CDC6 silencing or overexpression. All data are presented as the mean ± SD from three independent experiments. Statistical significance was determined as *P < 0.05, **P < 0.01, ***P < 0.001.

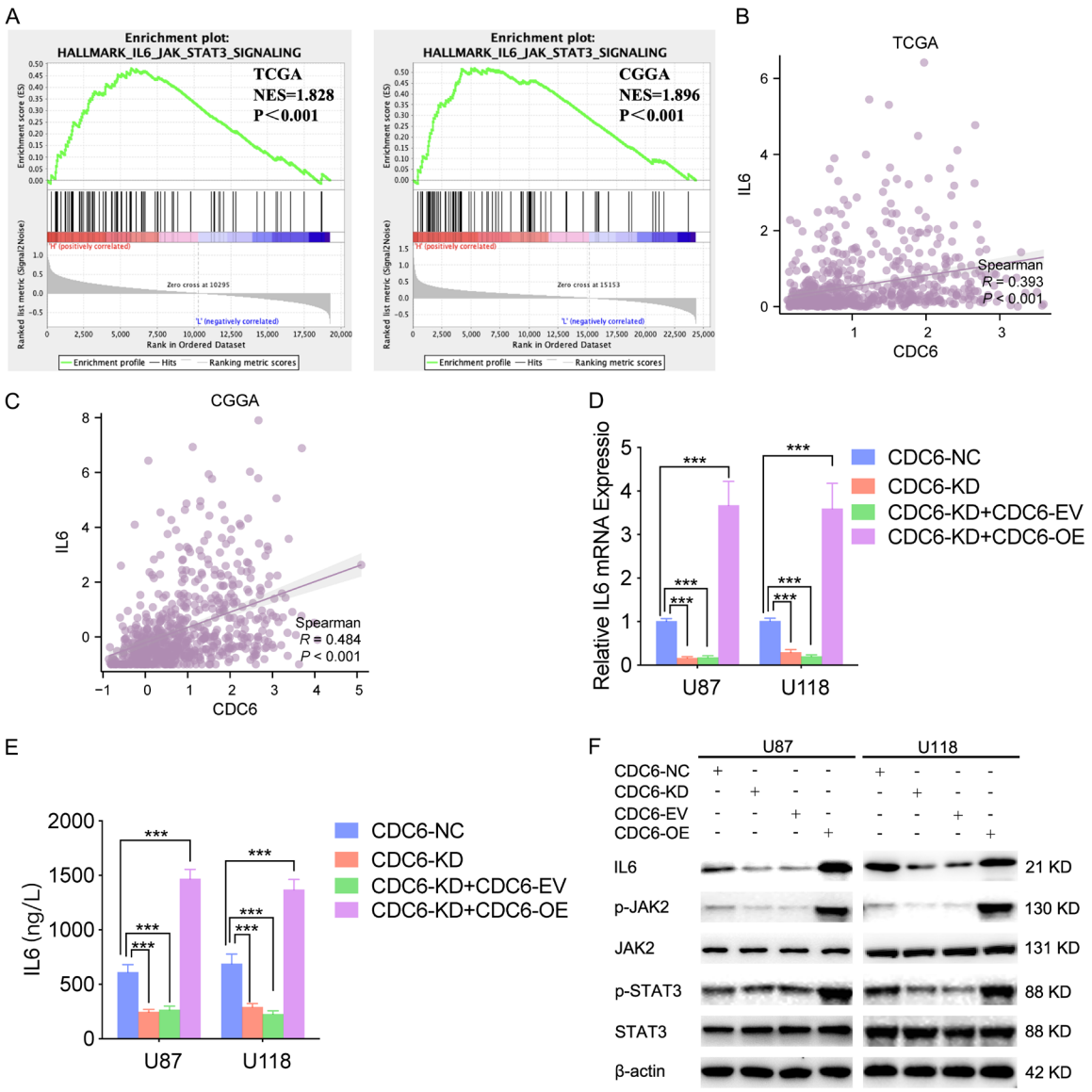

Figure 3. CDC6 knockdown inhibited the IL6/JAK2/STAT3 signaling. A: GSEA analysis revealed enrichment of the IL6/JAK2/STAT3 signaling pathway in TCGA and CGGA datasets with high CDC6 expression. B, C: Correlation analysis demonstrated the correlation between the expression levels of CDC6 and IL6 in TCGA and CGGA datasets. D, E: qPCR and ELISA were employed to detect changes in IL6 mRNA levels and secretion levels in U87 and U118 cells after CDC6 silencing or overexpression. F: Western blotting was performed to assess changes in the levels of IL6 and its downstream molecules, p-JAK2, and p-STAT3, in U87 and U118 cells after CDC6 silencing or overexpression. All data are presented as the mean ± SD from three independent experiments. Statistical significance was determined as *P < 0.05, **P < 0.01, ***P < 0.001.

marked increase in the IL6 level. Western blotting further confirmed that CDC6 silencing resulted in significant decreases in the IL6, p-JAK2, and p-STAT3D expression levels. Conversely, CDC6 overexpression significantly increased the levels of these molecules (**Figure 3F**). These findings confirm that CDC6 promotes the expression and secretion of IL6, thereby activating the IL6-mediated JAK2/STAT3 signaling pathway.

CDC6 promoted the malignant phenotype of gliomas via IL6/JAK2/STAT3 signaling

To confirm whether CDC6 promotes the malignant progression of glioma through the activation of the IL6/JAK2/STAT3 signaling pathway, we treated CDC6-overexpressing glioma cells with neutralizing anti-IL6 antibodies. MTS assays revealed a significant increase in absorbance values in U87 and LN229 cells after

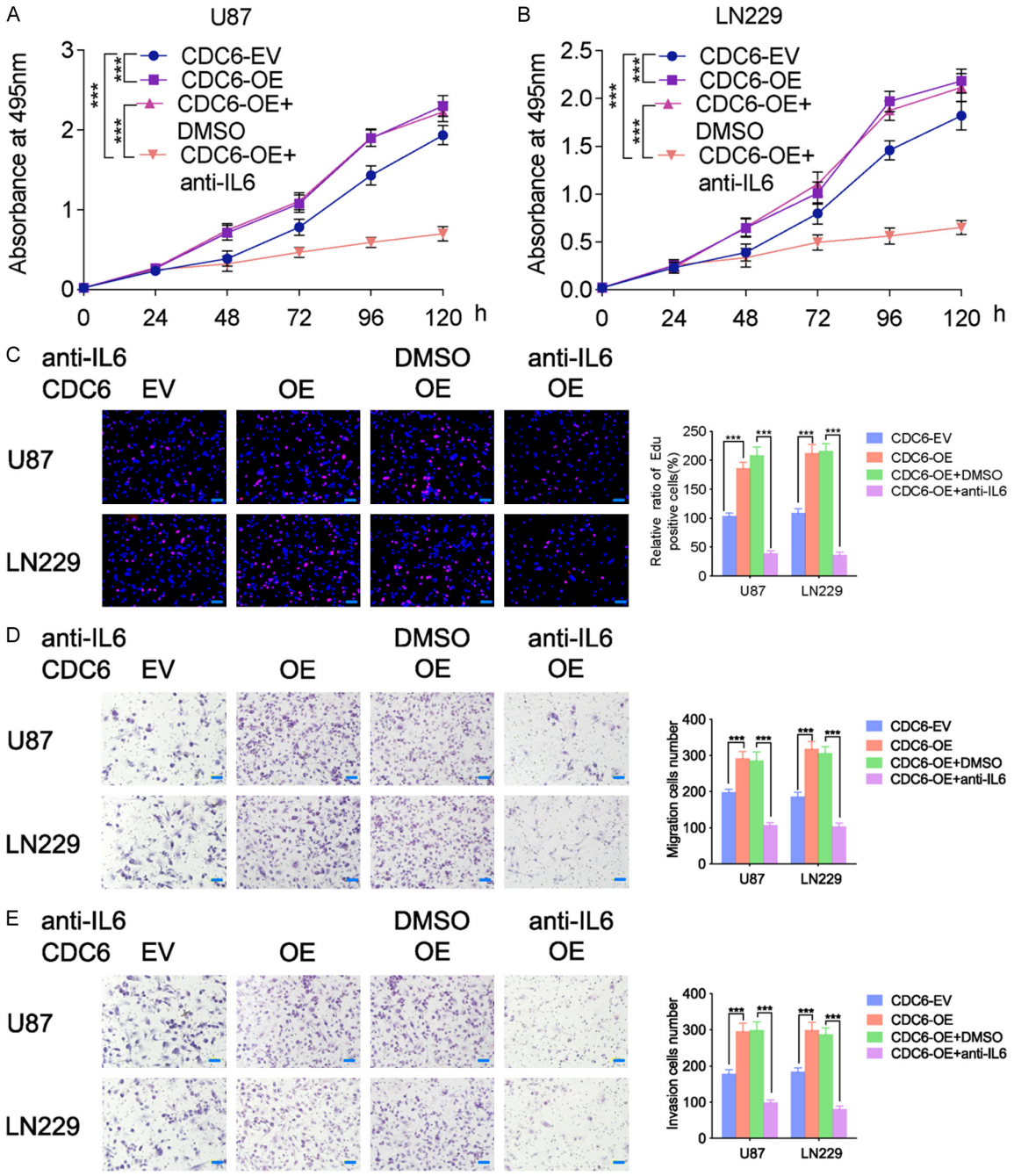

Figure 4. CDC6 promoted the malignant pheonotype of gliomas via IL6/JAK2/STAT3 signaling. A, B: MTS assays were conducted to assess the cell viability of glioma cell lines U87 and LN229 overexpressing CDC6 after treatment with anti-IL6. C: EdU assays were performed to examine the cell proliferation activity of glioma cell lines U87 and LN229 overexpressing CDC6 after treatment with anti-IL6. D: Migration assays were carried out to measure the number of migrated cells in glioma cell lines U87 and LN229 overexpressing CDC6 after treatment with anti-IL6. E: Transwell assays were employed to determine the number of invasive cells in glioma cell lines U87 and LN229 overexpressing CDC6 after treatment with anti-IL6. All data are presented as the mean ± SD from three independent experiments. Statistical significance was determined as *P < 0.05, **P < 0.01, ***P < 0.001.

CDC6 overexpression, while the absorbance values significantly decreased after treatment with anti-IL6 antibodies (**Figure 4A**, **4B**). EdU assays showed a significant increase in the percentage of EdU-positive U87 and LN229 cells after CDC6 overexpression, whereas the per-

centage of EdU-positive U87 and LN229 cells significantly decreased after anti-IL6 treatment (**Figure 4C**). Transwell and migration assays demonstrated a significant increase in the number of invading and migrating U87 and LN229 cells after CDC6 overexpression, while these numbers significantly decreased after treatment with anti-IL6 antibodies (**Figure 4D**, **4E**). These results provide full evidence that CDC6 promotes the activity, proliferation, invasion, and migration of glioma cells through the activation of the IL6/JAK2/STAT3 signaling pathway.

E2F8 can transcriptionally upregulate CDC6 expression

We further analyzed the potential upstream mechanisms leading to the overexpression of CDC6 in gliomas. Considering the crucial role of transcription factors in gene expression regulation, we initially examined the correlations between all the transcription factors and CDC6 expression in the TCGA and CGGA cohorts, as shown in Supplementary Tables 1 and 2. Notably, E2F8 presented the highest correlation with CDC6. GSEA revealed significant enrichment of E2F_Targets in patients with high CDC6 expression (**Figure 5A**). **Figure 5B** and **5C** illustrate the correlation between E2F8 and CDC6 expression in the TCGA and CGGA cohorts, with correlation coefficients exceeding 0.8, indicating a highly significant correlation. Next, we separately overexpressed or silenced E2F8 in U87 and LN229 cells, and through qPCR (**Figure 5D**, **5E**) and western blotting (**Figure 5F**, **5G**), we observed significant upregulation of CDC6 expression after E2F8 overexpression and significant downregulation of CDC6 expression after E2F8 silencing. **Figure 5H** presents the motif pattern of E2F8 as a transcription factor, on the basis of which luciferase reporter assays were designed (**Figure 5I**). The results show that E2F8 overexpression significantly increased luciferase activity in the CDC6 wild-type group (**Figure 5J**, **5K**), while silencing CDC6 resulted in a significant decrease in luciferase activity in the CDC6 wild-type group (**Figure 5L**, **5M**), with no significant change in the CDC6 mutant-type group. ChIP assays further revealed that E2F8 overexpression led to a significant increase in DNA enrichment in the CDC6 promoter region (**Figure 5N**), whereas E2F8 silencing resulted in a significant decrease in DNA enrichment in the CDC6 promoter region (**Figure 5O**). Taken together, these results provide ample evidence that E2F8 is a potential transcription factor that regulates CDC6 and may transcriptionally upregulate E2F8 expression in gliomas.

E2F8 promoted the malignant phenotype of gliomas in vitro via CDC6

Based on these results, we further investigated whether E2F8 could promote the malignant progression of gliomas by transcriptionally upregulating CDC6. We silenced CDC6 in E2F8-overexpressing glioma cells. MTS assays revealed a significant increase in the absorbance values of U87 and LN229 cells after E2F8 overexpression, while absorbance values significantly decreased upon CDC6 silencing (**Figure 6A**, **6B**). EdU assays demonstrated a significant increase in the percentage of EdU-positive U87 and LN229 cells after E2F8 overexpression, whereas the percentage of EdU-positive cells significantly decreased following CDC6 silencing (**Figure 6C**). Transwell and migration assays detected a significant increase in the invasive and migratory capacities of U87 and LN229 cells after E2F8 overexpression, while these capacities significantly decreased upon CDC6 silencing (**Figure 6D**, **6E**). These results provide compelling evidence that E2F8 can promote the proliferation, invasion, and migration of glioma cells by transcriptionally upregulating CDC6.

CDC6 promoted glioma tumorigenesis in vivo

While in vitro experiments have provided ample evidence that CDC6 promotes glioma cell activity, proliferation, invasion, and migration through the activation of the IL6-mediated JAK2/STAT3 signaling pathway, there is a lack of support from in vivo experimental results. To address this, we established an intracranial tumor formation model in nude mice for observation. We found that mice implanted with U87 cells overexpressing CDC6 had significantly larger tumor volumes in the mouse brain than did the control group (**Figure 7A**, **7B**), and the survival time of these mice was shorter than that of the control group (**Figure 7C**). However, we also implanted U118 cells with CDC6 knockdown in nude mice and observed the opposite results (Supplementary Figure 1A-C). Further immunohistochemical

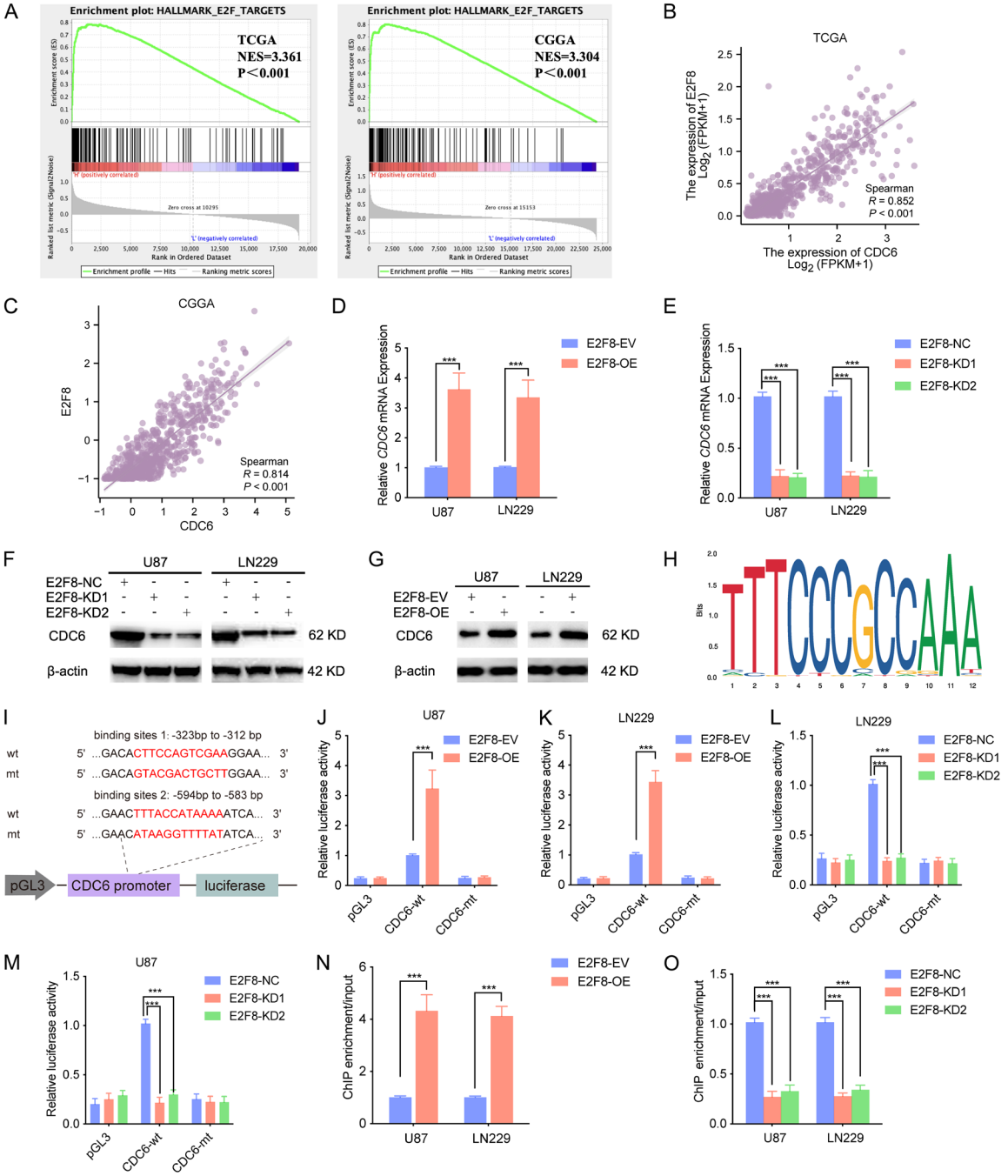

Figure 5. E2F8 can transcriptionally upregulate CDC6 expression. A: GSEA analysis revealed enrichment of the E2F_Targets signaling pathway in TCGA and CGGA datasets with high CDC6 expression. B, C: Correlation analysis demonstrated the correlation between the expression levels of CDC6 and E2F8 in TCGA and CGGA datasets. D, E: qPCR was performed to detect changes in CDC6 expression levels in U87 and LN229 cells after overexpressing or silencing E2F8. F, G: Western blotting was conducted to assess changes in CDC6 expression levels in U87 and LN229 cells after overexpressing or silencing E2F8. H: The Jaspar database predicted E2F8 as a transcription factor binding motif in the promoter region. I: A schematic diagram illustrating the predicted binding sites and mutation sites of E2F8 in the CDC6 promoter region for the luciferase reporter gene experiment. J-M: Luciferase reporter assays analyzed the changes in CDC6 luciferase activity levels in U87 and LN229 cells after overexpressing or silencing E2F8. N, O: ChIP assays analyzed the changes in the enrichment level of the CDC6 promoter region in U87 and LN229 cells after overexpressing or silencing E2F8. All data are presented as the mean ± SD from three independent experiments. Statistical significance was determined as *P < 0.05, **P < 0.01, ***P < 0.001.

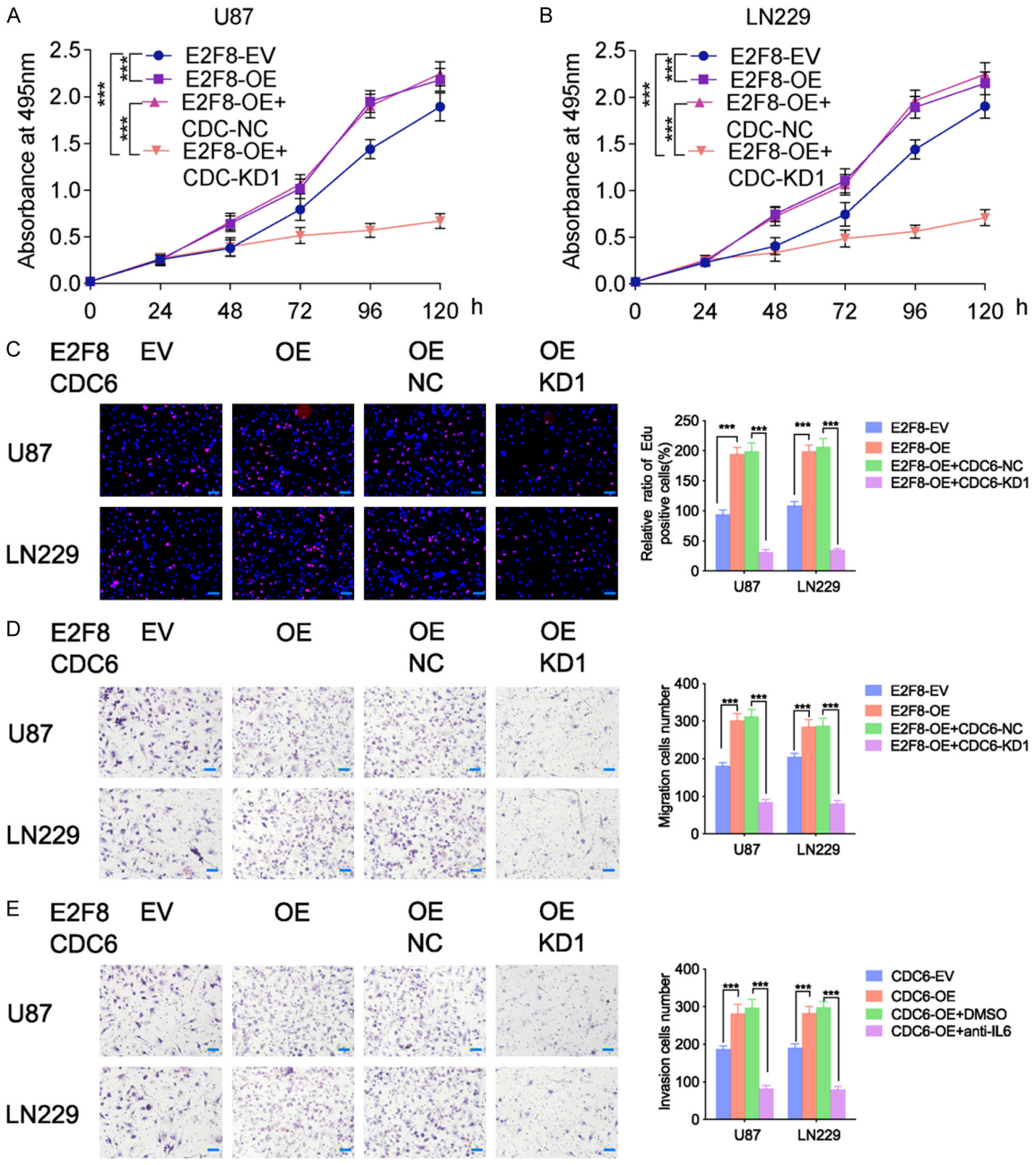

Figure 6. E2F8 promoted the malignant pheonotype of gliomas in vitro via CDC6. A, B: MTS assays were performed to assess the cell viability of glioma cell lines U87 and LN229 overexpressing E2F8 after CDC6 silencing. C: EdU assays were conducted to examine the cell proliferation activity of glioma cell lines U87 and LN229 overexpressing E2F8 after CDC6 silencing. D: Migration assays were carried out to measure the number of migrated cells in glioma cell lines U87 and LN229 overexpressing E2F8 after CDC6 silencing. E: Transwell assays were employed to determine the number of invasive cells in glioma cell lines U87 and LN229 overexpressing E2F8 after CDC6 silencing. All data are presented as the mean ± SD from three independent experiments. Statistical significance was determined as *P < 0.05, **P < 0.01, ***P < 0.001.

analysis of the formed tumor tissues revealed that the staining intensities of CDC6, Ki-67, and IL6 in the CDC6-overexpressing group were significantly greater than those in the control group (**Figure 7D-G**), while the staining intensities of these genes were significantly weaker in the CDC6-knockdown group than in the control group (Supplementary Figure 1D-G).

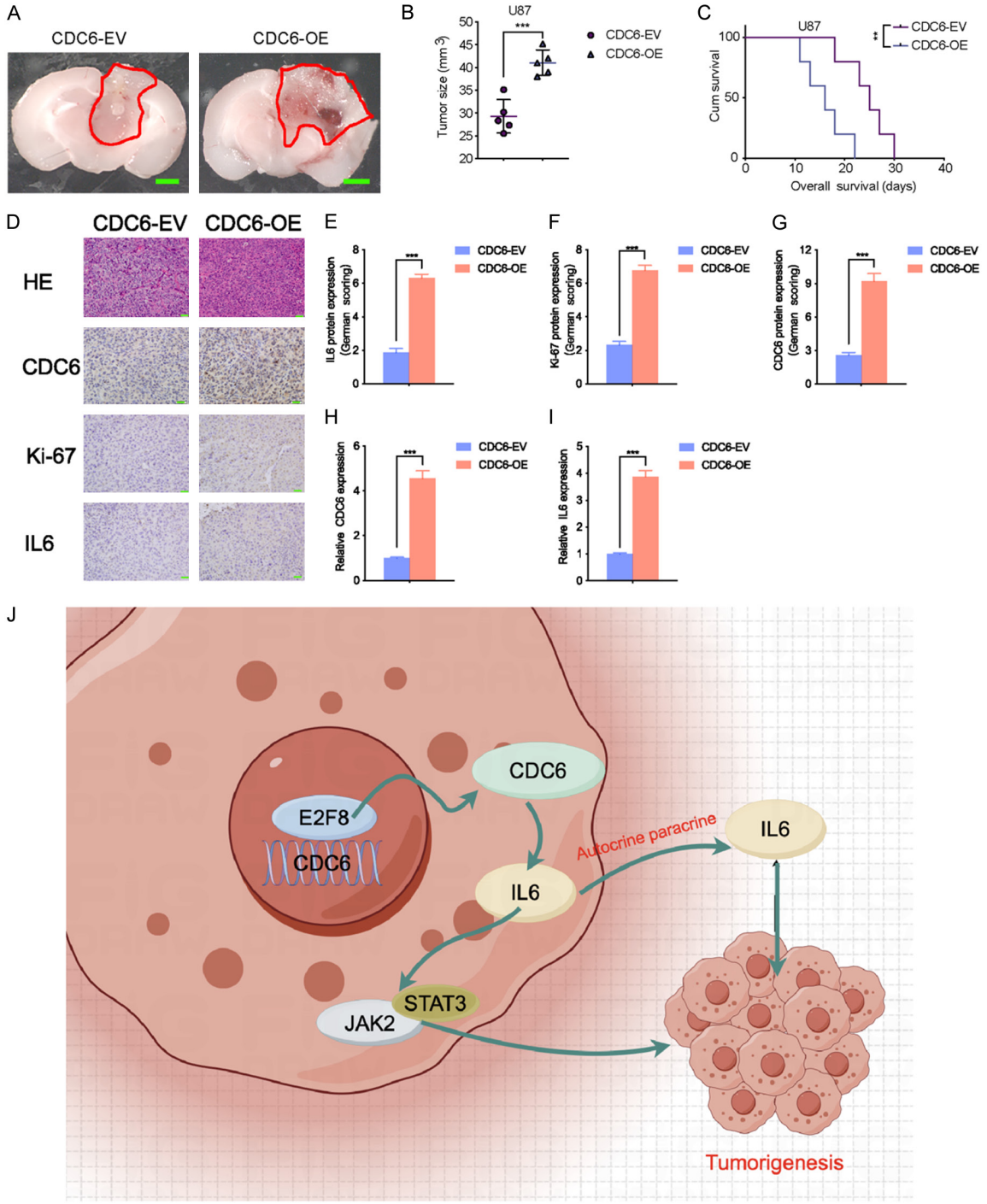

Figure 7. CDC6 promoted the tumorigenesis of gliomas in vivo. A: Representative images display the maximum cross-sectional area of tumors formed in the nude mouse brains after intracranial inoculation of U87 cells overexpressing CDC6 or the control group. B: Quantitative statistical analysis of tumor volumes formed in the nude mouse brains after intracranial inoculation of U87 cells overexpressing CDC6 or the control group. C: Survival analysis of mice after intracranial inoculation of U87 cells overexpressing CDC6 or the control group, assessing the survival time. D: Representative immunohistochemical images show the staining intensity of CDC6, Ki-67, and IL6 in tumor tissues formed intracranially in mice. E-G: Quantitative analysis of immunohistochemistry using the German immunohistochemical scoring system to assess the staining intensity of CDC6, Ki-67, and IL6 in tumor tissues formed intracranially in mice. H, I: qPCR was conducted to detect the expression levels of CDC6 and IL6 in tumor tissues formed intracranially in mice. J: A schematic diagram reveals that E2F8 can transcriptionally upregulate CDC6, activating the IL6-mediated JAK2/STAT3 signaling pathway to promote the malignant progression of gliomas. All data are presented as the mean ± SD from three independent experiments. Statistical significance was determined as *P < 0.05, **P < 0.01, ***P < 0.001.

qPCR analysis of the tumor tissues confirmed higher expression levels of CDC6 and IL6 in the CDC6-overexpressing group than in the control group (**Figure 7H**, **7I**), and the opposite results were also found after CDC6 knockdown (Supplementary Figure 1H, 1I). These results provide conclusive evidence of the role of CDC6 in promoting glioma formation in the mouse brain. A schematic diagram illustrating the specific upstream and downstream mechanisms of CDC6 in gliomas is depicted in **Figure 7J**.

Discussion

Gliomas, originating from neural glial cells, are primary intracranial tumors of various types, with glioblastoma multiforme (GBM) being the most common and malignant type [15]. GBM poses significant therapeutic challenges and has a grim prognosis. Molecular targeted therapy has emerged as a focal point of research and involves the mutation and overexpression of genes such as EGFR, IDH, and MGMT [16]. Treatment strategies targeting these molecular markers, including anti-EGFR drugs and IDH inhibitors, aim to increase treatment efficacy and mitigate patient side effects. Nevertheless, molecular targeted therapy remains under continuous research and optimization [1]. The quest for novel glioma-related genes is a promising avenue for advancing molecular targeted treatments [17]. Unveiling the molecular mechanisms of glioma can reveal new therapeutic targets, improve treatment outcomes and provide more promising individualized therapeutic options, thereby improving the prognosis of glioma patients [18].

The CDC6 gene, encoding the cell division cycle 6 protein, is commonly associated with the degree of malignancy of tumors and poor prognosis in patients, potentially serving as a tumor biomarker and providing crucial information for clinical assessment and treatment decisions [19]. Our prior bioinformatics study revealed notable overexpression of CDC6 in gliomas, closely linked to unfavorable patient outcomes [12]. These findings in clinical samples from glioma patients reaffirmed a significant upregulation of CDC6 in glioma tissues, strongly correlated with tumor grade, with the highest expression observed in glioblastomas.

The overexpression of CDC6 is associated with abnormal proliferation of cancer cells. During the G1/S transition of the cell cycle CDC6 forms complexes with other proteins, activating Cyclin-CDK complexes, driving cells into the S phase, and promoting DNA replication and cell proliferation [20]. Excessive expression of CDC6 can lead to cell cycle dysregulation, inducing uncontrolled proliferation of cancer cells [21]. For example, in breast cancer, aberrant CDC6 overexpression can drive excessive DNA replication and proliferation, facilitating tumor development [22]. In this study, we conducted silencing and overexpression experiments in glioma cell lines U87 and LN229, confirming that CDC6 overexpression promotes the activity and proliferation of glioma cells.

Furthermore, CDC6 plays a crucial role in tumor cell invasion and migration. Its overexpression is closely associated with the invasive behavior of malignant tumors [23]. CDC6 may activate intracellular signaling pathways, promoting changes in cell polarity and membrane skeleton dynamics and thereby enhancing the migratory capacity of tumor cells [24]. For example, in lung adenocarcinoma, elevated CDC6 expression promotes the invasion, migration, and metastasis of lung cancer cells [6]. In colorectal cancer, aberrant CDC6 overexpression facilitates the invasion, migration, and epithelial-mesenchymal transition (EMT) of colon cancer cells [25]. In this study, we conducted silencing and overexpression experiments in glioma cell lines U87 and LN229, confirming the effective promotion of invasion and migration in both cell lines.

While our previous findings confirmed the role of CDC6 in promoting the malignant phenotype of gliomas, consistent with other tumors, the specific molecular biology mechanisms involved await further exploration. Through gene set enrichment analysis (GSEA), we identified a correlation between CDC6 overexpression and the IL6-mediated JAK2/STAT3 signaling pathway [26]. The relationship between IL6 and the JAK2/STAT3 pathway in gliomas is well recognized [27]. Elevated IL6 expression in glioma tissues and the JAK2/STAT3 pathway, a major downstream pathway of IL6, are closely associated with malignant biological behaviors, including cell proliferation, survival, and invasion [28]. In our study, silencing CDC6 resulted

in significant downregulation of IL6 and its downstream molecules JAK2 and STAT3, whereas overexpression had the opposite effect. Functional rescue experiments further confirmed that the promotion of glioma cell proliferation, invasion, and migration induced by CDC6 overexpression could be blocked by IL6-neutralizing antibodies. Thus, our findings affirm the involvement of CDC6 via the IL6-mediated JAK2/STAT3 signaling pathway in the malignant progression of glioma cells.

Additionally, we further analyzed the reasons behind the abnormal overexpression of CDC6 in gliomas. Given the crucial role of transcription factors in promoting the aberrant expression of oncogenes, we focused on analyzing transcription factors significantly correlated with CDC6 expression. E2F8 emerged as one such relevant transcription factor, and through Jaspar database analysis we identified E2F8 binding sites in the CDC6 promoter region. Subsequent luciferase reporter gene assays, western blotting, and qPCR confirmed that E2F8 can transcriptionally upregulate CDC6. E2F8 plays a crucial role in gliomas [29]. As a key factor in cell cycle regulation, E2F8 is normally responsible for preventing cells from entering the S phase. However, abnormal expression of E2F8 in gliomas may lead to cell cycle dysregulation, prompting tumor cells to prematurely enter the S phase and accelerating DNA replication and cell proliferation [30]. Furthermore, elevated expression of E2F8 is closely associated with the occurrence and development of gliomas, potentially contributing to uncontrolled proliferation and infiltration of tumor cells [31, 32]. Our study also confirmed that E2F8 promotes glioma cell proliferation, invasion, and migration, which can be blocked by silencing CDC6.

In summary, although previous bioinformatics analyses have reported the expression and prognostic role of CDC6 in gliomas, our study further employs molecular biology experiments at the cellular, xenograft, and clinical tissue levels. We thoroughly confirmed the abnormal overexpression of CDC6 in gliomas, with the highest expression observed in glioblastomas, demonstrating its role in promoting glioma cell activity, proliferation, invasion, and migration. Additionally, our analysis of the upstream and downstream mechanisms of CDC6 in gliomas revealed that the transcription factor E2F8 is a crucial factor contributing to the abnormal overexpression of CDC6. Furthermore, we found that CDC6 can promote the malignant progression of glioma cells by activating the IL6-mediated JAK2/STAT3 signaling pathway. This study provides further confirmation and refinement of the role of CDC6 in gliomas, offering crucial evidence to support the molecular targeted therapy of CDC6 in gliomas.

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Disclosure of conflict of interest

None.

Abbreviations

GBM, glioblastoma multiforme; CNS, Central Nervous System; TMZ, Temozolomide; CDC6, Cell Division Cycle protein 6; TCGA, The Cancer Genome Atlas; CGGA, Chinese Glioma Genome Atlas; H&E, hematoxylin and eosin; IHC, Immunohistochemistry; qRT-PCR/qPCR, Real-Time Quantitative Reverse Transcription PCR.

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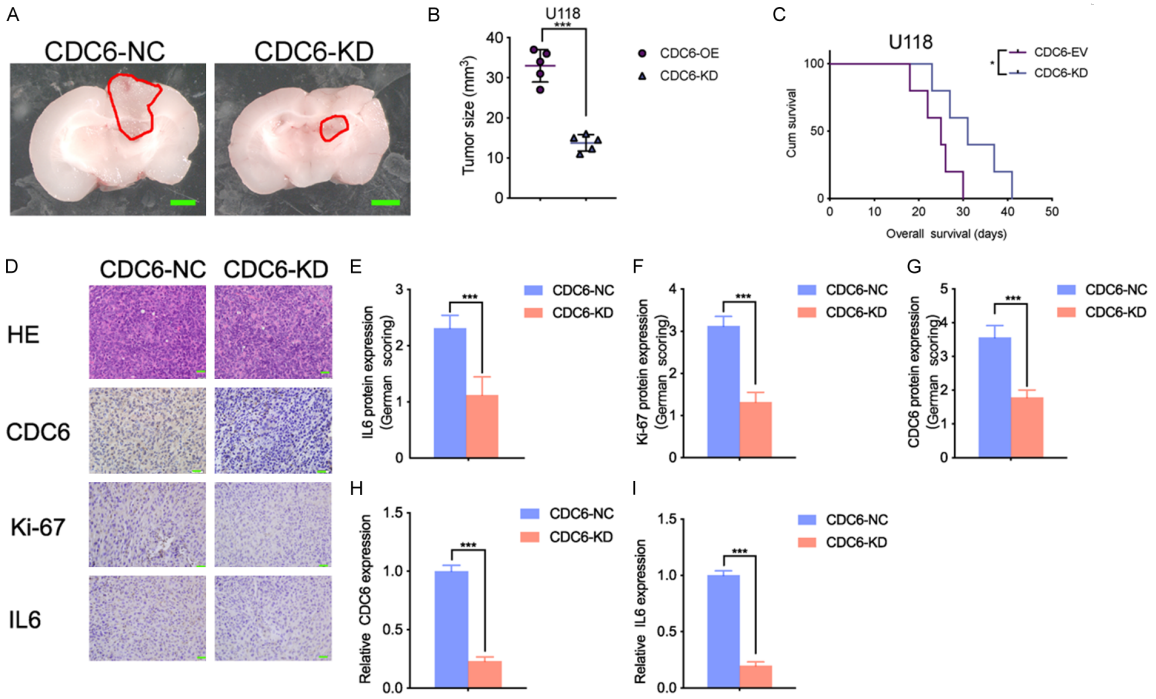

Supplementary Figure 1. CDC6 knockdown inhibits the tumorigenesis of gliomas in vivo. A: Representative images display the maximum cross-sectional area of tumors formed in the nude mouse brains after intracranial inoculation of U118 cells with CDC6 knockdown or the control group. B: Quantitative statistical analysis of tumor volumes formed in the nude mouse brains after intracranial inoculation of U118 cells with CDC6 knockdown or the control group. C: Survival analysis of mice after intracranial inoculation of U118 cells with CDC6 knockdown or the control group, assessing the survival time. D: Representative immunohistochemical images show the staining intensity of CDC6, Ki-67, and IL6 in tumor tissues formed intracranially in mice. E-G: Quantitative analysis of immunohistochemistry using the German immunohistochemical scoring system to assess the staining intensity of CDC6, Ki-67, and IL6 in tumor tissues formed intracranially in mice. H, I: qPCR was conducted to detect the expression levels of CDC6 and IL6 in tumor tissues formed intracranially in mice. All data are presented as the mean ± SD from three independent experiments. Statistical significance was determined as *P < 0.05, **P < 0.01, ***P < 0.001.