WAC-A New GBM Tumour Suppressor- Induces GBM Cell Apoptosis and Promotes Autophagy

Yixuan Wang
Renmin Hospital of Wuhan University: Wuhan University Renmin Hospital
Si Zhang
Renmin Hospital of Wuhan University: Wuhan University Renmin Hospital
Qian Sun
Renmin Hospital of Wuhan University: Wuhan University Renmin Hospital
Fan'en Yuan
Renmin University of China
Linyao Zhao
Renmin Hospital of Wuhan University: Wuhan University Renmin Hospital
Zhang Ye
Renmin Hospital of Wuhan University: Wuhan University Renmin Hospital
Yong Li
Renmin Hospital of Wuhan University: Wuhan University Renmin Hospital
Ronggui Wang
Renmin Hospital of Wuhan University: Wuhan University Renmin Hospital
Hongxiang Jiang
Renmin Hospital of Wuhan University: Wuhan University Renmin Hospital
Ping Hu
Renmin Hospital of Wuhan University: Wuhan University Renmin Hospital
Daofeng Tian
Renmin Hospital of Wuhan University: Wuhan University Renmin Hospital
Baohui Liu (bliu666@whu.edu.cn)
Renmin Hospital of Wuhan University: Wuhan University Renmin Hospital https://orcid.org/0000-0001-5821-6945

Research Article

Keywords: WAC, GBM, Apoptosis, Proliferation, Autophagy

Posted Date: August 13th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-771107/v1

Abstract

WAC is closely related to the occurrence and development of tumors. However, its role in human glioblastoma (GBM) and its potential regulatory mechanisms have not been investigated. This study demonstrated that WAC is downregulated in GBM, and its low expression predicts a poor prognosis. We investigated the effect of WAC on the proliferation of glioma cells through the CCK-8 assay, EdU incorporation and cell formation. The effects of WAC on apoptosis and autophagy in glioma were demonstrated by flow cytometry, TUNEL detection, immunofluorescence, q-PCR, WB and scanning electron microscopy. We found that overexpression of WAC inhibited proliferation of glioma cells, promoted apoptosis and induced autophagy. Therefore, WAC is likely to play a role as a new regulatory molecule in glioma.

Introduction

The most common tumor of the human brain is glioblastoma multiforme (GBM), a glial cell-derived tumour (Glioma) that has a high malignant potential and tends to invade surrounding tissues [1]. They are classified into four grades (I-IV) according to their histopathological and molecular characteristics. Glioblastoma can arise from the beginning as a grade IV tumour (glioblastoma multiforme) or a low grade (grade II) or anaplastic glioma (anaplastic astrocytoma, grade III) and eventually malignant progression to secondary glioma [2]. Despite the widespread use of standard treatment strategies, including surgery, chemotherapy, radiation therapy and immunotherapy, the average patient survival is still less than 15 months [3, 4]. More importantly, the survival rate of patients with GBM is inversely proportional to tumour grade[1]. While molecular targeted therapy and other methods have attracted more and more attention, the research of molecular targeted therapy is still complex because the relevant mechanism of the occurrence and development of GBM is still not precise. Therefore, further basic research on the occurrence and development of GBM is becoming increasingly important.

Autophagy and apoptosis are critical self-destructive processes and can be used as internal equilibrium mechanisms to maintain the homeostasis of eukaryotic cells. To some extent, the tumor is a disease with too little apoptosis and too much proliferation, if the proliferation of tumour cells can be inhibited and apoptosis can be induced, the growth of tumour cells will stop to some extent [5]. As a highly conserved catabolic process that is ubiquitous in evolution, autophagy has been shown to play an essential role in both promoting and inhibiting glioma proliferation in recent years[6, 7].In promoting tumour, cytoplasmic consumed by autophagy body can be used as "biofuel" promote the proliferation of glioma cells, at the same time in the cell stress state (hungry), radiotherapy, and chemotherapy, autophagy may also inhibit tumour cell disruption, maintain material synthesis and metabolism in cells and thus played a "tumour cell protection" [8].In terms of tumour inhibition, autophagy protects cells from reactive oxygen species (ROS), necrosis, inflammation, genomic instability and metabolic changes by removing damaged proteins and organelles. Therefore, autophagy is also considered an inhibitor of tumour initiation[9]. Autophagy may have positive or negative effects on the prognosis of glioma.

WAC is a new member of WW-domain-containing splicing factors. The WW domain of WAC contains a central block of three tyrosine residues [10]. WW domain-containing proteins are commonly involved in ubiquitin-dependent protein degradation[11, 12], signal transduction[13], transcriptional regulation[14], and pre-mRNA splicing[15]. WAC is no exception in these respects. It has been reported that during gene transcription, WAC targets RNF20/40 and binds to the RNA polymerase II complex to conduct H2B ubiquitination at the active transcription site, thereby regulating transcription[16]. In addition, WAC regulates p53-induced p21 transcription and prevents the cell cycle from responding to genotoxic stress, thus playing an essential role in cell cycle checkpoint activation following DNA damage [17]. Under different conditions, WAC may have adverse effects on autophagy regulation by binding to different protein chaperones. It can positively regulate hunger-induced autophagy and promote the formation of autophagosomes, or indirectly inhibit autophagy and regulate energy-mediated mTORC1 activity[18, 19]. However, the expression pattern and pathological role of WAC in the progression of human GBM have not been studied, and relevant animal studies remain to be explored.

In this study, we first provide evidence that WAC is a tumor suppressor for human glioblastoma. WAC expression was significantly down-regulated in human glioblastoma, which was positively correlated with the survival time of patients. Overexpression of WAC can inhibit proliferation, promote apoptosis and induce autophagy of GBM cells. More importantly, the changes of apoptosis-related proteins and autophagy-related proteins also confirmed the regulatory effect of WAC. In conclusion, our findings reveal for the first time the role of WAC in GBM and provide an entry point and impetus for the study of the mechanism of WAC's regulation of GBM apoptosis and autophagy.

Materials And Methods

Bioinformatics

To illustrate the expression pattern and clinical features of WAC in GBM, we used the Gliovis portal (http://gliovis.bioinfo.cnio.es/). Data mining and analysis were performed from three different databases: The Cancer Genome Atlas (TCGA), Gravendell and Rembrandt.

Human tissues

Normal brain tissue and human GBM tissues were collected from the Department of Neurosurgery, Renmin Hospital of Wuhan University. Tumor specimen was confirmed by the pathologists of the Department of Pathology at the Renmin Hospital of Wuhan University. Non glioma specimens were collected from patients with various traumatic brain injuries during the course of surgery with informed consent. Tissue acquisition and use in this study were performed with patients signed informed consent and were approved by the institutional ethics committee of the medical department, Renmin Hospital of Wuhan University.

Cell culture

Human glioblastoma cell lines (U87 and A172) purchased from the Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), the cells were cultured in high-glucose DMEM (Gibco, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) and 1% penicillin/streptomycin (Thermo Fisher Scientific), and the incubating temperature was 37 °C, with 5% CO2.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from tissues and cells using Trizol reagent (Invitrogen, USA). Next, we used the PrimeScript RT reagent kit with gDNA Eraser (Takara, Tokyo, Japan) to prepare for cDNA, and real-time PCR was performed using SYBR Premix Ex Taq II (RR820A, Takara) according to the manufacturer's protocol. The comparative Ct method was adopted to compare the experimental and control groups, and GAPDH was used for normalization. The specific primer pairs were as follows: GAPDH (forward primer, 5´-ACAACTTTGGTATCGTGGAAGG-3'; reverse primer, 5´-GCCATCACGCCACAGTTTC-3'); WAC (forward primer, 5´-GTTCCACAGTCGCCAATACCTC-3'; reverse primer, 5´-GCAGATGGTCCAGCAGTAAGAA-3'); Beclin1(forward primer, 5´-GAGCCATTTATTGAAACTCCTCG-3'; reverse primer, 5'-CCCAGTGACCTTCAGTCTTCG-3'); p62 (forward primer, 5´-GGAAGGTGAAACACGGACACTT-3'; reverse primer,5´-CTCTTCTCCTCTGTGCTGGAACT-3'); LC3B (forward primer, 5'- GTTGGCACAAACGCAGGGTA-3'; reverse primer, 5´-ACACTGCTGCTTTCCGTAACAA-3').

DNA construction and transfection

WAC cDNA was subcloned with a Flag tag (Flag-WAC) into the PCDNA3.1. The cells were seeded into the 6-well plate one night before transfection, and transfected with the help of the transfection reagent Lipofectamine 3000 (L3000015, Thermo Fisher Scientific) according to the guidance of the reagent manufacturer.

Antibodies and reagents

The primary antibodies used were anti-WAC (bs-12787R, Bioss, China), anti-DYKDDDK/Flag-tag (ANT102, Antgene, China), anti-P62 (M162–3, Medical Biological Laboratories, Japan), anti-Beclin1 (11306–1-AP, Proteintech, USA), anti-LC3B (GB11124, Service, China), anti-BAX (50599–2-Ig, Proteintech, USA), anti-Bcl-2 (GTX100064, GeneTex, USA), anti-GAPDH (#5174, Cell Signaling Technology, USA).

Clone formation

U87 and A171 cells were digested with trypsin 24 h after transfection and inoculated into 6-well plates at a density of 1000 cells per well. The cells were then incubated in a humidified incubator at 37°C and 5% CO 2 until the clones were visible to the naked eye. The medium was then removed, the cells were washed with phosphate buffer (PBS), fixed with 4% paraformaldehyde, and stained with crystal violet. The number of colonies with ≥50 cells was counted under an inverted microscope (Olympus, Tokyo, Japan).

Cell count kit-8(CCK-8) assay

CCK-8 (SolarBio Biotech Corp., Beijing, China) was used to detect cell viability according to the manufacturer's instructions. In short, approximately 4×10^3 cells were plated into each well of the 96-well plate. At 24, 48 and 72 h after transfection, a 10% volume of CCK8 solution was added to each well and incubated in the dark for two hours. OD values were measured at 450 nm to determine cell viability. Three separate experiments were conducted.

EdU-DNA Synthesis Assay

Cell-Light EdU Apollo567 In Vitro Kit (RiboBio, Guangzhou, China) was used to study the DNA synthesis activity in WAC-treated cells. According to the reagent vendor's protocol, cells were seeded in 96-well plates at 5000 per well. Edu was added to the medium at 50 μl 48 h after transfection, followed 2 h later by fixing the cells in 4% paraformaldehyde for 30 min, permeated with 0.5% Trixon-X 100 for 10 min, and then stained with 10 μM Apollo 567 for 30 min. The cells were then restained with Hoechst 33342 to hide from light for 30 min. EdU was visualized using a fluorescence microscope (Olympus BX51, Japan).

Flow Cytometric analysis

Forty-eight hours after transfection, AN Annexin V-PE/7-AAD Kit (Becton Dickinson, New Jersey, USA) was used to measure the apoptosis rate of GBM cells. The cells were digested with EDTA-free trypsin, collected, and then washed three times with cold PBS. After samples were stained with 5 μl of Annexin V-PE and l of 7-AAD for 15 min in the dark, 400 μl of 1 x Binding Buffer was mixed into the mixture. Finally, apoptosis results were analyzed by Cytoflex Flow Cytometer (Beckman Coulter) and FlowJo 10.0.7 software.

TUNEL assay

Forty-eight hours after transfection, the cells were immobilized in 4% paraformaldehyde for 15 min. Next, we used the Tunel Kit (Roche Diagnostics, Mannheim, Germany) and followed the protocol provided by the reagent vendor to detect apoptosis. Finally, under the Olympus BX51 microscope (Olympus, Japan), TUNEL positive cells show green fluorescence. ImageJ software is used to count the TUNEL positive cells. The experiment was carried out three times independently.

Assay of green fluorescent protein-LC3 Puncta

WAC plasmids were transfected into GBM cells that stably expressed green fluorescent protein (GFP)-LC3. The cells were fixed with 4% paraformaldehyde 48 h after transfection. Then the GFP-LC3 spots in the cells were observed using confocal laser scanning microscopy (Olympus, Japan). Count the mean of GFP-LC3 points/cell in at least 100 cells.

Western blotting

The RIPA lysis buffer containing the protease inhibitor mixture (Beyotime, Shanghai, China) was used to extract the protein, and the protein concentration was determined using the BCA assay (Biosharp, China).

The sample and loading buffer were thoroughly mixed. Heat at 100°C for 5 minutes. Next, each sample of the same concentration was separated by 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to the PVDF membrane. Block with 5% skim milk at room temperature for 1 hour and incubate overnight with diluted primary antibody at 4°C. After three elutions with PBST to remove residual primary antibodies, the membrane was incubated with Alex Fluor 680/790 labeled secondary antibodies (Li-Cor Bioscience, USA) for 1 to 2 h. Finally, the proteins were visualized using the Li-Cor Odyssey Infrared Imaging System (Li-Cor Bioscience). The relative density of the strips was analyzed by Image J software version 1.8.0. GAPDH was used as an internal control.

Immunohistochemistry

After the tissue sections were dewaxed and hydrated, they were immersed in a microwave oven boiling Tris-EDTA buffer (pH 9.0) for antigenic repair and then incubated overnight with anti-WAC (1:100; Bioss) at 4°C. The next day, the slides were washed and incubated with the secondary antibody for 1 hour. Finally, DAB staining was used to detect the signal, and hematoxylin was restained. After dehydration, the film was sealed with neutral resin. Images were visualized by Olympus.

Transmission Electron microscopy (TEM)

Cells transfected with Flag-WAC or control plasmid were fixed with an electronic fixation solution containing 2.5% glutaraldehyde and post-fixed in 1% osmic acid. The fixed sample was then dehydrated using a graded series of ethanol (70-100%) and finally embedded in the EPON resin. An ultrafine slicer was used to cut ultrafine slices and was double-stained with uranium dioxide acetate and lead citrate. The stained sections were then observed using a transmission electron microscope (Hitachi HT7700, Tokyo, Japan).

Statistical analysis

GraphPad Prism 8.0 software was used to analyze and generate graphs in this study, and all data were presented as mean ± standard deviation. Student T-test determined differences between two groups, and One-way ANOVA determined differences between multiple groups. Kaplan-Meier and log-rank tests were used for survival analysis. $P < 0.05$ was considered statistically significant.

Result

WAC is downregulated and associates with prognosis in GBM

In order to clarify whether WAC is involved in the occurrence and development of GBM, we used RNAseq data from three public datasets to find that WAC mRNA expression was significantly lower in GBM than in non-tumor tissues (Fig1A), and WAC expression was also significantly lower in high-grade gliomas than in low-grade gliomas (FigS1). Based on clinical specimens, we examined WAC protein or mRNA expression levels in non-tumor tissues and GBM. Consistent with the bioinformatic results, both protein and mRNA expression of WAC was significantly lower in GBM than in non-tumor tissues (Fig1B,1C),

which was also confirmed by immunohistochemistry (Fig1D). To investigate the correlation between WAC and GBM invasiveness, we compared the expression of WAC under different IDH1/2 states. Compared with IDH1/2 mutant glioma in TCGA, WAC expression was significantly reduced in IDH1/2 wild-type glioma (Fig1E). This suggests that WAC expression is inextricably related to GBM malignancy. To test whether WAC can predict prognosis in patients with GBM, we performed survival analyses on three different data points using the Gliovis platform. The results showed that the expression of WAC was positively correlated with the survival time of patients (Fig1F). Taken together, these results suggest that WAC expression is downregulated in GBM and predicts a good prognosis.

WAC overexpression inhibited GBM cell proliferation in vitro

To investigate the biological role of WAC in GBM, a Flag-WAC overexpression vector was constructed and verified by Western blot (Fig2B). CCK-8 was used to detect the viability of U87 and A172 cells. The results showed that overexpression of WAC significantly inhibited the proliferation of GBM cells in vitro (Fig2A). In addition, the results of the colony formation also showed that the GBM cells transfected with Flag-WAC formed fewer and smaller colonies compared to the control group (Fig2C,2D). EdU incorporation assay was used to detect the effect of WAC on DNA synthesis in GBM cells. The results showed that WAC induced a significant decrease in EdU positive cells in U87 and A172 cells (Fig2E-2H). These results suggest that WAC inhibits GBM cell proliferation in vitro.

WAC overexpression induced GBM cell apoptosis in vitro

Based on the above observations, we used Annexin V-PE/7-AAD double staining to examine whether WAC induced apoptosis in glioma cells. The results demonstrated by flow cytometry showed that the overexpression of WAC strongly induced apoptosis of U87 and A172 cells (Fig3A-3D). In addition, TUNEL staining showed that the proportion of TUNEL positive cells (green) in the WAC overexpression group was significantly higher than that in the control group (Fig3E-3G). In summary, all the data suggest that WAC expression can positively regulate apoptosis of glioblastoma cells.

WAC overexpression enhanced GBM cell autophagy in vitro

Autophagy can protect cells from death but can also mediate cell death [20]. Further to investigate whether WAC plays a crucial role in GBM autophagy, we exogenously upregulated WAC in GBM cells stably expressing GFP-LC3B, an intracellular green fluorescent labelled recombinant protein for autophagosomes. We found that the number of GFP-LC3-rich subcellular granules around the nucleus in the Flag-WAC group was significantly more than that in the control group, indicating that the autophagy activity in cells was increased (Fig4A,4B). In addition, we observed the autophagy structure of glioma cells using transmission electron microscopy. The results showed that autophagy vacuoles were increased in the Flag-WAC group, and most autophagosomes contained residual digestive components, while the control group rarely showed this characteristic (Fig4C,4D).

WAC influences glioma malignancy through the classical signal axis

In order to further explore the regulatory effect of WAC, we detected the differences in the mRNA expression levels of autophagy-related markers by RT-PCR, which was consistent with the expected results WAC promoted Beclin1 and LC3b and inhibited P62 at the transcriptional level (Fig.4E,4F). More importantly, Western blotting results showed that the protein expressions of Beclin1 and LC3B were significantly increased after WAC overexpression, while the expression of P62 was suppressed. At the same time, the expression of pro-apoptotic protein Bax was also increased, while that of Bcl-2 was reversed. In conclusion, it is very likely that WAC directly or indirectly associates with Beclin1, LC3B, p62, Bax, Bcl-2, and regulates proteins involved in apoptosis and autophagy, which promotes apoptosis and autophagy progression and consequently inhibits glioblastoma development.

Discussion

Many studies provide compelling evidence that glioblastoma is the most common primary brain tumour type[21]. Gene therapy, which can provide a "cure" for some terminal or severely disabling diseases, has long been a source of fascination for clinicians and researchers because of its potential to treat diseases at their genetic roots[22]. For example, effective targets such as EGFR and BRAF have been found in glioblastoma [23, 24]. Nevertheless, even in this era of molecular medicine, it is still difficult to change the poor prognosis of GBM patients[25]. Therefore, novel therapeutic targets for GBM are urgently needed to advance gene therapy and mechanistic studies of GBM.

In this study, we found low expression of WAC in high-grade GBM, relative to low-grade GBM and normal brain tissue, based on data from three publicly available databases and our cohort of clinical samples. In addition, WAC expression was significantly associated with several critical clinicopathological parameters, including WHO II, III, and IV classifications and IDH mutant or wild types. Subsequently, Kaplan-Meier survival analysis showed that higher WAC expression was associated with higher patient survival. The present results suggest that the WAC may be involved in the development and progression of GBM.

Further functional studies, we showed that the up-regulation of WAC inhibited the proliferation of GBM cells in vitro. Cancer cell growth depends on a balance between proliferation and apoptosis[26]. Now that we have found the inhibitory effect of WAC on cell proliferation, we examined the effect of WAC on cell apoptosis by flow cytometry. Interestingly, our results showed that Flag-WAC promoted apoptosis in U87 and A172 cells. mitochondria-dependent apoptosis is one of the most important pathways to induce apoptosis. Bcl-2 family proteins are central regulators of intrinsic pathways that inhibit or promote changes in mitochondrial membrane permeability required for the release of Cyt-c and other apoptotic proteins[27]. Bax, a pro-apoptotic member of the Bcl-2 protein family, can form transmembrane pores large enough to allow the release of cytochrome C and activate mitochondrial permeability conversion pores[28]. Subsequently, the influence of WAC on Bcl-2 and Bax in the two cell lines was detected, and the expression of Bcl-2 was significantly decreased, while the expression of Bax was significantly increased compared with the control group. Therefore, the present data suggest that WAC regulates cell

proliferation and apoptosis by down-regulating Bcl-2 expression and upregulating the Bax, leading to tumor development.

In the following study, we found that WAC could also induce autophagy in glioma cells. Mechanically speaking, it induces autophagy at the transcription and protein levels. The mRNA and protein expressions of Beclin1, LC3B and P62 were detected. Autophagy and apoptosis have antagonistic or synergistic effects under certain conditions. Apoptosis and autophagy can co-occur, triggering cell death, while apoptosis can also accelerate the transformation of cells to autophagy death[29, 30]. So, WAC has been shown to inhibit GBM development through the synergy of these two pathways, but whether there is a certain relationship between WAC's regulation of apoptosis and autophagy in GBM cells needs further study in the future.

In conclusion, the current data suggest that WAC may be a potential tumour suppressor of GBM and may regulate apoptosis and autophagy-related proteins like Beclin1, LC3B, P62, Bax, and Bcl-2 promote apoptosis and autophagy processes, thereby inhibiting the development of GBM. Thus, we demonstrate that WAC is an expression pattern and regulatory role in GBM for the first time. It has been previously reported that WAC and GM130 interact with the ATG8 homologous molecule GABARAP and regulate its subcellular localization. GABARAPs are located in the pericentral matrix, and this dynamic pool contributes to autophagosome formation [19]. Whether WAC influences the occurrence of GBM through a series of complex regulatory mechanisms in this signaling pathway still needs further study.

Declarations

Funding

No fundings.

Conflicts of interest

The authors declare that they have no conflict of interest.

Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon request.

Authors’ contributions

Baohui Liu and Daofeng Tian designed the research. Yixuan Wang and Si Zhang carried out all the experimental work. Fan’en Yuan, Qian Sun, Linyao Zhao, Zhang Ye wrote the paper, Yong Li, Ronggui Wang Collected and assembled data, Ping Hu and Hongxiang Jiang analysed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The following information was supplied related to ethical approvals (i.e., approving body and any reference numbers): Institutional Ethics Committee of the Faculty of Medicine at Renmin Hospital of Wuhan University approval (2012LKSZ (010) H) to carry out the study within its facilities.

Consent for publication

Not applicable.

Acknowledgements

The authors thank the personnel in the department of neurosurgery and the central laboratory at the Renmin Hospital of Wuhan University.

References

1. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A et al. The 2007 WHO classification of tumours of the central nervous system. Acta neuropathologica. 2007;114(2):97-109. doi:10.1007/s00401-007-0243-4.
2. Biernat W, Tohma Y, Yonekawa Y, Kleihues P, Ohgaki H. Alterations of cell cycle regulatory genes in primary (de novo) and secondary glioblastomas. Acta neuropathologica. 1997;94(4):303-9. doi:10.1007/s004010050711.
3. Ostrom QT, Bauchet L, Davis FG, Deltour I, Fisher JL, Langer CE et al. The epidemiology of glioma in adults: a "state of the science" review. Neuro-oncology. 2014;16(7):896-913. doi:10.1093/neuonc/nou087.
4. Ostrom QT, Gittleman H, Stetson L, Virk SM, Barnholtz-Sloan JS. Epidemiology of gliomas. Cancer treatment and research. 2015;163:1-14. doi:10.1007/978-3-319-12048-5_1.
5. Ito M, Minamiya Y, Kawai H, Saito S, Saito H, Nakagawa T et al. Tumor-derived TGFbeta-1 induces dendritic cell apoptosis in the sentinel lymph node. Journal of immunology (Baltimore, Md : 1950). 2006;176(9):5637-43. doi:10.4049/jimmunol.176.9.5637.
6. Feng Y, He D, Yao Z, Klionsky DJ. The machinery of macroautophagy. Cell research. 2014;24(1):24-41. doi:10.1038/cr.2013.168.
7. Kimmelman AC, White E. Autophagy and Tumor Metabolism. Cell metabolism. 2017;25(5):1037-43. doi:10.1016/j.cmet.2017.04.004.
8. Abdul Rahim SA, Dirkse A, Oudin A, Schuster A, Bohler J, Barthelemy V et al. Regulation of hypoxia-induced autophagy in glioblastoma involves ATG9A. British journal of cancer. 2017;117(6):813-25. doi:10.1038/bjc.2017.263.
9. Choi AM, Ryter SW, Levine B. Autophagy in human health and disease. The New England journal of medicine. 2013;368(7):651-62. doi:10.1056/NEJMra1205406.
10. Xu GM, Arnaout MA. WAC, a novel WW domain-containing adapter with a coiled-coil region, is colocalized with splicing factor SC35. Genomics. 2002;79(1):87-94. doi:10.1006/geno.2001.6684.

11. Hein C, Springael JY, Volland C, Haguenauer-Tsapis R, André B. NPl1, an essential yeast gene involved in induced degradation of Gap1 and Fur4 permeases, encodes the Rsp5 ubiquitin-protein ligase. Molecular microbiology. 1995;18(1):77-87. doi:10.1111/j.1365-2958.1995.mmi_18010077.x.
12. Staub O, Dho S, Henry P, Correa J, Ishikawa T, McGlade J et al. WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na+ channel deleted in Liddle's syndrome. The EMBO journal. 1996;15(10):2371-80.
13. Maleszka R, Lupas A, Hanes SD, Miklos GL. The dodo gene family encodes a novel protein involved in signal transduction and protein folding. Gene. 1997;203(2):89-93. doi:10.1016/s0378-1119(97)00522-2.
14. Sudol M, Sliwa K, Russo T. Functions of WW domains in the nucleus. FEBS letters. 2001;490(3):190-5. doi:10.1016/s0014-5793(01)02122-6.
15. Kao HY, Siliciano PG. Identification of Prp40, a novel essential yeast splicing factor associated with the U1 small nuclear ribonucleoprotein particle. Molecular and cellular biology. 1996;16(3):960-7. doi:10.1128/mcb.16.3.960.
16. Zhang F, Yu X. WAC, a functional partner of RNF20/40, regulates histone H2B ubiquitination and gene transcription. Molecular cell. 2011;41(4):384-97. doi:10.1016/j.molcel.2011.01.024.
17. Abbas T, Dutta A. p21 in cancer: intricate networks and multiple activities. Nature reviews Cancer. 2009;9(6):400-14. doi:10.1038/nrc2657.
18. David-Morrison G, Xu Z, Rui YN, Charng WL, Jaiswal M, Yamamoto S et al. WAC Regulates mTOR Activity by Acting as an Adaptor for the TTT and Pontin/Reptin Complexes. Developmental cell. 2016;36(2):139-51. doi:10.1016/j.devcel.2015.12.019.
19. Joachim J, Jefferies HB, Razi M, Frith D, Snijders AP, Chakravarty P et al. Activation of ULK Kinase and Autophagy by GABARAP Trafficking from the Centrosome Is Regulated by WAC and GM130. Molecular cell. 2015;60(6):899-913. doi:10.1016/j.molcel.2015.11.018.
20. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nature reviews Molecular cell biology. 2007;8(9):741-52. doi:10.1038/nrm2239.
21. Jiang T, Mao Y, Ma W, Mao Q, You Y, Yang X et al. CGCG clinical practice guidelines for the management of adult diffuse gliomas. Cancer letters. 2016;375(2):263-73. doi:10.1016/j.canlet.2016.01.024.
22. Naldini L. Gene therapy returns to centre stage. Nature. 2015;526(7573):351-60. doi:10.1038/nature15818.
23. Klingler S, Guo B, Yao J, Yan H, Zhang L, Vaseva AV et al. Development of Resistance to EGFR-Targeted Therapy in Malignant Glioma Can Occur through EGFR-Dependent and -Independent Mechanisms. Cancer research. 2015;75(10):2109-19. doi:10.1158/0008-5472.Can-14-3122.
24. Ahn JH, Lee YW, Ahn SK, Lee M. Oncogenic BRAF inhibitor UAI-201 induces cell cycle arrest and autophagy in BRAF mutant glioma cells. Life sciences. 2014;104(1-2):38-46. doi:10.1016/j.lfs.2014.03.026.

25. Geraldo LHM, Garcia C, da Fonseca ACC, Dubois LGF, de Sampaio ESTCL, Matias D et al. Glioblastoma Therapy in the Age of Molecular Medicine. Trends in cancer. 2019;5(1):46-65. doi:10.1016/j.trecan.2018.11.002.
26. Wang C, Zhao N, Zheng Q, Zhang D, Liu Y. BHLHE41 promotes U87 and U251 cell proliferation via ERK/cyclinD1 signaling pathway. Cancer management and research. 2019;11:7657-72. doi:10.2147/cmar.S214697.
27. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. Science (New York, NY). 2004;305(5684):626-9. doi:10.1126/science.1099320.
28. Gómez-Crisóstomo NP, López-Marure R, Zapata E, Zazueta C, Martínez-Abundis E. Bax induces cytochrome c release by multiple mechanisms in mitochondria from MCF7 cells. Journal of bioenergetics and biomembranes. 2013;45(5):441-8. doi:10.1007/s10863-013-9508-x.
29. Doherty J, Baehrecke EH. Life, death and autophagy. Nature cell biology. 2018;20(10):1110-7. doi:10.1038/s41556-018-0201-5.
30. Denton D, Kumar S. Autophagy-dependent cell death. Cell death and differentiation. 2019;26(4):605-16. doi:10.1038/s41418-018-0252-y.

Figures

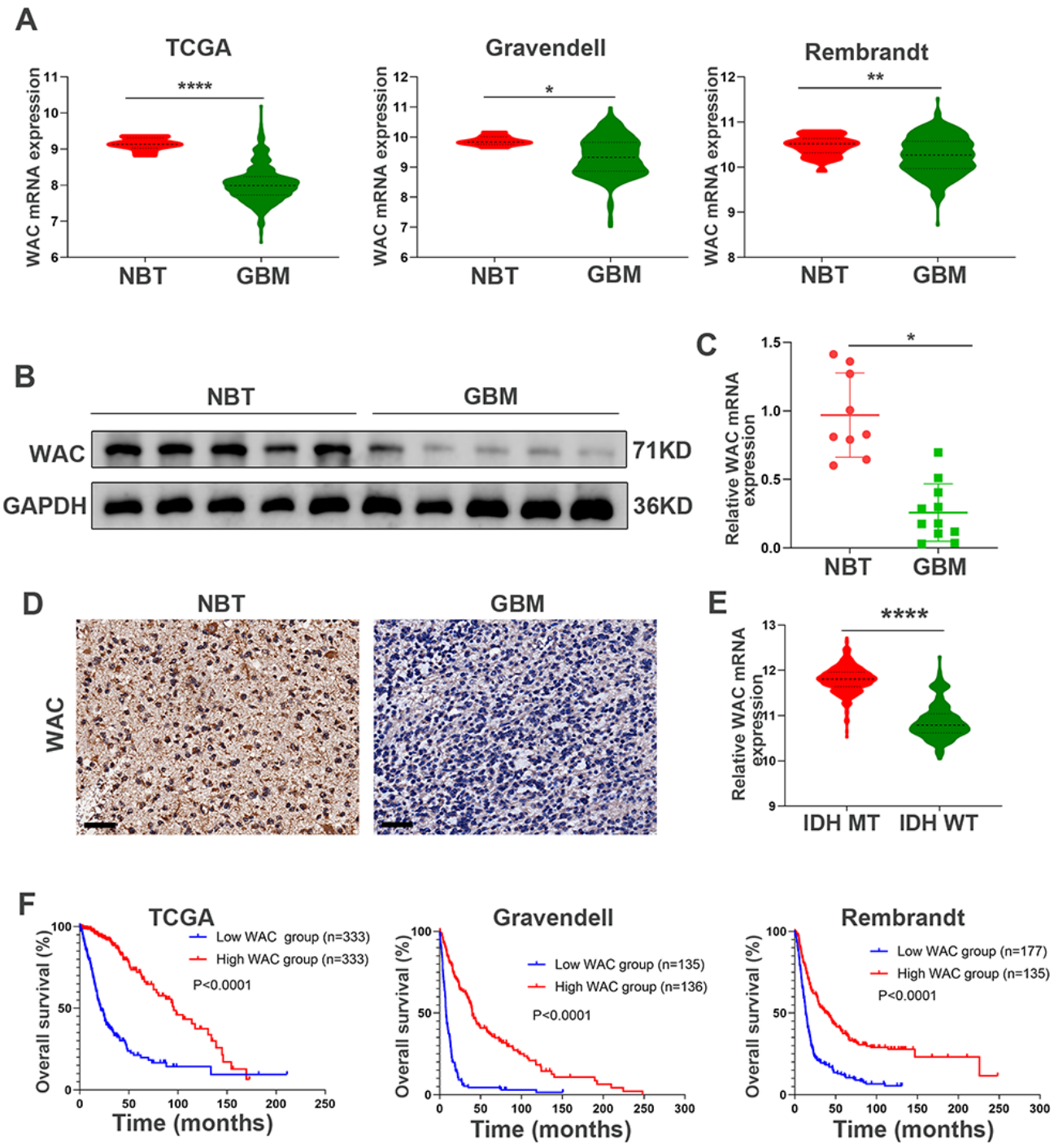

Figure 1

WAC is downregulated and associates with prognosis in GBM. (A) WAC mRNA expression in Normal brain tissue (NBT) and GBM in TCGA, Gravendell and Rembrandt datasets. (B-C) Western blot analysis and RT-PCR were performed to detect protein (NBT, n=5; GBM, n=5) and mRNA (NBT, n= 9; GBM, n=11) levels of WAC in NBT and glioma. (D)Representative images of IHC staining of WAC in in NBT and GBM, Scale bar, 50 μm. (E) WAC mRNA expression in patients with mutant (MT) and wildtype (WT) IDH1/2 in

TCGA datasets. (F) Kaplan-Meier survival analysis for WAC expression in all glioma patients in TCGA, Gravendell and Rembrandt datasets. *P < 0.05, **P < 0.01, and ***P < 0.001.

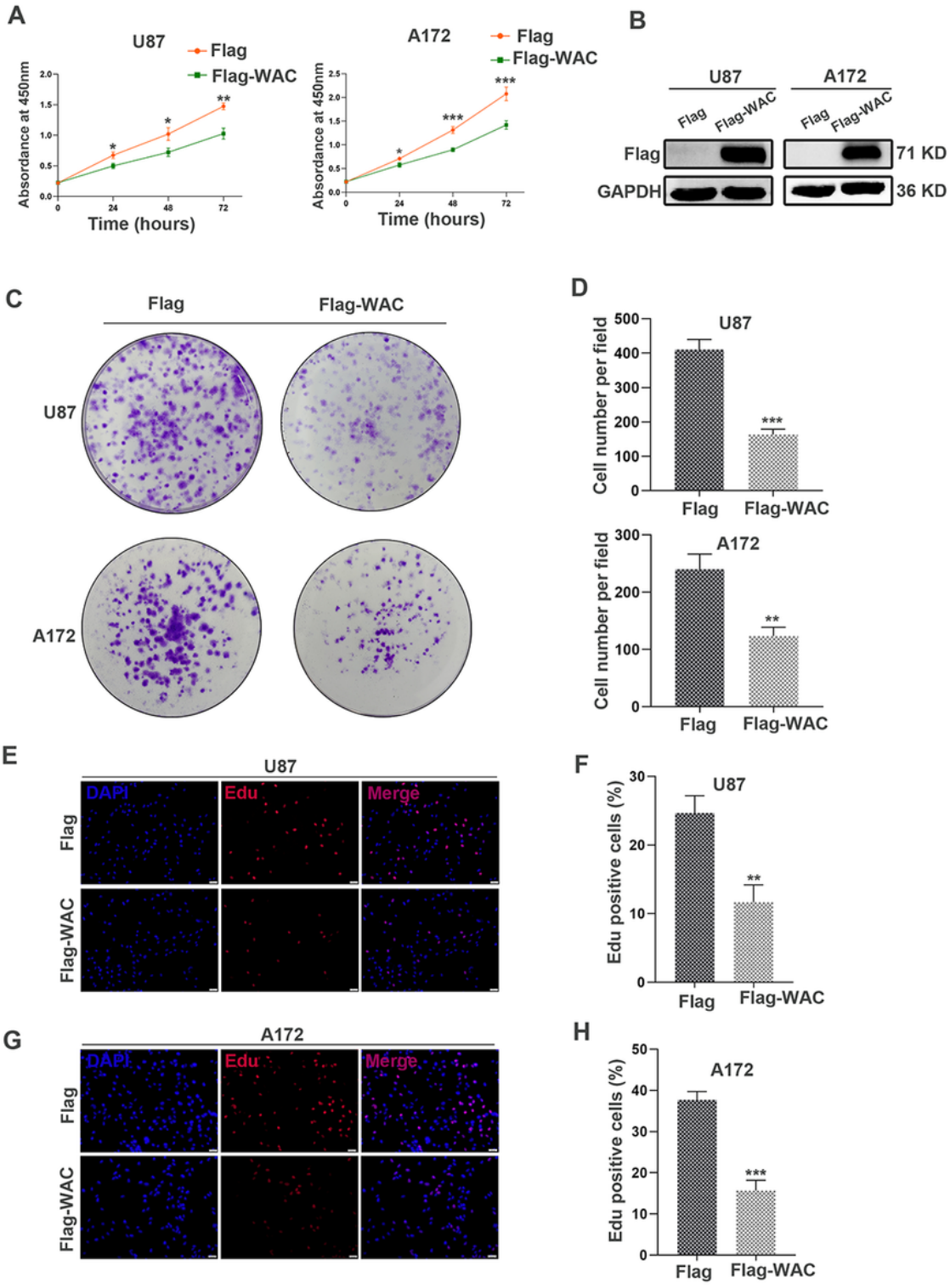

Figure 2

WAC overexpression inhibited GBM cell proliferation in vitro. (A) CCK-8 assay was used to detect the effect of WAC overexpression on U87 and A172 cell viability over a three-day period. (B)Western blot was used to detect the overexpression efficiency of WAC, GAPDH served as loading control. (C, D) Colony

formation assay was performed to measure clone formation of U87 and A172 cells after transfection. (E-H) EdU incorporation assay was used to measure DNA replication of U87 and A172 cells after transfection. Proliferative cell was labeled with EdU (red) and cell nuclei were stained with DAPI (blue). Scale bar, 200 μm. *P<0.05 **P<0.01 and ***P<0.0001. Experiment was repeated three times with similar results

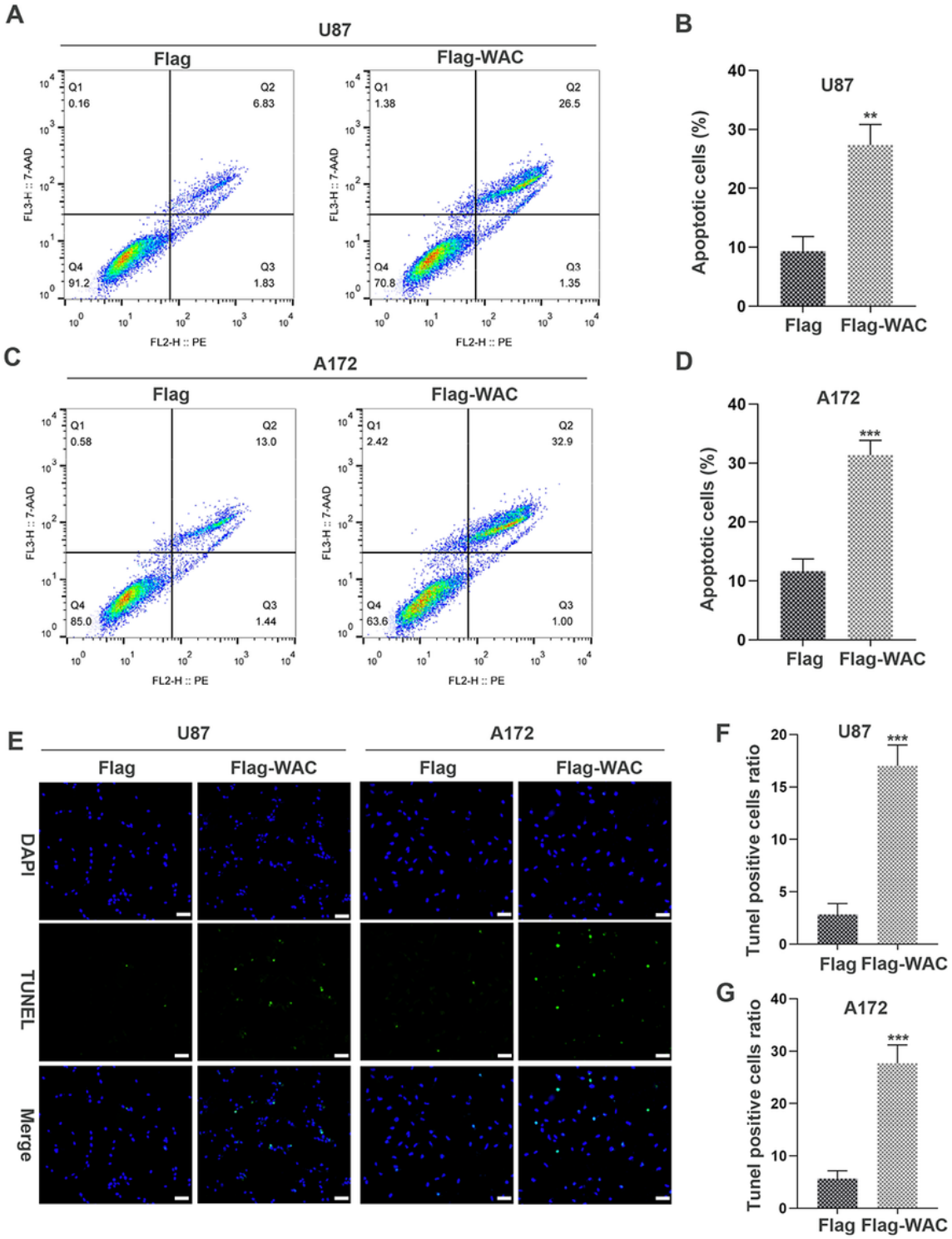

Figure 3

WAC overexpression induced GBM cell apoptosis in vitro. (A-D) Cell apoptosis was significantly increased after transfection with WAC measured by flow cytometry and results were statistically analyzed. (E-G) Representative images of TUNEL staining. Scale bars, 50 μm. For at least three independent experiments. **P < 0.01, ***P < 0.001.

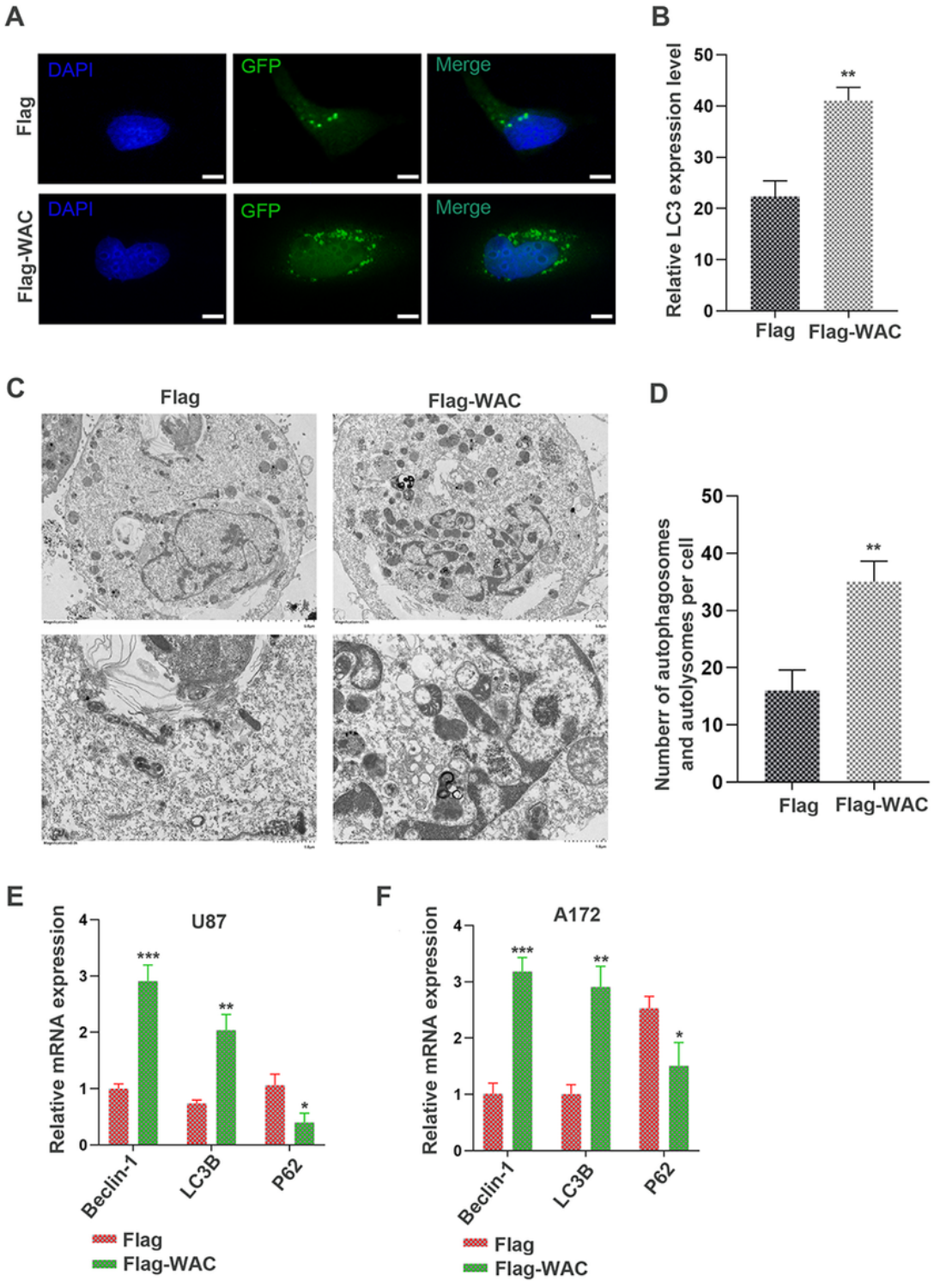

Figure 4

WAC overexpression enhanced GBM cell autophagy in vitro. (A, B) The numbers of GFP-LC3 puncta were quantified using confocal laser scanning microscopy in U87-MG cells transfected with GFP-LC3. Scale bars,10 μm. **P < 0.01. (C, D) Transmission Electron microscopy was used to detect autophagy activity in U87 cells after transfection with Flag-WAC and control Flag at 48 h. Scale bars, 5 μm at low magnification, 1 μm at high magnification. (E, F) RT-PCR was used to detect the effect of WAC overexpression on the mRNA expression of Beclin1, LC3B and P62 in two cell lines. *P<0.05 **P<0.01 and ***P<0.0001. Data represents mean ± SD.

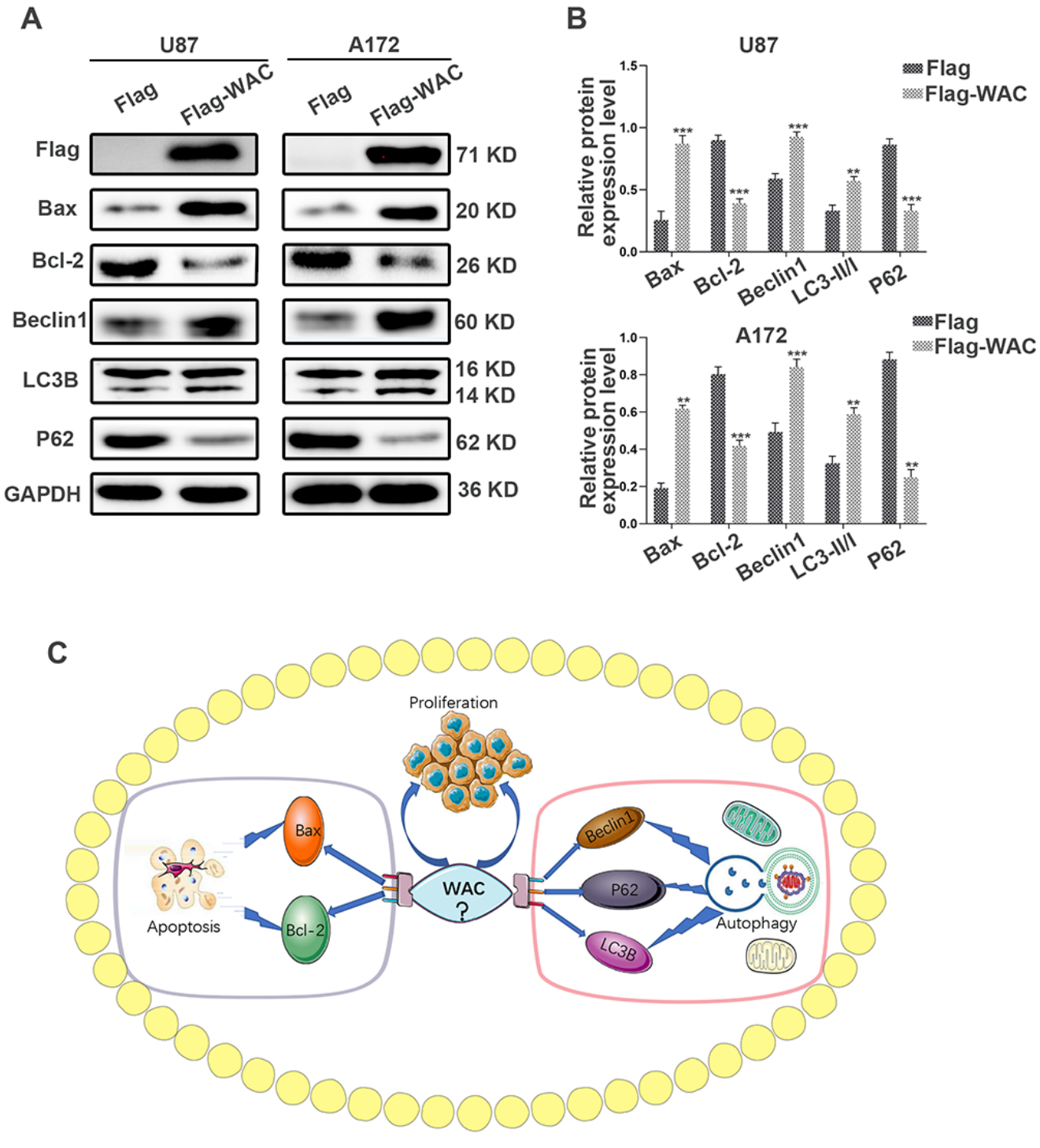

Figure 5

WAC influences glioma malignancy through the classical signal axis. (A, B) Effects of WAC overexpression on autophagy-related and apoptosis-related protein levels in GBM cells. (C) Mechanistic model for the WAC regulation of cell apoptosis and autophagy in GBM. **P < 0.01, ***P < 0.001. All bar plot data are the means ± SD.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigS1.png