Original Article
GOLPH3 promotes glioma progression by enhancing PHB2-mediated autophagy

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Abstract: Due to the hypoxia and nutrient deficiency microenvironment, malignant glioma exhibits high autophagy activity and autophagy plays a significant role in the occurrence and development of glioma. However, the potential molecular mechanism of autophagy in glioma remains unknown. In this study, we demonstrated that Golgi phosphorylation protein 3 (GOLPH3), a highly conserved protein basically concentrates in the trans-Golgi network, promoted glioma autophagy. Inhibiting autophagy by using chloroquine suppressed the stimulating effect of GOLPH3 on glioma malignant development both *in vitro* and *in vivo*. Mechanistically, GOLPH3 interacted with and recruited prohibitin-2 (PHB2), an autophagy receptor of mitochondrion, and LC3-II. PHB2 promoted cell autophagy and downregulation of PHB2 abolished the effect of GOLPH3 on autophagy. On the side, the relative mRNA and protein levels of GOLPH3 and PHB2 were positively associated with each other and both also correlated with autophagy in glioma tissues. Together, our results revealed that GOLPH3 promotes glioma progression by enhancing PHB2-mediated autophagy and inhibiting autophagy may benefit glioma patients with GOLPH3 high level. The novel GOLPH3-PHB2-autophagy axis maybe a potential and prospective therapeutic target for gliomas.

Keywords: Glioma, GOLPH3, PHB2, autophagy, proliferation

Introduction

Malignant glioma is a common and aggressive tumor in human central nervous system worldwide. Currently, the globally standard treatment for newly diagnosed malignant glioma patient is maximal surgical resection followed by systemic application of the alkylating agent temozolomide (TMZ) combined with adjuvant radiotherapy. However, the median survival time of glioblastoma patients was not significantly prolonged, which was still less than 15 months [1]. Therefore, it is very necessary to study its potential molecular mechanism and develop new therapy strategies for gliomas.

Autophagy is a conserved self-protecting mechanism for the clearance of unwanted cellular components like incorrectly folded proteins and damaged organelles. In this process, cytoplasmic components are captured in double-membrane vesicles, named autophagosome,

and delivered to lysosomes for decomposition and recycling [2, 3]. It has been reported that autophagy may promote or inhibit tumorigenesis, suggesting of a context-dependent role of autophagy in cancer. The fate of cancer cells determined by autophagy depends upon tumor type, stage and genetic background [4, 5]. Generally, in tumorigenesis stage, by clearing the damaged organelles or protein aggregates to keep hemostasis, autophagy suppresses tumor initiation. However, in established and rapid expanding tumors, via strengthening stress tolerance and providing more nutrient and energy, autophagy plays important roles in supporting tumor cell survival. Studies have indicated capsaicin can induce autophagy in U251 glioma cells [6]. Especially, cancers with activating KRAS or HRAS mutations are mainly dependent upon autophagy and have a high basal level of autophagy even in growth conditions [7]. Intriguingly, substantial preclinical evidence shows that autophagy inhibition by using

chloroquine (CQ) or hydroxychloroquine improves clinical outcomes in cancer patients [8]. Although the reported positive clinical outcomes are encouraging for the role of autophagy inhibition in cancer therapy, attention needs to be paid to understand the potential contexts in which autophagy inhibition will be beneficial and those in which it could be damaging [8].

Golgi phosphoprotein 3 (GOLPH3) is a highly conserved protein mainly concentrated in mature peripheral membrane of Golgi, Golgi secretory tubules and vesicles, endosomes and plasma membrane [9]. GOLPH3 is participated in substances and vesicular transportation between Golgi and plasma, the Golgi and mitochondria structure maintenance, protein glycosylation, the division channel and contraction ring formation during mitosis [9]. In addition, GOLPH3 is found up-regulated in a variety of human solid tumors and involved in tumor progression [10, 11]. In our systemic studies previously, we identified GOLPH3 fosters GBM malignant progress via inhibiting EGFR endocytosis, promoting Wnt2b recycling and activating YB-1 [12-14]. Unexpectedly, when we examined whether GOLPH3 affect the products of exosome with transmission electron microscopy (TEM) in our previous study [15], we found that autophagosomes increased dramatically after GOLPH3 over-expression in glioma cells (data not shown), indicating that GOLPH3 may promote autophagic activity. Actually, GOLPH3 has been reported to promote the transformation of LC3 I to II under the induction of stress in the oxygen-glucose deprivation/reperfusion model [16]. However, the role and mechanism of GOLPH3 on cancer autophagy were largely unknown.

Prohibitins (PHB1 and PHB2) are highly conserved protein, which exist in many kinds of cells, mainly in mitochondria, nucleus and plasma membrane [17, 18]. PHB1/2 are two highly homologous subunits of the eukaryotic mitochondrial PHB complex. It has been found that PHB1 and PHB2 are involved in transcriptional regulation, cell cycle regulation, cell surface signaling, cellular senescence, apoptosis, regulation of sister chromatid condensation, and mitochondrial pleiotropic effects [19]. It is reported that PHB2-induced mitochondrial autophagy suppresses tubular epithelial cell damage and NLRP3 inflammatory body activa-

tion by regulating mitochondrial dysfunction [20]. In addition, mitochondrial inner membrane protein PHB2 promotes mitophagy as a key mitochondrial autophagy receptor by interacting with LC3-II via its LC3-interacting region (LIR) after mitochondrial depolarization and proteasome dependent mitochondrial outer membrane rupture [21]. Furthermore, PHB1 is reported to regulate mitochondrial autophagy in colon cancer [22]. However, the regulatory mechanisms of PHB1/2 on autophagy remain unknown.

In this study, we demonstrate GOLPH3 promotes glioma cell autophagy via PHB2, but not PHB1. Blocking autophagy by using CQ abolished the stimulating effect of GOLPH3 on glioma progression both *in vitro* and *in vivo*. Lastly, the protein levels of GOLPH3 and PHB2 showed positively associated with autophagy in glioma tissues. In general, our study showed that GOLPH3 promotes glioma progression by enhancing cell autophagy which is mediated by PHB2. The novel GOLPH3-PHB2-autophagy axis maybe a potential and promising therapeutic target for gliomas.

Materials and methods

Cell culture

Human glioblastoma cell line U251 and U87 were purchased from the cell bank of the Shanghai Branch of the Chinese Academy of Sciences. Cell lines were cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco) and grown in a humidified incubator with 5% CO_2 at 37°C. Sometimes, cells were cultured in Earle's Balanced Salts Solution (EBSS), medium for nutritional deprivation (ND) treatment.

Antibodies and reagents

Primary antibodies and reagents were used as follows: rabbit anti-GOLPH3 (Abcam); mouse anti-β-actin (Millipore); mouse anti-PHB1 (Santa Cruz); mouse anti-PHB2 (Santa Cruz); rabbit anti-LC3 (Cell signaling); mouse anti-Flag (Sigma); Protein A/G Agarose (Roche); sliver stain kit (Pierce); CQ (Sigma); rapamycin (Selleck).

Plasmids and siRNA

The lentiviral core plasmid pWPXLd-GOLPH3 was constructed by our laboratory [12].

pcDNA3.1-Septin2, pcDNA3.1-ENO3, shPHB1, shPHB2, shNC were constructed by Shanghai Genechem Company. The PHB1 and PHB2 overexpression plasmids were kindly gifted by Professor Beth Levine (The University of Texas Southwestern Medical Center). The human GOLPH3 siRNA oligo was synthesized and sequenced by Shanghai GenePharma Technology Company.

Negative control siRNA oligo sense: 5'-UUCUC-CGAACGUGUCACGUdtdt-3'; Antisense: 5'-ACG-UGACACGUUCGGAGAAdtdt-3'; GOLPH3-siRNA oligo792 sense: 5'-GUUAAGAAAUGUACGGG-AATT-3' antisense: 5'-UUACAGAUUACCUUUC-UUGTT-3'.

Western blot

Protein lysates were harvested with RIPA lysate containing 1% cocktail and separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system. Then protein was electro-transferred to PVDF membranes (Millipore), and incubated with primary and secondary antibodies. Fluorescent signals were visualized with the Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.) and exposed by ChemiDoc Touch (BIO-RAD).

Co-immunoprecipitation (Co-IP)

U251 cells were lysed in IP lysis buffer (Beyotime) with 1% cocktail (CWBIO). Cell lysates (1.5 mg) were incubated with 1 μg of indicated antibodies and Protein A/G beads (Roche) overnight. Immunoprecipitation complexes were boiled and resolved by 12% SDS-PAGE for immunoblotting analysis.

Protein mass spectrometry

Separated protein mixture was detected at the protein platform of Institute of Biochemistry and Cell Biology, Shanghai Academy of Biological Sciences, Chinese Academy of Sciences.

Immunofluorescence

U251 cells were seeded on coverslips in 24 plates and fixed with 4% paraformaldehyde for 20 minutes after cells spreading. Next, the cells were infiltrated by using 0.5% Triton X-100 after washing with PBS. Blocked with 3% BSA buffer for 2 hours and incubated overnight in primary antibody. Then, the cells were incubated for 2 hours with second antibody and DAPI was used to stain the nucleus.

Transmission electron microscopy (TEM)

Transmission electron microscopy assay is based on the previous reports [23]. Cell mass were fixed by 2.5% glutaraldehyde for 2 h. After being washed with 0.1 M phosphoric acid rinse solution, the cell mass were fixed by 1% osmium acid for 2 h. The samples were then stained with 1% filtered uranyl acetate, dehydrated in increasing concentrations of ethanol, infiltrated and embedded in epoxy resin for 2-3 h in 37°C. Thereafter, the samples were conducted microtome slicing and stained with 3% uranyl acetate and lead citrate. This experiment was performed in the Center of TEM, Xuzhou Medical University.

EdU incorporation assay

The cell growth was estimated by EdU incorporation assay (RiboBio) according to the manufacturer's instruction and our previous study [12].

Tissue samples

The human glioma tissue samples were provided by the Affiliated Hospital of Xuzhou Medical University. This study was approved by the Ethics Committee of Xuzhou Medical University and informed consent was obtained from each patient in this study.

Public available clinical data analysis

Data of GOLPH3, PHB1/2 and LC3-II in TCGA were extracted from Betastasis (http://www.betastasis.com/) and those in CGGA were extracted from http://www.cgga.org.cn/.

Glioma intracranial mouse model

All experimental protocols were carried out according to Xuzhou Medical University guidelines for animal research and were approved by Institutional Animal Use Committee. Five-week-old male nude mice, purchased from the GemPharmatech Co., Ltd., were used to establish intracranial glioma xenografts. GOLPH3-overexpressing or Vector U87 cells labeled with luciferase were used for the mouse xenograft

model (n=8 per group). A total of 8×10^5 cells were intracranially injected into each mouse. The mice were then treated with intraperitoneal injection of PBS or CQ (25 mg kg^{-1} d^{-1}) every two days, which was lasted for 4 weeks. Bioluminescence imaging was taken on day 7, 14 and 21 after implantation to monitor intracranial tumor growth. Lastly, Kaplan-Meier survival curves were plotted to show survival.

Statistical analysis

The results were representative of experiments repeated at least three times. The results were statistically processed using software SPSS18.0. Statistical comparisons were performed using Student's t-test with two tails, ANOVA or nonparametric test for multiple comparisons followed by Tukey t-test for post hoc pairwise comparisons. We assessed differences in the human glioma data using the Pearson correlation test. Survival analysis was conducted using the Kaplan-Meier model with a two-sided log-rank test. *P* values less than 0.05 were considered statistically significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Results

GOLPH3 promotes autophagy of glioma cells

We previously reported that GOLPH3 is up-regulated in glioma samples and promotes glioma progression [12, 25]. Similarly, by analyzing the TCGA and CGGA datasets, we found that the GOLPH3 mRNA levels in glioma were up-regulated significantly (**Figure 1A**, **1B**). In addition, patients with high GOLPH3 level exhibit worse prognosis (**Figure 1C**).

To investigate the effect of GOLPH3 on autophagy, we generated GOLPH3 down-regulation (**Figure 1D**) and over-expression (**Figure 1E**, **1F**) U251 GBM cells via lentivirus system. As shown in **Figure 1D** and **1E**, according to percentage of GFP positive cells and western blot results, the efficiency of GOLPH3 downregulation and overexpression was very high. In addition, the exogenous GFP tagged GOLPH3 exhibited specific localization in the cytoplasm (**Figure 1F**), indicating that the U251 cells with downregulation and overexpression of GOLPH3 were successfully prepared.

During the development of autophagy, the autophagy-related proteins undergo characteristic changes, among which LC3 is the most widely studied. Upon induction, the LC3 precursor was processed into LC3-I, which subsequently was modified to form LC3-II with phosphatidylethanolamine binding to the surface of the autophagic vacuole membrane. Therefore, LC3-II is localized to the pre-autophagic and autophagic bodies and is widely regarded as a marker of autophagy [24]. We interestingly found that the level of LC3-II was significantly decreased after GOLPH3 stably down-regulation with or without Rapmycin or ND induction (**Figure 2A**, **2B**). On the contrary, the level of LC3-II was markedly increased after GOLPH3 overexpression with or without Rapmycin induction (**Figure 2A**). Furthermore, down-regulation of GOLPH3 induced LC3-II decrease obviously abolished after autophagy inhibitor CQ treatment (**Figure 2B**). Because the stable GOLPH3 down-regulation glioma cells harboring GFP tag, which cannot be used to detect the GFP-LC3 clusters, we therefore down-regulated GOLPH3 with small interfering RNA (**Figure 2C** left). We found that GOLPH3 down-regulation decreased the GFP-LC3 clusters (autophagic bodies) with or without ND challenge (**Figure 2C** right).

Moreover, the results of TEM, the gold standard method for autophagy detection, showed that down-regulation of GOLPH3 reduced the number of autophagosomes and autolysosomes, while overexpression of GOLPH3 increased it in U251 cells with or without Rapmycin induction (**Figure 2D-F**). Collectively, our data strongly suggest that GOLPH3 promotes autophagy in U251 cells.

Autophagy partially mediates the promoting effect of GOLPH3 on glioma progression

Due to the hypoxia and nutrient deficiency microenvironment, GBM has high autophagy activity [26] and autophagy plays an important role in the malignant progress of GBM [27-29]. We therefore performed EdU assay to determine whether autophagy is crucial for the stimulating effect of GOLPH3 on GBM cells growth. We discovered overexpression of GOLPH3 promoted GBM cell growth, while autophagy inhibitor CQ treatment partially abolished the promotion effect of GOLPH3 (**Figure 3A**, **3B**), indi-
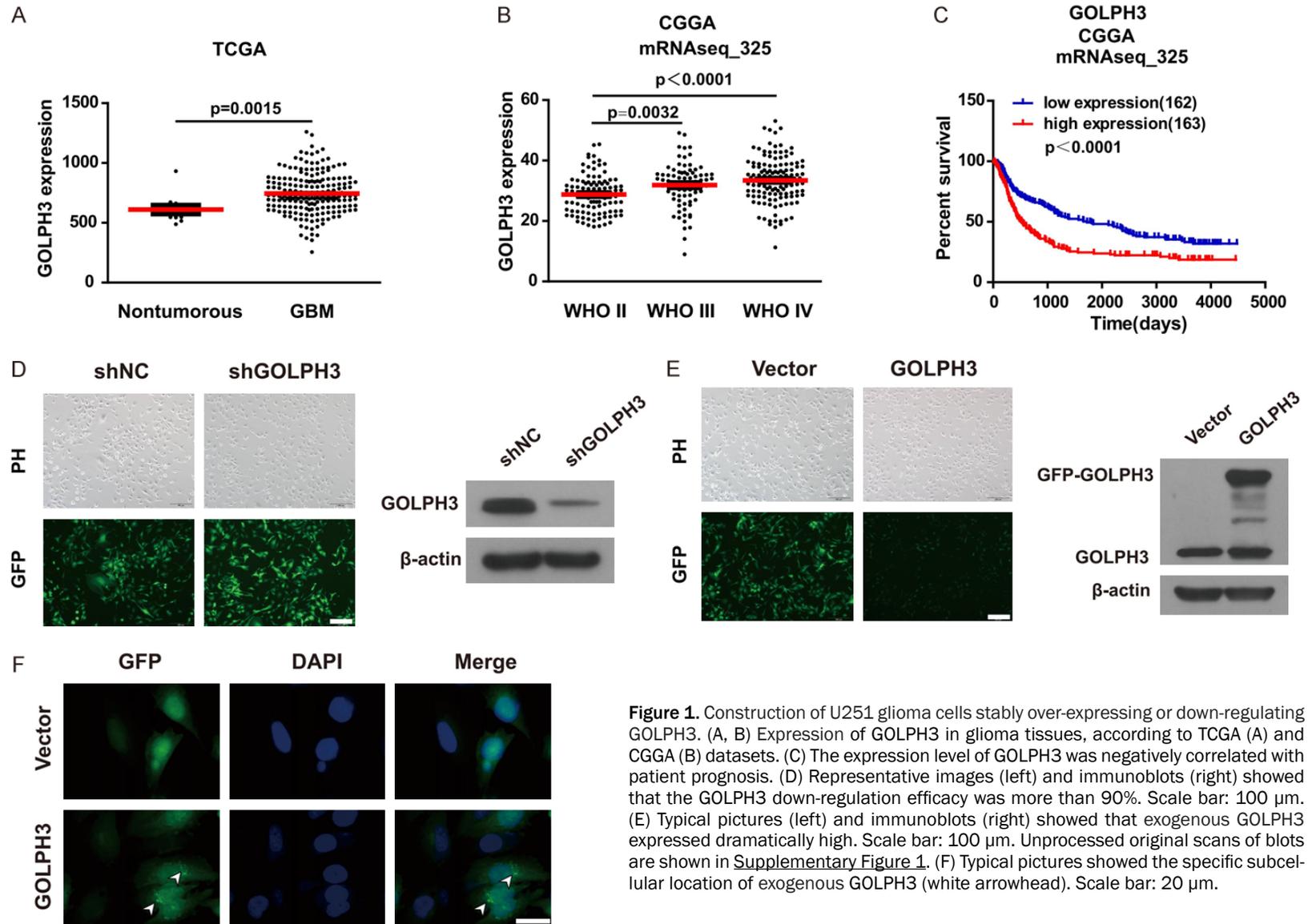
Figure 1. Construction of U251 glioma cells stably over-expressing or down-regulating GOLPH3. (A, B) Expression of GOLPH3 in glioma tissues, according to TCGA (A) and CGGA (B) datasets. (C) The expression level of GOLPH3 was negatively correlated with patient prognosis. (D) Representative images (left) and immunoblots (right) showed that the GOLPH3 down-regulation efficacy was more than 90%. Scale bar: 100 μm. (E) Typical pictures (left) and immunoblots (right) showed that exogenous GOLPH3 expressed dramatically high. Scale bar: 100 μm. Unprocessed original scans of blots are shown in Supplementary Figure 1. (F) Typical pictures showed the specific subcellular location of exogenous GOLPH3 (white arrowhead). Scale bar: 20 μm.
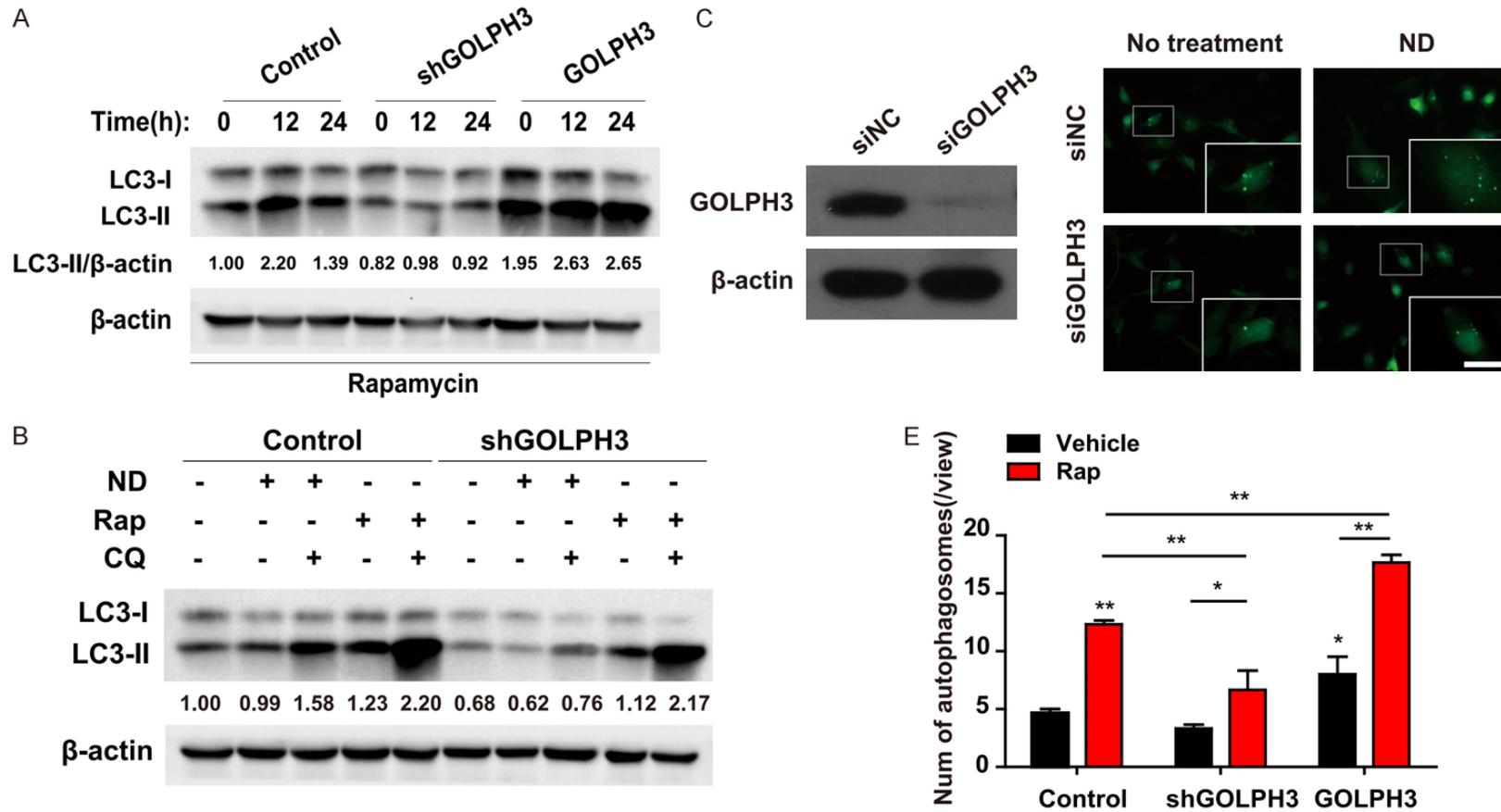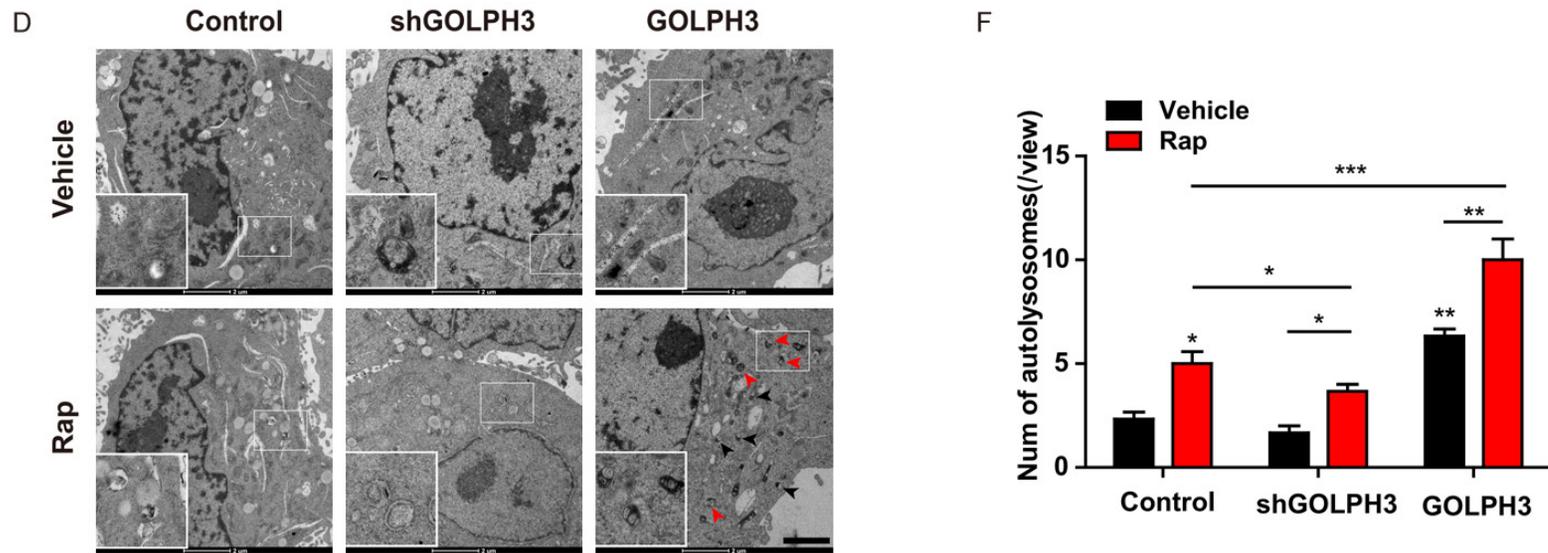
Figure 2. GOLPH3 promotes the autophagy of U251 cells. (A) Representative immunoblots showed that, compared with the control group, LC3-II level decreased significantly after GOLPH3 down-regulation, while increased after GOLPH3 over-expression with or without Rapamycin induction. Unprocessed original scans of blots are shown in Supplementary Figure 1. (B) Representative immunoblots showed that the decreased LC3-II level induced by GOLPH3 down-regulation can be restored after CQ treatment. Unprocessed original scans of blots are shown in Supplementary Figure 1. (C) Representative immunoblots showed the high reducing efficiency of siGOLPH3 in U251 glioma cells (left). (right) Representative images showed that the number of autophagic bodies (GFP-LC3 aggregates) decreased after GOLPH3 down-regulation either under basal conditions or ND induction (EBSS starvation for 4 h). Scale bar: 20 μm. (D) Representative transmission electron microscopy images showed that down-regulation of GOLPH3 decreased the number of autophagic bodies, while over-expression of GOLPH3 increased it in glioma cells with or without rapamycin treatment for 12 h. Scale bar: 2 μm. Red arrowhead indicated autophagosomes; Black arrowhead indicated autolysosomes. (E, F) Statistical analysis of autophagosomes (E) and autolysosomes (F). ND: Nutrition deprivation. Rap: Rapamycin. CQ: chloroquine.
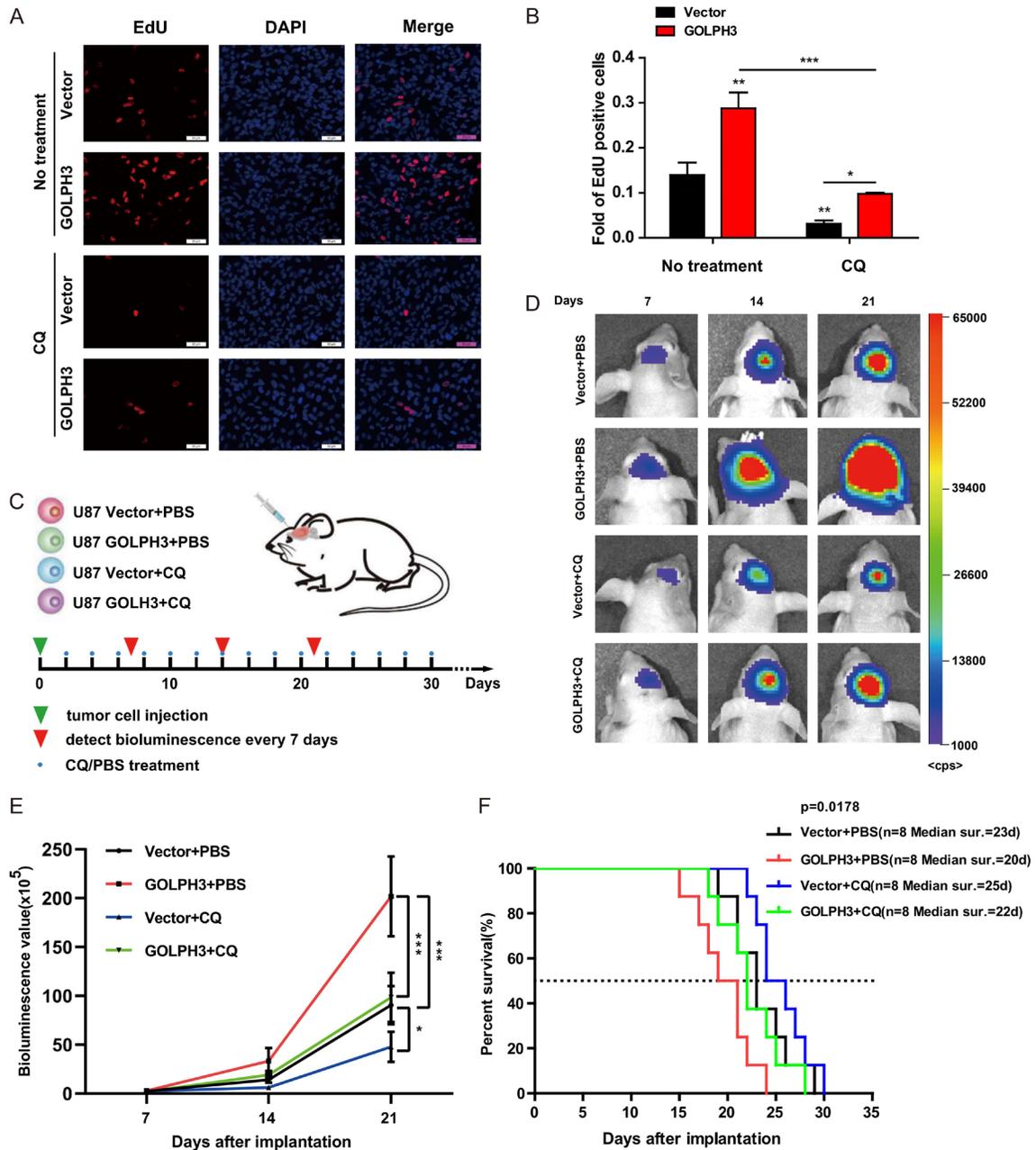
Figure 3. Autophagy partially mediates the promoting effect of GOLPH3 on glioma progression. A. Typical pictures of EdU assay showed that inhibiting autophagy by CQ abolished the promoting effect GOLPH3 on U251 cell growth. DAPI (blue) was used to stain nucleus and EdU (red) showed the incorporated cells. Scale bar, 50 μm. B. Quantitative analysis of the relative cell proliferation rate. C. Schematic illustration of the in vivo experimental workflow. Mice were intracranially injected with GOLPH3 over-expression or control U87-GFP-luci cells and subsequently treated by intraperitoneal injection of CQ or PBS after implantation (n=8 per group). D. Typical bioluminescence images. E. Quantitative analysis of the photon flux. Data are presented as mean ± SEM (n=8 mice for each group, two-way ANOVA test). F. Kaplan-Meier curve showed the overall survival time of glioma-bearing mice (n=8 mice for each group, Kapla-Meier model with two-sided log-rank test).

cating that autophagy is crucial for the promotion effect of GOLPH3 on glioma cell progression.

In addition, we sought to further examine whether autophagy functions as a key factor in promotion effect of GOLPH3 on glioma progres-

sion in vivo. Orthotopic glioma nude mouse models were constructed by intracranially injecting GOLPH3-overexpressing or Vector U87 cells labeled with luciferase (n=8 per group). Two days later, CQ (25 mg kg^{-1} d^{-1}) or PBS was intraperitoneally injected into nude mice every two days, which was lasted for 4 weeks (**Figure 3C**). The bioluminescence imaging analyses exhibited that CQ treatment slowed down the tumor growth and abolished the promoting effect of GOLPH3 on tumor growth (**Figure 3D**, **3E**). Significantly, survival curve analysis exhibited that CQ treated mice had much longer survival time than PBS treated mice (**Figure 3F**). In aggregate, these results suggest autophagy plays a pivotal role in the promotion effect of GOLPH3 on glioma progression *in vitro* and *in vivo*.

Mass spectrometric analysis of proteins interacting with GOLPH3

Next, we explored the molecular mechanism of GOLPH3 on autophagy by identifying GOLPH3-interacting proteins using mass spectrometric analysis after immunoprecipitating exogenous GOLPH3 (**Figure 4A**). After a series of screening and analysis, 42 proteins were identified, which bound to GOLPH3 with 1.5-fold higher than those bound to IgG control (**Figure 4B**). According to the main function of these GOLPH3 interacting proteins, we found that GOLPH3 was involved in various cellular activities, such as metabolism, cell morphology, transcription, cell cycle, and protein synthesis and modification (**Figure 4C**).

According to the literature, we chose Septin 2, ENO3 and prohibitin (PHB1/2), which were reported to be involved in cell autophagy [21, 30, 31] to explore the molecular mechanism of GOLPH3 in autophagy. As shown in **Figure 4D-F**, after over-expressing Flag-Septin2 or Flag-ENO3 into U251 cells (**Figure 4D**), both of Flag-Septin2 and Flag-ENO3 interacted with endo-GOLPH3, either examined by forward or reverse co-IP experiment (**Figure 4E** and **4F**), indicating the credibility of protein mass spectrometry results. Unfortunately, examined by immunofluorescence, GOLPH3 showed few co-localization with Septin2 or ENO3 (**Figure 4G**). However, endogenous PHB1/2 not only interacted with endogenous GOLPH3, but also co-localized with GOLPH3 very well (**Figure 4H**, **4I**),

indicating that PHB1/2 may mediate the promoting effect of GOLPH3 on autophagy in glioma cells.

PHB2 is required for the promoting effect of GOLPH3 on autophagy

To address the above question, we firstly examined the possibility that GOLPH3 regulates PHB1/2. The results showed that overexpression of GOLPH3 increased both PHB1 and PHB2 protein levels (**Figure 5A**). However, over-expression of PHB1 or PHB2 could not change the GOLPH3 protein level (**Figure 5B**). Therefore, PHB1 and PHB2 are the downstream molecules regulated by GOLPH3.

Next, we over-expressed PHB1 or PHB2 into glioma cells to determine the effect of PHB1/2 on autophagy. The results showed that there was no significant difference in the protein levels of LC3-II between the PHB1 over-expression and the control cells with or without ND induction (**Figure 5C**). However, over-expressing PHB2 greatly elevated the levels of LC3-II (**Figure 5D**), indicating that PHB2, but not PHB1, is involved in positive regulation of autophagy in glioma cells.

To investigate whether GOLPH3 promotes autophagy via PHB2 mediation, we constructed shPHB2 stably expressing glioma cells and found that PHB2 knocking-down decreased the protein level of LC3-II (**Figure 5E**, **5F**), in line with the previous report [21]. In addition, the level of LC3-II was reduced after PHB2 knocking-down in GOLPH3 over-expression cells, indicating that down-regulation of PHB2 abolished the promoting effect of GOLPH3 in autophagy (**Figure 5F**). Furthermore, to identify how GOLPH3 interacts with PHB2 affected the cell autophagy, we performed co-IP experiment to test whether GOLPH3 could recruit PHB2 and LC3 to promote autophagy. We found that endogenous PHB2, but not the normal control IgG, interacted with LC3-II and the interaction increased after GOLPH3 over-expression (**Figure 5G**), indicating that GOLPH3 recruited and enhanced the interaction between PHB2 and LC3-II and then to promote autophagy. Therefore, our results suggested that PHB2 mediates the promoting effect of GOLPH3 on autophagy in glioma cells.
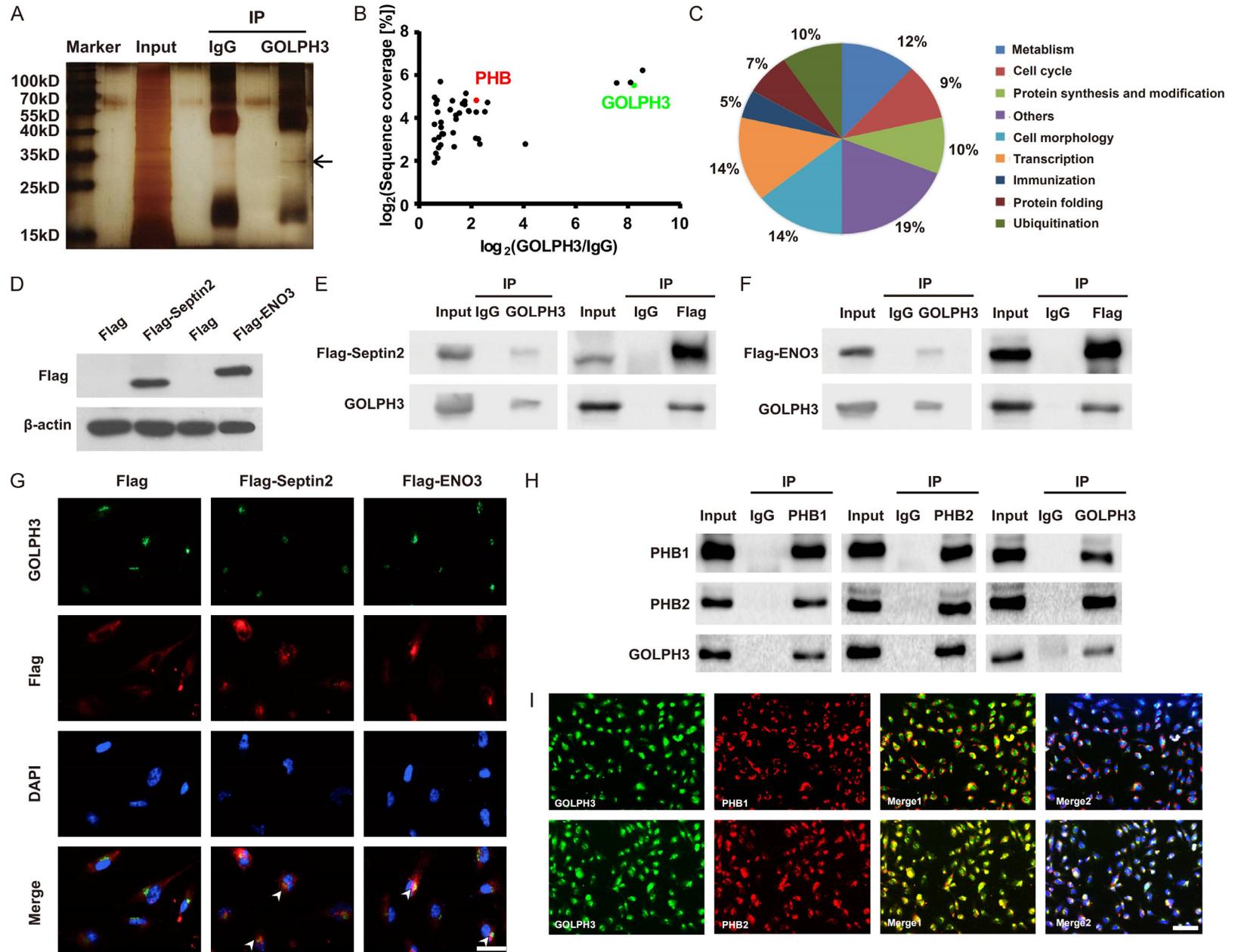
Figure 4. GOLPH3 interacts with PHB1/2. A. Silver staining of gel after immunoprecipitating GOLPH3. The arrow pointed specific band around 34 kDa was presumed to be GOLPH3. B. Mass spectrometry analysis showed that there were 42 proteins bound to GOLPH3 with more than 1.5 fold higher level than that bound to control IgG. C. The biological function distribution of the GOLPH3 bound proteins. D. Flag-Septin2 and Flag-ENO3 were over-expressed successfully in U251 cells. Uncropped images of blots are shown in Supplementary Figure 1. E, F. Forward and reverse Co-IP experiment proved that both Flag-Septin2 and Flag-ENO3 interacted with GOLPH3. Uncropped images of blots are shown in Supplementary Figure 1. G. GOLPH3 (green) showed few co-localization with Septin2 and ENO3 (white arrowhead). H. Forward and reverse Co-IP experiment showed that both PHB1/2 interacted with GOLPH3. Uncropped images of blots are shown in Supplementary Figure 1. I. GOLPH3 co-localized with PHB1/2 very well. Scale bar: 100 μm.

The correlation of PHB2, GOLPH3 and autophagy in human glioma tissues

To examine whether GOLPH3 and PHB2 were associated with autophagy in gliomas, we analyzed the TCGA and CGGA datasets. We found that the mRNA levels of PHB2 in gliomas were up-regulated significantly (**Figure 6A**, **6B**). In addition, patients with high PHB2 levels exhibited worse prognosis in CGGA database (**Figure 6C**), similar to that of GOLPH3 (**Figure 1C**). Notably, significant positive correlations were found between the protein levels of GOLPH3 and PHB2 in glioma samples (r=0.5060, $P<0.0001$, **Figure 6D**). Interestingly, the levels of LC3-II also showed positive correlation with GOLPH3 (r=0.4704, $P<0.0001$, **Figure 6E**) and PHB2 (r=0.4723, $P<0.0001$, **Figure 6F**) respectively in TCGA dataset. Similar results were obtained in CGGA dataset (**Figure 6G-I**). Excitingly, we found the positive correlation of GOLPH3, PHB2 and LC3-II with each other at protein levels in our glioma samples (**Figure 6J-M**). Taken together, these results indicated the expression of GOLPH3 and PHB2 are associated with autophagy for glioma patients.

Discussion

Autophagy is a self-protection mechanism of cells, which plays a significant role in cell self-renewal as well as homeostasis maintenance. Although it prevents tumorigenesis, it promotes established tumor progression and was involved in tumor chemo- and radio-therapy resistance [32]. The first clinical trial of CQ on tumor was performed in GBM patients [27] and obtained positive clinical results [8]. However, the molecular mechanism of autophagy remains largely unknown and attention should be paid to understand the potential environment in which autophagy inhibition will be beneficial [8]. We discovered GOLPH3 enhanced autophagy in glioma cells and then promoted cell growth in this study. The effect of GOLPH3 on autophagy was mediated by PHB2, one receptor of mitophagy.

GOLPH3 promotes cell proliferation through enhancing growth-factor-induced the mammalian target of rapamycin (mTOR) signaling and alters the response of cancer cells to rapamycin, an mTOR inhibitor *in vivo* [10]. Our previous studies have shown GOLPH3 inhibits endocytosis and degradation of EGFR and thereby activating the PI3K-AKT-mTOR signaling to promote GBM cell proliferation [12]. We further discovered although GOLPH3 does not affect the number of exosomes, it regulates the miRNA secretion in exosomes [15]. In 2016, Li. et al reported that GOLPH3 promotes transformation of LC3-I to LC3-II by up-regulating ROS level in mouse neuroblastoma cells after oxygen-glucose deprivation induction [16]. Wang et al reported that GOLPH3 over-expression inhibits apoptosis induced by paclitaxel in HeLa cells via promoting autophagy [33]. In this study, we discovered GOLPH3 promoted autophagy of GBM cells after induction either by starvation or by rapamycin, in line with the above studies. In addition, CQ treatment partially blocked the promotion effect of GOLPH3 on glioma progression both *in vitro* and *in vivo*. According to the literature, autophagy promotes established tumor progression by providing energy to tumor cells under hypoxia and nutrient deficiency microenvironment. Therefore, we deduce that GOLPH3 promotes glioma growth by enhancing autophagy to provide energy to tumor cells.

Prohibitins, including PHB2 and its homologue PHB1, are scaffold proteins that regulate many signaling pathways controlling cell survival, metabolism and inflammation [34, 35]. Both PHB1/2 can regulate the development of autophagy [21, 22]. Although both PHB1 and PHB2 interacted with GOLPH3, only PHB2 was
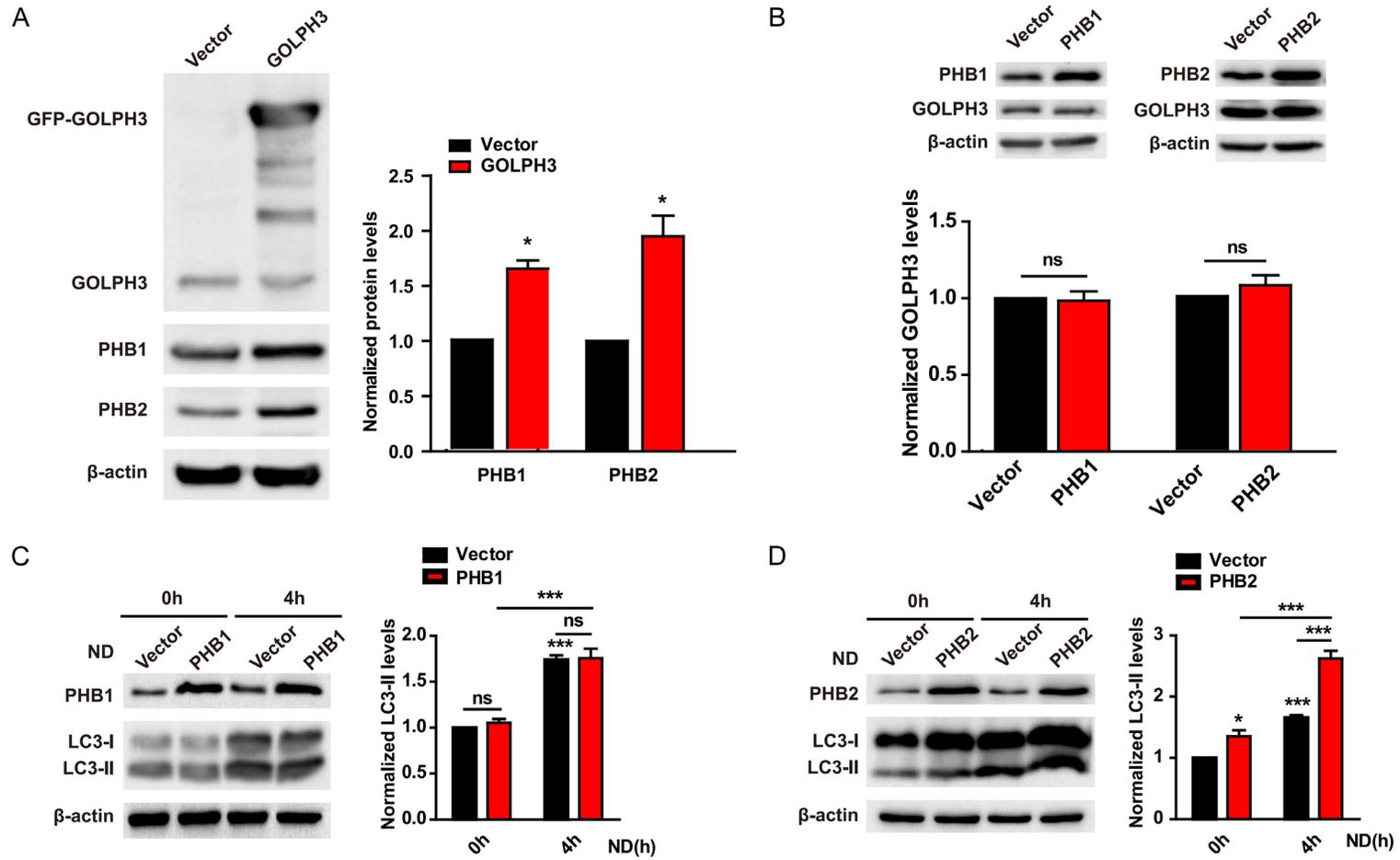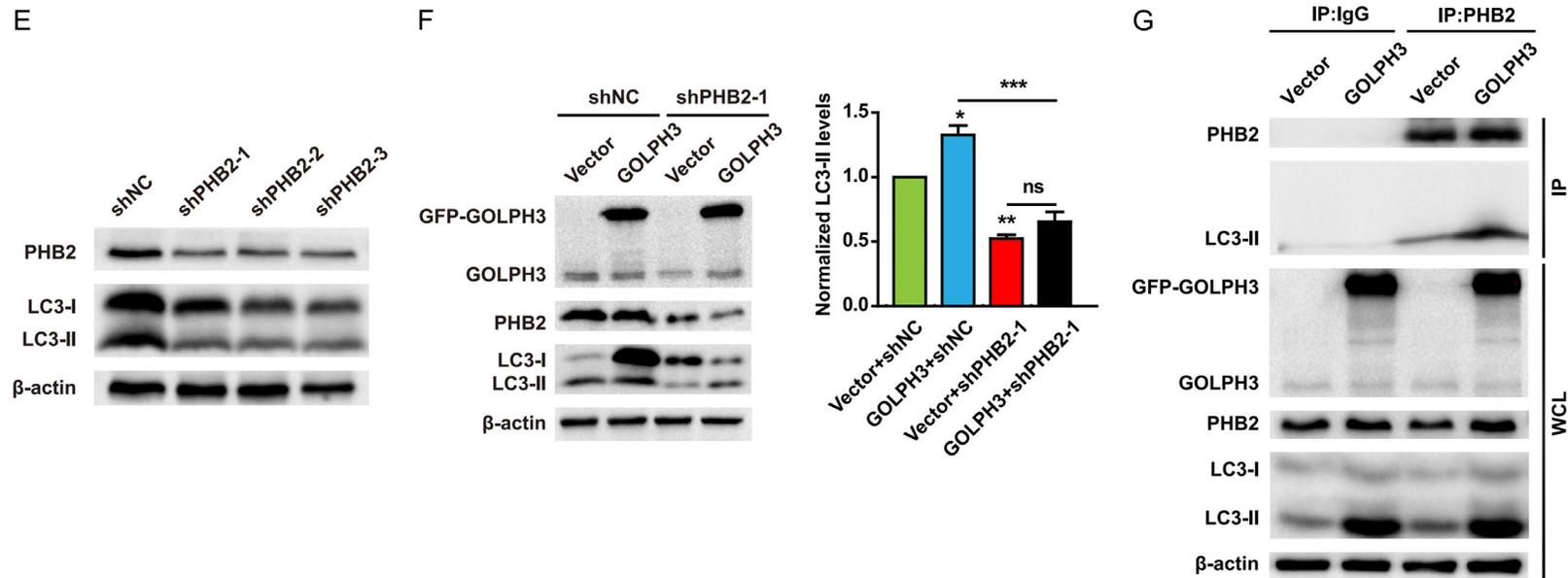
Figure 5. PHB2 is required for the promoting effect of GOLPH3 on autophagy. A. Typical immunoblots (left) and quantitative results (right) showed the protein levels of PHB1/2 increased after GOLPH3 over-expression. Uncropped images of blots are shown in Supplementary Figure 1. B. The protein level of GOLPH3 showed unchanged after either over-expression of PHB1 or PHB2. Uncropped images of blots are shown in Supplementary Figure 1. C. Representative immunoblots (left) and quantitative results (right) showed that the LC3-II level increased significantly after ND induction, while PHB1 over-expression did not affect them with or without ND induction. Uncropped images of blots are shown in Supplementary Figure 1. D. Representative immunoblots (left) and quantitative results (right) showed that the LC3-II level increased significantly after ND induction and PHB2 over-expression further increase them with or without ND induction. Uncropped images of blots are shown in Supplementary Figure 1. E. Representative immunoblots showed the down-regulation efficacy of three shPHB2 in U251 glioma cells and the levels of LC3-II significantly decreased after PHB2 down-regulation. Uncropped images of blots are shown in Supplementary Figure 1. F. Representative immunoblots (left) and quantitative results (right) showed the increase of LC3-II level induced by GOLPH3 over-expression was abolished after down-regulation of PHB2. Uncropped images of blots are shown in Supplementary Figure 1. G. Representative immunoblots showed that GOLPH3 promotes the binding of PHB2 with LC3-II. The normal IgG was used as IP control. Uncropped images of blots are shown in Supplementary Figure 1.
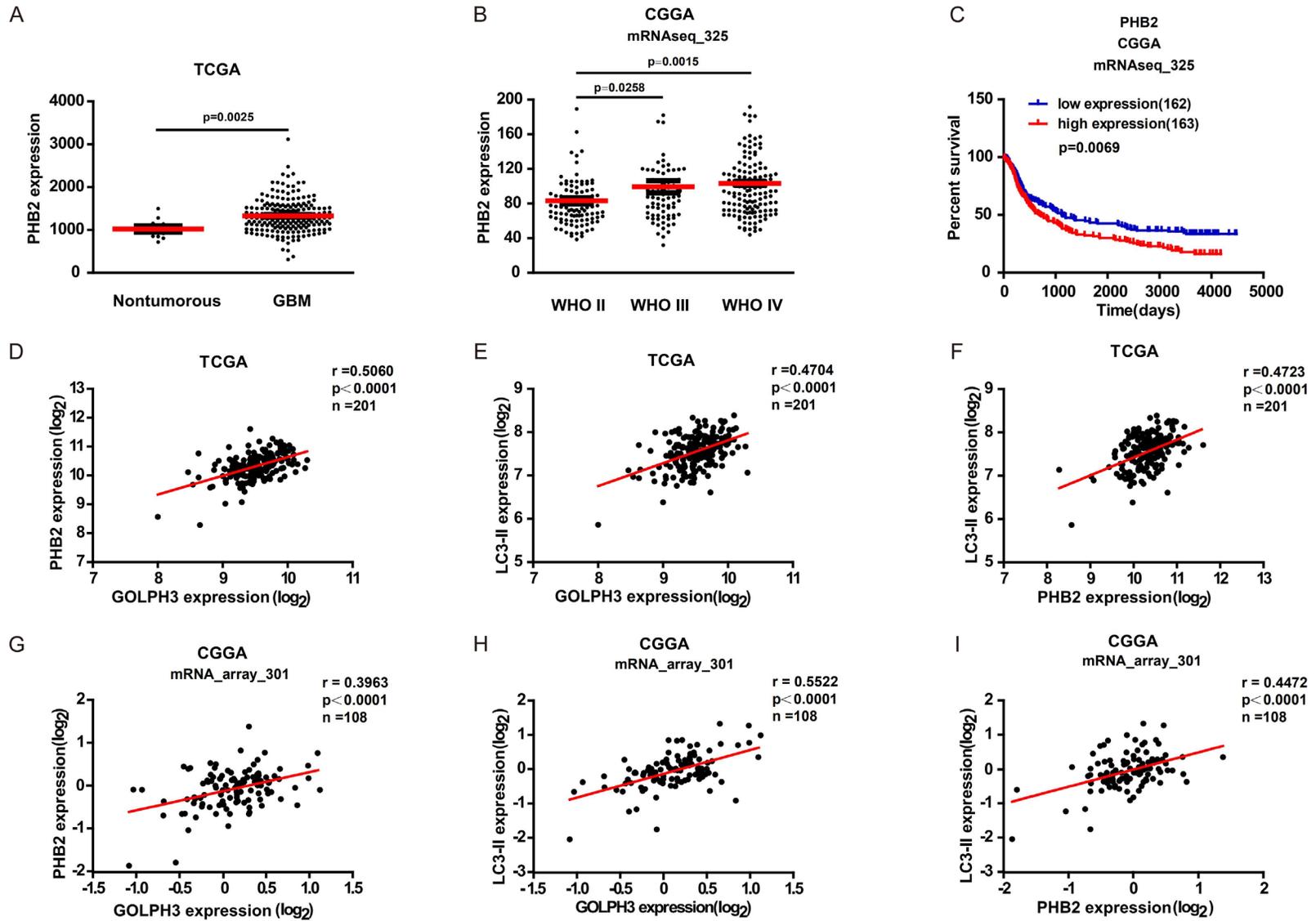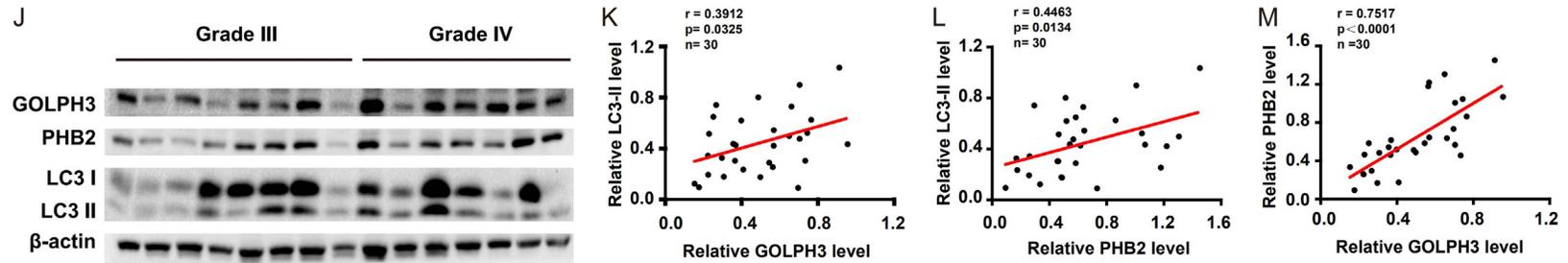
Figure 6. The correlation of GOLPH3, PHB2 and autophagy in glioma tissues (A&B) Expression of PHB2 in glioma tissues, according to TCGA (A) and CGGA (B) datasets. (C) According to CGGA dataset, the expression level of PHB2 was negatively correlated with patient prognosis. (D, E, G, H) GOLPH3 positively correlated with PHB2 (D, G) and LC3-II (E, H) in TCGA (D, E) and CGGA (G, H) datasets. (F, I) The correlation analysis of PHB2 with LC3-II in TCGA (F) and CGGA (I) datasets. (J) Representative immunoblots showed the protein levels of GOLPH3, PHB2, LC3-II extracted from glioma tissues. Uncropped images of blots are shown in Supplementary Figure 1. (K-M) Quantitative analysis of the correlation of GOLPH3 with PHB1/2 or LC3-II.

found to promote the autophagy of glioma cells in our system. Moreover, down-regulation of PHB2 not only inhibited autophagy but also abolished the promoting effect of GOLPH3 on autophagy. It is reported that even PHB1/2 are localized in the mitochondrial membrane, PHB2 has been shown to contain a non-cleavable mitochondrial targeting sequence at the N-terminus. On the contrary, human PHB1 has no typical mitochondrial targeting sequence. This difference maybe the reason for that PHB2, but not PHB1, promotes the autophagy.

Besides mitochondria, PHB2 also presents in the nucleus, endoplasmic reticulum, and plasma membrane [18]. Similarly, besides trans-Golgi network, GOLPH3 is also located at mitochondria and maintained mitochondrial homeostasis by regulating lipid shuttle during mitochondrial stress [36]. Previously, we found GOLPH3, as a scaffold protein, interacts with JAK2-STAT3 to promote GBM cell proliferation [25]. Wang reported that Bax inhibitor-1 interacts with and recruits PHB2 into mitochnodria to preserves mitochondrial homeostasis in acute kidney injury [37]. We thus deduce GOLPH3 maybe act as a scaffold protein to recruit PHB2 to promote autophagy. As expected, we found that GOLPH3 over-expression recruited and enhanced PHB2 and LC3-II interaction, which may promote autophagy consequently.

Some studies reported the relationship between PHB and cancer, such as gastric cancer, papillary thyroid carcinoma, esophageal squamous cell carcinoma, colorectal cancer, high-grade breast cancer, human prostate cancer, human bladder cancer and blood tumor [9]. The expression of PHB2 protein and mRNA increased in human ovarian cancer tissues. In our study, both the levels of GOLPH3 and PHB2 were up-regulated in glioma tissues and exhibited positive correlation with each other. Furthermore, both proteins also positively correlated with LC3-II, the molecular marker of autophagy, indicating that both proteins are involved in autophagy and then glioma malignant progression.

In summary, we found that Glogi protein GOLPH3 promotes glioma autophagy and the subsequent malignant progression. Mechanistically, the effect of GOLPH3 on autophagy was mediated by PHB2, the mitochnodrial autopha-

gy receptor. Our study demonstrates that autophagy plays a pivotal role in the development of GBM with GOLPH3 high level and inhibiting autophagy may benefit this type of gliomas. In addition, GOLPH3-PHB2-autophagy axis maybe a potential therapeutic target of gliomas.

Acknowledgements

Disclosure of conflict of interest

None.

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GOLPH3 promotes glioma autophagy through PHB2
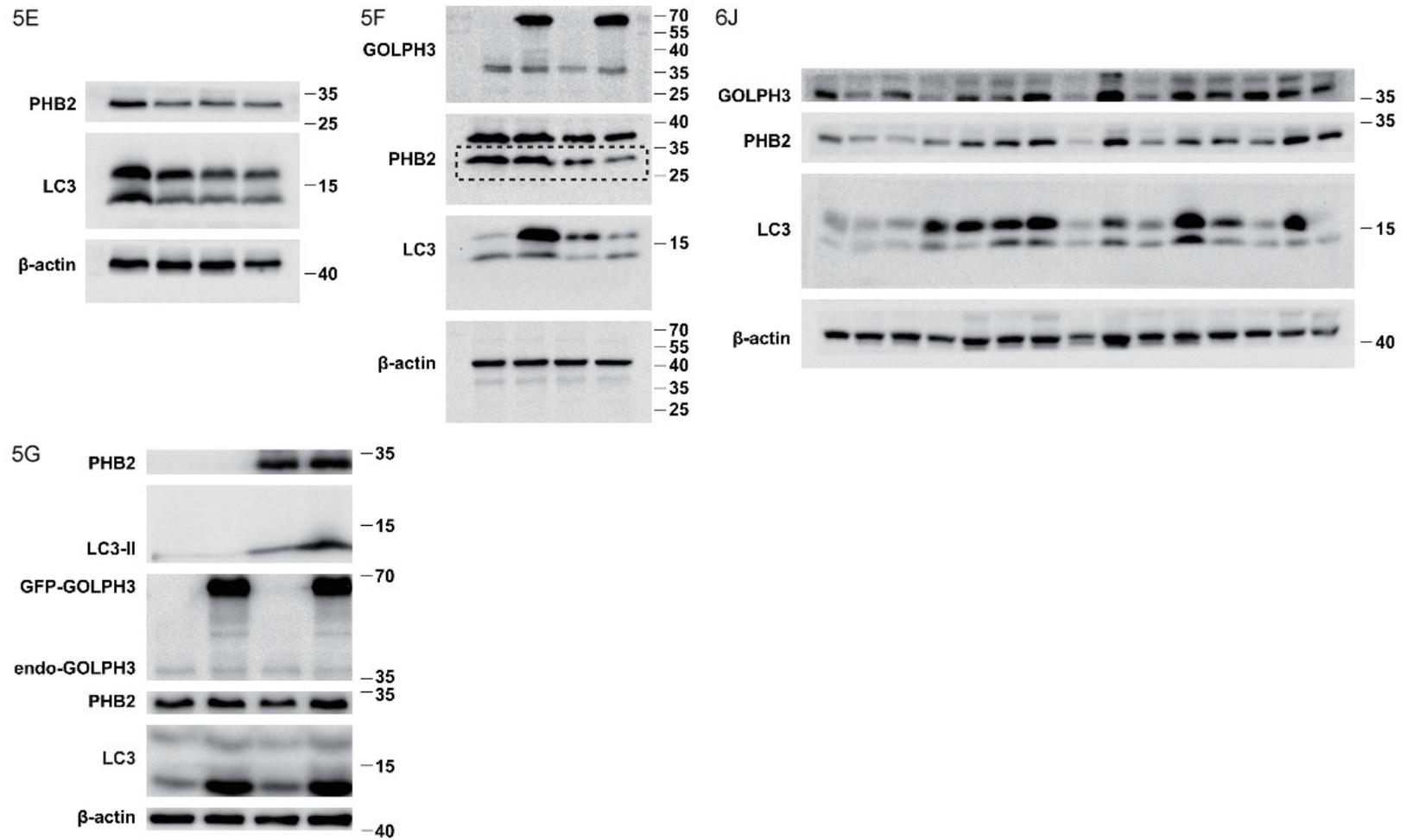
Supplementary Figure 1. Supplementary Figure Uncropped images of blots. Uncropped images of western blots shown in each figures were provided. Rectangles in 4H, 5C, 5F indicated the cropping of blots.