

## Original Paper

# Golgi Phosphoprotein 3 Promotes Wls Recycling and Wnt Secretion in Glioma Progression

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Xiuping Zhou<sup>a,b,d</sup> Rutong Yu<sup>a,b,d</sup><sup>a</sup>Institute of Nervous System Diseases, Xuzhou Medical University, Xuzhou, <sup>b</sup>Brain Hospital, Affiliated Hospital of Xuzhou Medical University, Xuzhou, <sup>c</sup>The Graduate School, Xuzhou Medical University, Xuzhou, <sup>d</sup>Jiangsu Center for the Collaboration and Innovation of Cancer Biotherapy, Cancer Institute, Xuzhou Medical University, Xuzhou, China**Key Words**

Glioma • GOLPH3 • Wls Recycling • Wnt secretion • Proliferation

**Abstract**

**Background/Aims:** Golgi phosphoprotein 3 (GOLPH3) plays pro-malignancy roles in several types of cancer. However, the molecular mechanism underlying GOLPH3 promoting tumor progression remains poorly understood. **Methods:** The expression of GOLPH3 and Wntless (Wls) in glioma tissues was examined by western blotting and immunohistochemistry. EdU incorporation assay and colony formation assay was used to examine the cell growth ability. The effect of GOLPH3 on Wls recycling, Wnt secretion and  $\beta$ -catenin activity was detected using western blotting, immunofluorescence, RT-PCR, ELISA or luciferase assay. **Results:** The protein levels of GOLPH3 and Wls were upregulated and positively correlated with each other in human glioma tissues. The promoting effect of GOLPH3 on glioma cell proliferation was partially mediated by Wls. In addition, GOLPH3 interacted with Wls and GOLPH3 down-regulation drove Wls into lysosome for degradation, inhibiting its recycling to golgi and the plasma membrane. Importantly, GOLPH3 down-regulation inhibited Wnt2b secretion and decreased  $\beta$ -catenin level and transcription activity. **Conclusions:** This study provides a brand new evidence that GOLPH3 promotes glioma cell proliferation by facilitating Wls recycling and Wnt/ $\beta$ -catenin signaling. Our findings suggest a rationale for targeting the GOLPH3-Wls-Wnt axis as a promising therapeutic approach for glioblastoma.

**Introduction**

Gliomas, the largest group of primary brain tumors, are histologically classified as grade I-IV by World Health Organization. Grade I and II are low-grade gliomas, while high-grade gliomas (grade III and IV) are malignant and aggressive with extremely proliferative and

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invasive capacity [1]. Despite the advances in surgery and adjuvant therapy, the overall prognosis for patients with malignant gliomas remains dismal, emphasizing the need for an in-depth understanding of the molecular pathogenesis and finding key molecular targets for the effective therapy [2].

Golgi phosphoprotein 3 (GOLPH3, also referred to as GPP34, GMx33, MIDAS and Vps74p) is a highly conserved protein rich at the trans golgi network (TGN) and is implicated in antero- and retro-grade golgi trafficking and glycosylation [3-9]. In addition, GOLPH3 is involved in the DNA-PK-GOLPH3-MYO18A pathway to increase cell survival after DNA damage [10, 11]. Interestingly, recent studies indicated that GOLPH3 was highly expressed in malignant tumors, including breast, gastric, hepatocellular, glioma and non-small cell lung cancers, and was closely associated with poor prognosis of the tumor [12-18]. Our previous study reports that GOLPH3 is involved in glioma cell migration and invasion by regulating AKT-mTOR-YB1 pathway [19]. In addition, GOLPH3 was regulated by protein kinase D2 and was involved in glioma cell proliferation by using transient gain-of or loss-of function approaches [20]. Furthermore, we recently found that GOLPH3 promotes glioma progression via inhibiting endocytosis and degradation of EGFR, thereby activating PI3K-AKT-mTOR signaling pathway [21]. Interestingly, we and others [12] found that GOLPH3 interacts with VPS35, the main subunit of retromer complex. We therefore wonder whether GOLPH3 promotes glioma progression through regulating retrograde golgi trafficking.

Abnormal Wnt/ $\beta$ -catenin signaling in adults may contribute to disease such as glioblastoma, including cell proliferation [22] and invasion [23]. However, in spite of the importance of Wnt signaling in tumorigenesis, there is still short of druggable targets in the Wnt pathway to potentially inhibit tumor growth [24]. Wntless (Wls, also known as Evi and GPR177), the chaperone protein of Wnt secretion, is a highly conserved 7-pass transmembrane protein and is located at the compartment of the secretory pathway including the golgi apparatus, endosomes, and plasma membrane [25, 26]. As a Wnt cargo receptor, Wls shuttles palmitoylated Wnts from the endoplasmic reticulum to the plasma membrane. After exocytosis of Wnt, the unloaded Wls protein on the plasma membrane will subsequently be internalized into early endosome and followed by two different fates. One is delivered to lysosomes for degradation [27-29], and the other is retrieved from early endosome to TGN, or directly to cell membrane for reusing. It is generally well accepted that membrane trafficking from early endosome to TGN or plasma membrane is driven by the retromer complex, which includes five proteins, namely SNX1/2, SNX5/6, Vps26, Vps29, and Vps35 in mammalian cells [30-33]. It was shown that inhibition of retromer function through depleting its subunit VPS35 destabilizes Wls by impeding its recycling after internalization and that retromer-mediated recycling of the Wls is essential for proper secretion of the Wnt morphogens. Given that GOLPH3 interacted with VPS35, it is rational that manipulation of GOLPH3 may affect Wls recycling, Wnt secretion and function, leading to cancer-relevant activities [6]. However, up to date, the direct evidence supporting this hypothesis still lacks. In this study, we found that GOLPH3 forms a protein complex with Wls and VPS35, facilitates Wls recycling, promotes Wnt secretion, and then activates  $\beta$ -catenin, leading to glioma cell proliferation.

## Materials and Methods

### *Glioma and nontumorous samples*

A total of 24 human glioma samples and 12 nontumorous brain tissues (decompressive surgery) for western blotting examination were obtained from Affiliated Hospital of Xuzhou Medical University. All of the glioma samples used in this study were astrocytomas, which was histologically diagnosed according to the World Health Organization grading system. All the glioma and nontumorous brain tissues had been collected immediately after surgical resection and stored in  $-80^{\circ}\text{C}$ . Written informed consent was obtained from each patients prior to this study. All experimental protocols were approved by the Medical Ethical Committee of Xuzhou Medical University.

## Cell culture

HEK 293T, human glioblastoma cell line U251 and U87 were purchased from Shanghai Cell bank, Type Culture Collection Committee, Chinese Academy of Sciences. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% fetal bovine serum (BioInd) and grown in a humidified incubator with 5% CO<sub>2</sub> at 37°C.

## Antibodies and reagents

The following antibodies were used: rabbit anti-GOLPH3, rabbit anti-GOLPH3L, mouse anti-LAMP1, mouse anti-TGN46 (Abcam); rabbit anti-Wls, rabbit anti-GFP, rabbit anti-cyclin D1 (Santa Cruz); mouse anti-β-actin (Millipore); mouse anti-V5 (Invitrogen); rabbit anti-Wnt2b, rabbit anti-GSK3β, rabbit anti-phospho-GSK3β, rabbit anti-active-β-catenin, rabbit anti-GAPDH (Cell signaling); rabbit anti-β-catenin (Novus); Leupeptin (Amresco); SYBR Green Master (Roche); Dual-Luciferase Reporter (Promega); Enzyme linked immunosorbent assay (ELISA) kit for Wnt2b (Cloud-Clone).

## Lentiviral vectors and transient small interfering RNA transfection

To obtain high GOLPH3 and Wls down-regulation efficacy in U251 and U87 cells, we generated lentivirus based short hairpin RNA (shRNA) targeting human GOLPH3 and Wls with pLL3.7 as the backbone, which has GFP tag. Cells were infected with lentivirus containing GOLPH3 shRNA or Wls shRNA or a control scrambled short hairpin RNA (scramble). The shRNA sequences (5'-3') targeting human GOLPH3 and Wls were listed below. Target sequences are underlined.

shGOLPH3: gttaagaaatgtacgggaattcaagagattcccgtacatttcttaactttttc (Forward);

tcgagaaaaagtttaagaaatgtacgggaatctcttgaattcccgtacatttcttaaca (Reverse).

shWls: tggacattgccttcaagctattcaagagatagcttgaaggcaatgtctttttc (Forward);

tcgagaaaaaggacattgccttcaagctatcttgaatagcttgaaggcaatgtcca (Reverse).

To obtain GOLPH3 over-expression U251 and U87 cells, the GOLPH3 cDNA was inserted into the pWPXLd backbone (with GFP tag) at BamH I and Mlu I sites. The viruses were propagated in 293T cells by co-transfecting corresponding plasmids with the helper plasmids. Glioma cells were infected with lentivirus containing pWPXLd-GOLPH3 or pWPXLd.

For over-expression of Wls, the Wls cDNA was inserted into the pEGFP-N1 plasmid at Bgl II and Hind III sites. Cell transfection was performed with Polyjet (SignaGen) as described in the manufacturer's protocol. In some experiments, glioma cells were transiently transfected with human GOLPH3 siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions [20]. siRNA duplexes (Shanghai GenePharma Co.) targeting human GOLPH3 were listed below. GOLPH3-siRNA: 5'-guuaagaauguacgggaatt-3'.

## EdU incorporation assay

The effects of GOLPH3 and Wls on the proliferation of U251 and U87 cells were measured by 5-ethynyl-20-deoxyuridine (EdU) incorporation assay (RiboBio) according to the manufacturer's protocol and previous study [21]. Briefly, the cells were cultured in triplicate in 96-well plate (7×10<sup>3</sup> cells per well) for 48 h and then exposed to 50 μM of EdU for additional 2 h at 37°C. Then, the cells were fixed with 4% paraformaldehyde for 20 min and treated with 0.5% Triton X-100 for 20 min at room temperature. After being washed with PBS for three times, the cells were reacted with 100 μL of 1×Apollo® reaction cocktail for 30 min. Subsequently, the DNA contents of cells were stained with 100 μL of Hoechst 33342 (5 μg/mL) for 20 min and visualized under the Olympus IX-71 inverted microscope (Olympus Corporation).

## Plate colony formation assay

Six hundred cells were seeded in 60 mm dish and cultured for 12 days. The cells were fixed with methanol and stained with 0.05% crystal violet to assess colony staining. After being photographed with a camera, colonies containing more than 50 cells were counted.

## Western blotting analysis

Protein lysates were subjected to 10% SDS-PAGE and then transferred to 0.45 μm pore size PVDF membrane (Millipore). After blocking, the membrane was probed with primary antibodies at 4°C overnight and secondary antibodies at room temperature for 2 h. Bound antibodies were detected by the Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Scientific Inc) and exposed to x-ray films. Band densities

were quantified by using ImagePro Plus Software (Media Cybernetics, Inc) and the densitometric results were shown.

#### Co-immunoprecipitation

One microgram of total protein was incubated with 1µg of indicated antibodies and Protein A agarose (Roche) at 4 °C overnight with rocking. The beads were washed with NP-40 buffer and then centrifuged in 1000g for 2 min at 4°C for 5 times. The beads and antigen banding antibodies complex were boiled and detected by western blotting.

#### Reverse transcription-polymerase chain reaction (RT-PCR) or quantitative real-time PCR (qPCR)

For RT-PCR, total RNA of the cells was extracted using the TRIzol Reagent (Tiangen Biotech Co) and the cDNA was synthesized using reverse transcription reagents (Roche Applied Science) according to the instructions. The primer sequences were as follows.

β-actin: 5'-catgtactgtctatccaggc-3' (forward); 5'-ctccttaatgtcagcagcat-3' (Reverse).

GOLPH3: 5'-tgtaagtcagatgtccaacagg-3' (forward); 5'-tcaccattgtcaagaacgg-3' (Reverse).

Wls: 5'-tcattgatttcaggtgtttcg-3' (forward); 5'-gcatgaggaaactgaacctaaaa-3' (Reverse). The products were 249 bp, 316 bp and 117 bp respectively.

For qPCR, amplifications were carried out by using the Applied Bio-systems 7500. To quantify gene expression changes, the  $2^{-\Delta\Delta C_t}$  method was used to calculate relative fold-changes after normalizing to the value of β-actin. The mean value of nontumorous groups was set as 1.0 and all values were normalized by the means of the nontumorous groups. Primers for β-catenin were designed by ourselves and the rest were obtained from studies, specifically Wnt2b, Wnt3, Wnt3a, Wnt5a, Wnt5b, Wnt7b, Wnt11 and β-actin [34], cyclin D1[35] and C-MYC[36]. All qPCR primers were synthesized by Sangon Biotech (Shanghai, China) and listed in Table 1.

#### Immunohistochemistry

Paraffin-embedded pathological sections were from the Department of pathology, Affiliated Hospital of Xuzhou Medical University (12 of Grade II glioma tissue, 5 anaplastic astrocytomas of grade III, 7 glioblastomas of grade IV). Nontumorous brain specimens (n=8) were acquired from patient under-going surgery for internal decompression in cerebral trauma and were confirmed the absence of tumor. The sections (5µm thick) were deparaffinized in xylene and rehydrated through graded ethanol, and subsequently microwaved for antigen retrieval. Endogenous peroxidase was blocked in 3% hydrogen peroxide for 15 min and then incubated at 4°C overnight with anti-GOLPH3 antibody (1:200) and anti-Wls antibody (1:100). The bound antibodies were detected by use of the streptavidin-peroxidase kit (Zhongshan Golden bridge Bio.). Later, the slides were counterstained with hematoxylin, dehydrated with ethanol and xylene, and covered with coverslips. The photos were taken under an Olympus IX-71 inverted microscope and the results were presented as the percentage of the glioma cells with positive staining.

**Table 1.** Primers used for qPCR. F, forward; R, reverse

Gene	Primer Sequence	Gene	Primer Sequence
Wnt2b-F	5'-gccgtgtcatgctcagaa-3'	Wnt7b-F	5'-cgcctcatgaacctgcata-3'
Wnt2b-R	5'-gtggactaccctgctgatg-3'	Wnt7b-R	5'-gctgcatccggctcctcta-3'
Wnt3-F	5'-ctcgtggctaccaattt-3'	Wnt11-F	5'-tgtgctatggcatcaagtgg-3'
Wnt3-R	5'-gccagagatgtgtactgctg-3'	Wnt11-R	5'-cagtgtgctgctggttcag-3'
Wnt3a-F	5'-catgaaccgccacaacaac-3'	CyclinD1-F	5'-tcaaatgtgtgcagaaggagggt-3'
Wnt3a-R	5'-tggcacttgcaactgaggt-3'	CyclinD1-R	5'-gacaggaagcgggtccaggtta-3'
Wnt5a-F	5'-attgtactgcaggtgtacctaaac-3'	C-MYC-F	5'-cagctgcttagacgctggatt-3'
Wnt5a-R	5'-ccccttataaatgcaactgttc-3'	C-MYC-R	5'-gtagaatacggctgcaccga-3'
Wnt5b-F	5'-ctgctgctgctgttcagc-3'	β-actin-F	5'-ccaaccgcgagaagatga-3'
Wnt5b-R	5'-caccgggttcaaagctaag-3'	β-actin-R	5'-ccagaggcgtacagggtatg-3'

### *Immunofluorescence*

After being fixed, permeabilized and blocked, the cells were incubated with the primary antibody for 2 h at room temperature, followed by incubating with Alexa-conjugated secondary antibody. The nuclei were labeled with DAPI and cells were embedded in glycerin and photographed by Olympus IX-71 inverted microscopy or ZEISS LSM880. To elucidate the co-localization of proteins, the confocal images were quantified using line profile showing the overlap of signal peaks.

### *Enzyme linked Immunosorbent Assay (ELISA)*

The U251 cells ( $1 \times 10^6$ ) were cultured in 6-well plate and the supernatant was collected 24h after plating. Wnt2b concentration was determined using Quantitine ELISA kits according to the manufacturer's instructions.

### *Luciferase assay*

The U251 cells ( $2 \times 10^5$ ) were seeded in a 24-well plate. Twenty-four hours later, cells were transfected with 1 $\mu$ g of reporter (FOPFLASH or TOPFLASH) and 0.05 $\mu$ g of internal control plasmid (pRL-TK). At 48 h after transfection, luciferase activities were measured using the dual-luciferase reporter assay system according to the manufacturer's instructions.

### *Statistical analysis*

The results were representative of experiments repeated at least three times and presented as the mean  $\pm$  SEM. Statistical comparisons were performed using Student's *t*-test with two tails or ANOVA for multiple comparisons. P values less than 0.05 were considered statistically significant (\* $P < 0.05$ , \*\* $P < 0.01$ ).

## Results

### *Up-regulation and positive clinical relevance of GOLPH3 and Wls in human glioma tissues*

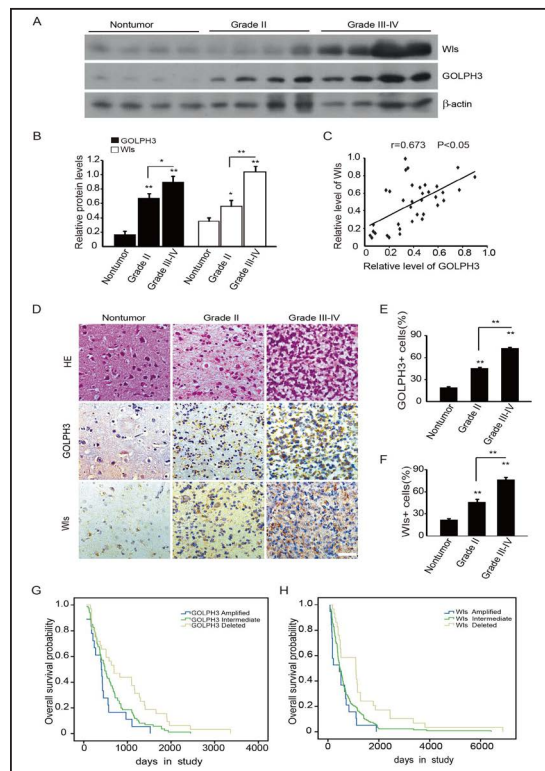
Firstly, we examined the expression level and clinical relevance of GOLPH3 and Wls in glioma samples. As is shown in Fig. 1A and 1B, the protein level of GOLPH3 and Wls in glioma samples was approximately 3-4 fold higher than that of nontumorous tissues. Interestingly, both the protein levels of GOLPH3 and Wls increased with the increase of the grade of gliomas, and exhibited positive correlation with each other ( $r = 0.673$ ;  $P < 0.05$ ; Fig. 1C). Similarly, the percentage of GOLPH3 and Wls positive cells in glioma tissues were higher than that of nontumorous tissues (Fig. 1D-1F). In addition, by measuring the percentage of GOLPH3 and Wls positive cells, we found that the higher the grade of glioma, the higher the level of GOLPH3 or Wls (Fig. 1D-1F). Moreover, according to the data from the REMBRANDT Database, higher levels of GOLPH3 and Wls were associated with shorter overall survival of glioma patients (Fig. 1G and 1H). The results about GOLPH3 was consistent with our previous report [21].

### *GOLPH3 promotes the proliferation of human glioma cells*

Our previous study reported that GOLPH3 down-regulation inhibits glioma cell proliferation by using siRNA approach [21]. In this study, we down-regulated GOLPH3 expression by using lentiviral based shRNA in U251 and U87 cells (for all online suppl. material, see [www.karger.com/doi/10.1159/000491618](http://www.karger.com/doi/10.1159/000491618), Suppl. Fig. 1A, sFig. 1A) to confirm its role in glioma progression. Notably, GOLPH3 shRNA did not affect the protein level of GOLPH3 paralogue GOLPH3L[37] (sFig. 1B-1D) and could be rescued by GOLPH3<sup>Res</sup> (shRNA-resistant mutant of GOLPH3), indicating the specificity of GOLPH3 shRNA[19, 21]. Furthermore, the high down-regulation efficiency of GOLPH3 shRNA was also confirmed by immunofluorescence assay (see online suppl. material, sFig. 1E).

Thereafter, the effect of GOLPH3 down-regulation on glioma cell proliferation was examined by EdU incorporation assay and plate colony formation assay. As is shown (see online suppl. material) in sFig. 2A, 2B, 2D, 2E, both the EdU positive cells and colonies of GOLPH3 down-regulation group decreased. On the contrary, over-expression of GFP-GOLPH3

**Fig. 1.** Up-regulation and positive clinical relevance of GOLPH3 and Wls in human glioma tissues (A) Representative blots of GOLPH3 and Wls from nontumorous and human glioma tissues.  $\beta$ -actin served as an internal control. (B) The statistical analysis of relative level of GOLPH3 and Wls on nontumorous brain tissues (n=12) and glioma tissues (n=24). (C) The correlation of GOLPH3 expression with Wls in nontumorous and glioma tissues.  $r=0.673$ ;  $P<0.05$ . (D) Representative images of GOLPH3 and Wls from nontumorous (n=8) and gliomas (n=24) determined by immunohistochemistry. Scale bar: 50  $\mu$ m. (E&F) Quantitative analysis of the percentage of GOLPH3 and Wls positive cells. The level of GOLPH3 or Wls in glioma tissues was higher than that of nontumorous brain and the number of positive cells gradually increased with the increase of grade of glioma. (G&H) Higher levels of GOLPH3 (G) and Wls (H) expression were associated with shorter overall survival of glioma patients from the REMBRANDT Database.



(see online suppl. material, sFig. 3A, 3B), which exhibited cluster-like signals specifically located around the nuclear (see online suppl. material, sFig. 3C), significantly increased the EdU incorporation and colony formation ability of glioma cells (see online suppl. material, sFig. 2A, 2C, 2D, 2F).

#### *Wls promotes the proliferation of human glioma cells*

It is shown that Wls promotes glioma cell proliferation and invasion through regulating Wnt signaling and up-regulation of interleukins and other pro-oncogenic factors [38]. Consistently, after down-regulation of Wls (see online suppl. material, sFig. 4A-4C), both the EdU positive cells and colonies decreased (see online suppl. material, sFig. 5A-5D), in line with the previous report [38]. On the contrary, over-expression of Wls, which was specifically located at the perinuclear and the plasma membrane (see online suppl. material, sFig. 6A and 6B), promoted glioma cell proliferation (see online suppl. material, sFig. 7A-7D). The above results demonstrate that Wls promotes glioma cell proliferation, similar to GOLPH3.

#### *Wls partially mediates the effect of GOLPH3 on glioma cell proliferation*

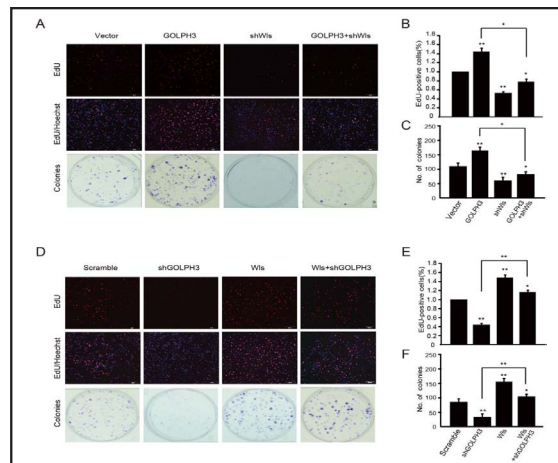
Next, we set up different co-transfection experiments (see online suppl. material, sFig. 8A and 8B) to examine whether the effect of GOLPH3 on glioma cell growth is mediated by Wls. As is shown in Fig. 2A and 2B, down-regulation of Wls alone inhibited EdU incorporation and over-expression of GOLPH3 alone promoted it, in line with our previous results (see online suppl. material, sFig. 5 and sFig. 2). Interestingly, down-regulation of Wls partially abolished glioma cell proliferation induced by GOLPH3 over-expression. The plate colony formation assay also showed similar results (Fig. 2A, 2C). On the contrary, Wls over-expression partially rescue the inhibition effect induced by GOLPH3 down-regulation on the cell proliferation (Fig. 2D-2F). Taken together, these results suggest that GOLPH3 promotes glioma cell growth partially mediated by Wls.

#### *GOLPH3 associates with Wls and regulates Wls at the post-transcription level*

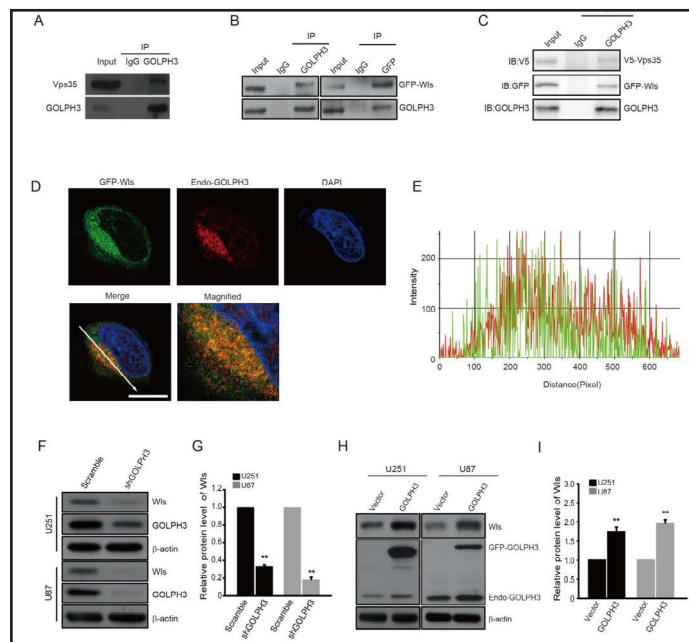
GOLPH3 plays a vital role in protein trafficking, receptor recycling and glycosylation, and its most notable interacting protein is Vps35, a highly conserved member of the cargo-

recognition complex of the retromer [12]. Previous studies have found that Wls interacts with Vps35 and its protein level decreased after Vps35 down-regulation [27, 29]. We also found that GOLPH3 was associated with Vps35 and Wls respectively (Fig. 3A and 3B). Furthermore, the endogenous GOLPH3 formed a protein complex with GFP-Wls and V5-Vps35 (Fig. 3C). Similarly, examined by immunofluorescence and quantified with line profile, endogenous GOLPH3 co-localized with exogenous GFP-Wls (Fig. 3D, 3E). Interestingly, the protein level of Wls decreased significantly after GOLPH3 down-regulation, while it increased after GOLPH3 over-expression (Fig. 3F-3I). However, the mRNA level of Wls shows no change after either down-regulation or over-expression of GOLPH3 (see online suppl. material, sFig. 9A-9C). The above results indicate that GOLPH3 regulates Wls at the post-transcriptional level.

**Fig. 2.** Wls partially mediates the effect of GOLPH3 on glioma cell proliferation (A) Representative images of EdU incorporation assay or colonies after infecting cells with indicated lentivirus. (B&C) Quantification of EdU positive cells (B) and colonies (C). (D) Representative images of EdU incorporation assay or colonies after infecting cells with indicated lentivirus. (E& F) Quantification of EdU positive cells (E) and colonies (F). Scale bar, 100  $\mu$ m. \* P<0.05; \*\* P<0.01.



**Fig. 3.** GOLPH3 regulates Wls at the post-transcription level (A) Interaction of GOLPH3 and Vps35. Glioma U251 cell lysates were subjected to immunoprecipitation with anti-GOLPH3 and immunoblot with the indicated antibodies. (B) Interaction of GOLPH3 and GFP-Wls. Glioma U251 cells were transfected with GFP-Wls. Cell lysates were subjected to immunoprecipitation with anti-GOLPH3 or anti-GFP and immunoblot with the indicated antibodies. (C) Complex formation between GOLPH3, V5-Vps35 and GFP-Wls. Glioma U251 cells were transfected with GFP-Wls and V5-Vps35. Cell lysates were subjected to immunoprecipitation with anti-GOLPH3 and immunoblot with the indicated antibodies. (D&E) GOLPH3 was co-localized with GFP-Wls or endogenous Wls in glioma U251 cells. Cells were subjected to immunofluorescence staining after with (up panel) or without GFP-Wls (bottom panel) transfection (D). The image was quantified with line profile method showing the overlap of signal peaks (E). Scale bar, 20  $\mu$ m. (F-I) GOLPH3 knocking down decreased the protein level of Wls (F&G), while GOLPH3 over-expression increased it (H& I).



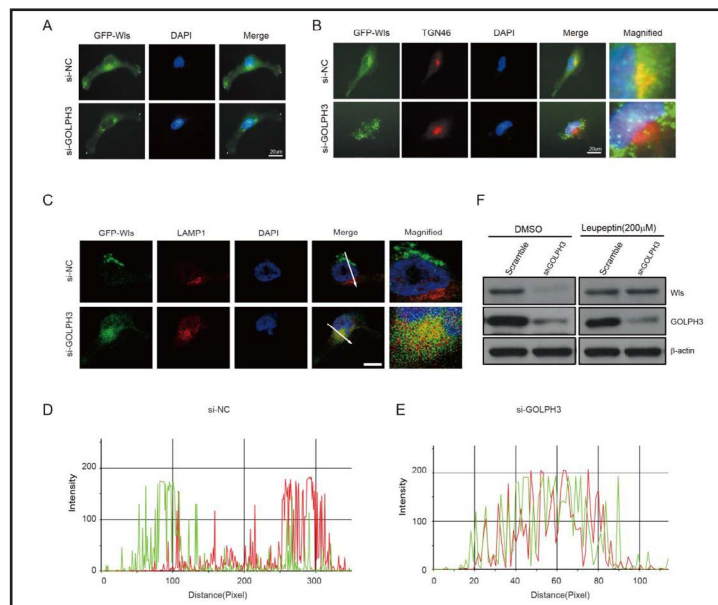
*GOLPH3 down-regulation inhibits Wls recycling*

Because Vps35 interacts with Wls and Vps35 knocking-down promotes Wls to be delivered into lysosomes for degradation [25, 29], we wonder whether GOLPH3 down-regulation will affect Wls trafficking. Interestingly, we found that the protein level of GFP-Wls located at the cell membrane and TGN, marked as TGN46, decreased after down-regulating GOLPH3 (Fig. 4A and 4B). However, the level of GFP-Wls located at lysosome, marked as LAMP1, significantly increased after down-regulating GOLPH3 (Fig. 4C-E). The above findings indicated that GOLPH3 down-regulation inhibited Wls recycling to golgi and cell membrane for reusing and promoted Wls trafficking to lysosome for degradation. Furthermore, after treatment with lysosome inhibitor Leupeptin (200 μM) for 6 hours, the decreased Wls protein level caused by GOLPH3 depletion restored to the original level (Fig. 4F), suggesting that down-regulating GOLPH3 really promoted Wls degradation in lysosome.

*GOLPH3 regulates Wnt2b secretion and β-catenin activity*

Wls, as a Wnt cargo receptor, carries and sends Wnt proteins from TGN to the cell surface for secretion. Considering our finding that GOLPH3 regulated the protein trafficking of Wls, we wonder whether GOLPH3 can regulate Wnt secretion. To address whether and which Wnt's secretion is regulated by GOLPH3, we screened the literature and found that, in the 19 Wnt subfamily members, Wnt2b, Wnt3, Wnt3a, Wnt5a, Wnt5b, Wnt7a and Wnt7b were reported to be expressed in gliomas [34, 39, 40]. Examined by qPCR, we found that the mRNA levels of Wnt2b, Wnt5a, Wnt5b and Wnt7b were expressed in glioma U251 and/or U87 cells (Fig. 5A and 5B). In addition, over-expression of GOLPH3 did not affect the mRNA levels of Wnt2b, Wnt5a, Wnt5b and Wnt7b (Fig. 5C). However, GOLPH3 down-regulation decreased the mRNA and protein expression of Wnt2b (Fig. 5D, 5E). Furthermore, examined by ELISA, the concentration of Wnt2b in the culture medium decreased after GOLPH3 down-regulation (Fig. 5F). It is well known that, without Wnt signal, cytoplasmic β-catenin was phosphorylated by glycogen synthase kinase 3β (GSK3β), targeted for degradation and therefore could not be translocated into the nucleus to activate the transcription of c-MYC and cyclin D1[41]. In line with the above reports, we found that the protein levels of phospho-GSK3β, active-β-catenin, β-catenin and cyclin D1 decreased after depletion of GOLPH3 (Fig. 6A). Interestingly, GOLPH3 depletion cells (GFP positive cells) showed lower levels of β-catenin than those of the normal cells (GFP negative cells) in the same field (Fig. 6B). Consistently, the nuclear β-catenin levels, the mRNA level of β-catenin target gene c-MYC and cyclin D1 decreased after GOLPH3 down-regulation (Fig. 6C and 6D). Consequently,

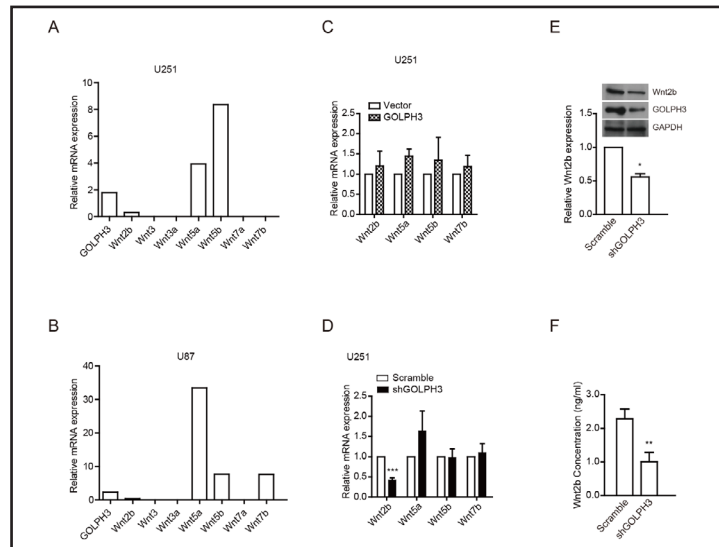
**Fig. 4.** GOLPH3 down-regulation inhibits Wls recycling (A-E) Knockdown of GOLPH3 inhibited the recycling of Wls to cell membrane (A) and TGN (B), increased its localization in lysosomes (C) in U251 cells. The image was quantified with line profile method showing the overlap of signal peaks (D&E). Cells were stained with indicated antibody after si-NC or si-GOLPH3 transfection for 48h. TGN46, TGN marker; LAMP1, lysosome marker. Scale bar, 20μm. (F) Lysosome inhibitor Leupeptin abolished GOLPH3 depletion induced Wls degradation in lysosomes. \*\* P<0.01.



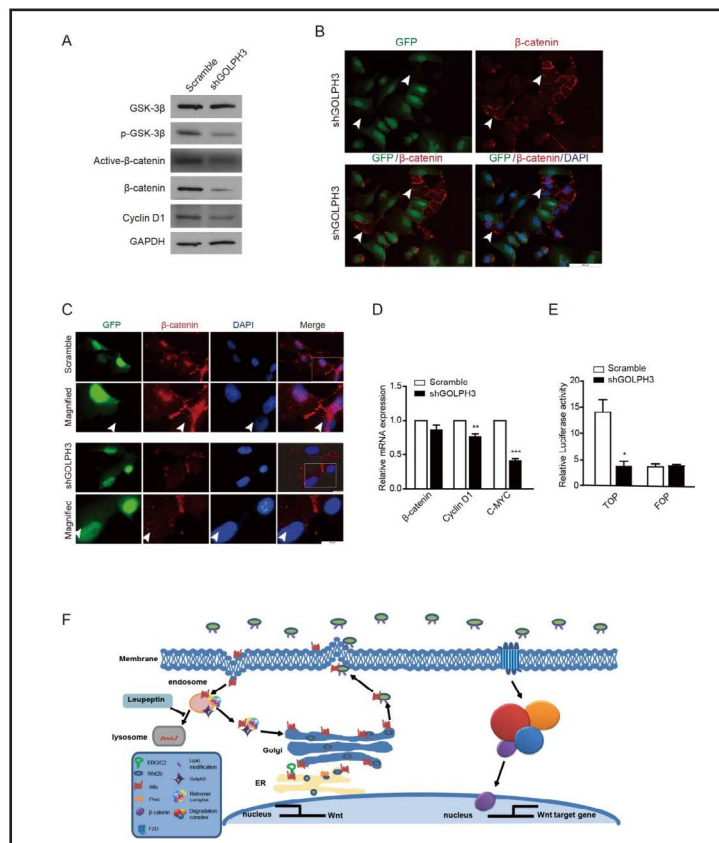


examined by luciferase reporter assay, the transcription activity of  $\beta$ -catenin also decreased after GOLPH3 down-regulation (Fig. 6E). The above results demonstrate, for the first time, that depletion of GOLPH3 results in impaired Wls recycling and Wnt2b secretion, and then decreases Wnt2b/ $\beta$ -catenin/Cyclin D1 signaling axis in the context of glioma.

**Fig. 5.** GOLPH3 regulates Wnt2b secretion (A&B) Relative mRNA levels of GOLPH3 and indicated Wnt family members in U251 (A) and U87 (B) glioma cells. (C&D) Relative mRNA levels of indicated Wnt family members in U251 glioma cells after GOLPH3 over-expression (C) or down-regulation (D). (E&F) GOLPH3 depletion decreased Wnt2b protein level (E) and secretion (F). The culture medium of U251 cells infected with shGOLPH3 was collected 24h after plating and Wnt2b concentration was determined by ELISA.



**Fig. 6.** GOLPH3 regulates  $\beta$ -catenin activity. (A) Representative blots of GSK-3 $\beta$ , p-GSK-3 $\beta$ , active- $\beta$ -catenin,  $\beta$ -catenin and cyclin D1 after GOLPH3 depletion. (B&C) Representative images of U251 cells stained with  $\beta$ -catenin after shGOLPH3 lentivirus infection. White arrowhead indicated the  $\beta$ -catenin signals. (D) Relative mRNA levels of  $\beta$ -catenin, cyclin D1 and C-MYC after GOLPH3 down-regulation in U251 glioma cells. (E) Relative  $\beta$ -catenin transcription activity after GOLPH3 down-regulation in U251 glioma cells, examined by dual luciferase assay. (F) Working model (please see the detail described in the 'discussion' section).



## Discussion

Based on the above results, a possible model is depicted in Fig. 6F. The GOLPH3-Wls-VPS35 complex forms a dynamic recycling. In this complex, the retromer component VPS35 is responsible for Wls recycling from the cell plasma membrane to TGN and Wls is responsible for helping Wnt secretion. Here, as a scaffold protein, GOLPH3 facilitates the interaction between Vps35 and Wls, thereby helps Wls retrieval from endosome to TGN and Wnt secretion. Disruption of any factor in this dynamic balance will cause abnormal Wnt secretion and consequently abnormal cell growth. Therefore, the high level of GOLPH3 in glioblastoma may promote the interaction of Vps35 and Wls to facilitate Wls recycling and Wnt secretion, which then causes  $\beta$ -catenin activation and target genes transcription.

Studies about the mechanism of GOLPH3 in cancer biology are mainly focused on the following signal pathways, such as mTOR-YB1, GSK3 $\beta$ -FOXO1 and AKT-mTOR, but rarely attach importance to its biological roles of golgi trafficking, receptor recycling and glycosylation in cancers [12, 17, 19]. It is reported that GOLPH3 interacts with and mediates the golgi localization of POMGnT1[42], a mammalian glycosyltransferase, which promotes glioblastoma progression [43]. In addition, GOLPH3 plays an important role in integrin-mediated cell migration via the up-regulation of sialylation [8], which suggests that GOLPH3 might regulate the progression of the tumors through its biological roles of glycosylation. Although it is generally accepted that the GOLPH3 has been implicated in retrograde trafficking of vesicles routed towards the Golgi [9], some studies reported that many receptors (e.g. transferrin receptor) recycle from perinuclear/trans Golgi region via recycling endosomes rather than getting into the constitutive secretory traffic [44, 45]. In addition, Tan et al. reported that Golgi-mediated vesicle trafficking may occur and it is independent of GOLPH3 in some cancer types, such as in lung cancer EMT and metastasis [46]. In our present study, we identified that GOLPH3 formed a protein complex with Wls and Vps35, and GOLPH3 down-regulation promoted Wls into the lysosome for degradation, but not retrieved to TGN or recycled to cell membrane. In the current study, we have no further evidence showing whether Wls was retrieved to TGN, or recycled to cell membrane directly from early endosome in the presence of GOLPH3. However, our results indicate that GOLPH3 is necessary for Wls retrieval or recycling, which supports the role of GOLPH3 in vesicles retrograde trafficking. Furthermore, considering Wls mediated the promotion effect of GOLPH3 on glioma progression, our study reported, for the first time, that GOLPH3 may promote tumor progression by helping the recycling of transmembrane proteins. As for the detailed mechanism, we guess that GOLPH3, as a non-kinase non-transcriptional molecule, might act as a scaffold protein to facilitate the interaction between Wls and retromer component Vps35, therefore to promote Wls recycling.

Recently, Wls is reported to promote the progression of tumors mainly by enhancing the secretion of Wnts and thereby affecting its downstream signals [38, 47]. Here, we also found that Wls is up-regulated in glioma tissues and promotes glioma cell growth, in line with the previous study [38]. Over-expression of GOLPH3 could activate AKT to inhibit GSK-3 $\beta$  and further activate the Wnt signaling and promote proliferation in human colon cancer cells [48]. In this study, we found that, by facilitating the recycling of Wls, GOLPH3 regulates Wnt2b secretion, which further inhibited GSK-3 $\beta$  to activate  $\beta$ -catenin signaling. Our study provides the direct evidence that GOLPH3 regulates Wnt secretion. In the current study, we only examined the secretion of Wnt2b, which is a key extra-epithelial Wnt ligand capable of promoting Wnt/ $\beta$ -catenin signaling and shows mRNA level change after GOLPH3 knocking down. Whether GOLPH3 also regulates other Wnt family protein secretion deserves further study.

Exosomes, a tiny extracellular particles, play more and more important roles in cancer progression [49, 50]. Gross et al. shows that Wnts are secreted in exosomes in *Drosophila* and human cells. They demonstrate that exosomes carry Wnts on their surface to induce Wnt signalling activity in target cells [51]. Since GOLPH3 may facilitate cargo sorting from the Golgi, it will be interesting to explore whether GOLPH3 regulates Wnt secretion as exosome.

In conclusion, we report here that GOLPH3 promotes the glioma cell proliferation by facilitating Wls recycling, Wnt2b secretion and then  $\beta$ -catenin activation. This study provides a brand new evidence that GOLPH3 promotes tumor progression through regulating protein recycling and secretion, suggesting a rationale for targeting the GOLPH3-Wls-Wnt axis as a promising therapeutic approach for glioblastoma.

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## Disclosure Statement

The authors declare that they have no competing interests.

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