Overexpression of GOLPH3 Promotes Proliferation and Tumorigenicity in Breast Cancer via Suppression of the FOXO1 Transcription Factor

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Translational relevance

Golgi phosphoprotein 3 (GOLPH3), a trans-Golgi matrix membrane protein, plays a role in protein trafficking, receptor recycling and glycosylation. GOLPH3 has also been linked to tumorigenesis. In this study, we found that GOLPH3 mRNA and protein expression were significantly upregulated in human breast cancer tissues. High levels of GOLPH3 expression were associated with advanced clinical stage and poorly differentiated tumors. GOLPH3 was an independent prognostic factor, along with clinical stage, T classification, M classification and Ki67 expression. High levels of GOLPH3 expression were associated with poorer overall survival in breast cancer. Furthermore, we demonstrated that overexpression and silencing of GOLPH3 promoted and inhibited, respectively, breast cancer cell proliferation and tumorigenicity in vitro and in vivo. Together, these results suggest that GOLPH3 overexpression promotes progression of human breast cancer and GOLPH3 has potential as a novel prognostic biomarker and therapeutic target for the treatment of human breast cancer.
Abstract

**Purpose:** Golgi phosphoprotein 3 (GOLPH3) has been reported to be involved in various biological process. The clinical significance and biological role of GOLPH3 in breast cancer, however, remains unknown.

**Experimental Design:** Expression of GOLPH3 in normal breast cells, breast cancer cells and six paired breast cancer and adjacent non-cancerous tissues were quantified using real-time PCR and western blotting. GOLPH3 protein expression was analyzed in 258 archived paraffin-embedded breast cancer samples using immunohistochemistry (IHC). The role of GOLPH3 in breast cancer cell proliferation and tumorigenicity was explored *in vitro* and *in vivo*. Western blotting and luciferase reporter analyses were employed to investigate the effect of GOLPH3 overexpression and silencing on the expression of cell cycle regulators and FOXO1 transcriptional activity.

**Results:** GOLPH3 was significantly upregulated in breast cancer cells and tissues, compared to normal cells and tissues. Immunohistochemical analysis revealed high expression of GOLPH3 in 133 of the 258 (51.6%) breast cancer specimens. Statistical analysis showed a significant correlation of GOLPH3 expression with advanced clinical stage and poorer survival. Overexpression and ablation of GOLPH3 promoted and inhibited, respectively, the proliferation and tumorigenicity of breast cancer cells *in vitro* and *in vivo*. GOLPH3 overexpression enhanced AKT activity and decreased FOXO1 transcriptional activity, downregulated cyclin-dependent kinase inhibitor p21\(^{\text{Cip1}}\), p27\(^{\text{Kip1}}\) and p57\(^{\text{kip2}}\) and upregulated the CDK regulator cyclin D1.

**Conclusion:** Our results suggest that high GOLPH3 expression is associated with poor overall survival in breast cancer patients, and that GOLPH3 overexpression increases the proliferation and tumorigenicity of human breast cancer cells.
Introduction

Breast cancer is the most common cancer amongst females and the second leading cause of cancer-related deaths in females. Over 1 million women are diagnosed and more than 400 000 women die from breast cancer every year (1). Mounting evidences indicate that induction of the neoplastic phenotype is invoked for the early events leading to increased tumorigenicity and metastasis. Thus, it is of great clinical value to identify potential early biomarkers to improve the diagnosis and prognostic assessment of breast cancer.

Golgi phosphoprotein 3 (GOLPH3), also known as GPP34, is a newly identified membrane protein in the trans-Golgi matrix, which plays a role in anterograde and retrograde Golgi trafficking (2-4). Recently, GOLPH3 has been shown to be involved in tumorigenesis. GOLPH3 localizes in human chromosome 5p13, a region that is frequently amplified in multiple solid tumor types (5-7). It has been reported that overexpression of GOLPH3 promotes cell transformation via enhancing the activity of the serine/threonine kinase mammalian target of rapamycin (mTOR) (8, 9). GOLPH3 overexpression also drove the transformation of primary cell lines and enhances mouse xenograft tumor growth in vivo. In contrast, silencing of GOLPH3 expression abrogates the transformation and proliferation of tumor cell lines in which GOLPH3 is amplified (9, 10). The clinical significance and biological role of GOLPH3 in breast cancer, however, remains largely unknown.

In this study, we found that GOLPH3 expression was significantly upregulated in breast cancer cells and tissues, and was associated with the clinical features of breast cancer. Furthermore, we demonstrated that GOLPH3 can promote proliferation and tumorigenicity in breast cancer cells in vitro and in vivo. Moreover, the biological behavior of GOLPH3 was mechanistically associated with suppression of FOXO1 transcriptional activity, via activation of the AKT signaling pathway. Our results demonstrate that GOLPH3 functions as an oncoprotein in breast cancer progression, and may provide a new therapeutic target for the treatment of
human cancer.
Materials and methods

Cell lines. Human mammary epithelial cells (HMECs) were established according to a previous report (11). Breast cancer cell lines, including MDA-MB-231, MDA-MB-415, T47D, MCF-10a, BT474, MCF-7, and Bcap37 were cultured in DMEM medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (HyClone, Logan, UT, USA).

Vectors and retroviral infection. An GOLPH3 expression construct was generated by subcloning PCR-amplified full-length human GOLPH3 cDNA into the pMSCV plasmid. For depletion of GOLPH3, two human GOLPH3-targeting siRNA sequences were cloned into pSuper-retro-puro to generate pSuper-retro-GOLPH3-RNAi(s), respectively, and the sequences are RNAi#1: GCATGTTAAGGAAACTCAGCC; RNAi#2: GCAGCGCCTCATCAAGAAAG T (synthesized by Invitrogen). Retroviral production and infection were performed as previously described (12). Stable cell lines expressing GOLPH3 or GOLPH3 shRNAs were selected for 10 days with 0.5 μg/ml puromycin. The reporter plasmid for quantitatively detecting the transcriptional activity of FOXO1 was generated in the pGL3-Enhancer plasmid (Promega, Madison, WI, USA) as described previously (13).

Patient information and tissue specimens. This study was conducted on a total of 258 paraffin-embedded, archived breast cancer samples, which were histopathologically and clinically diagnosed at the Sun Yat-sen University Cancer Center from 1998 to 2004. Clinical and clinicopathological classification and stage were determined according to the American Joint Committee on Cancer (AJCC) criteria. The histological grade was determined according to the Elston–Ellis modification of the Scarff–Bloom–Richardson (SBR) system. For the use of these clinical materials for research purposes, prior consent of the patients and approval from the Institutional Research Ethics Committee were obtained. Clinical information of the samples is summarized in the Supplemental Table 1.
**Immunohistochemistry (IHC).** The IHC procedure and the scores of GOLPH3 expression were performed as previously reported (14). IHC staining was quantitatively analyzed with the Axio Vision Rel.4.6 computerized image analysis system assisted with the automatic measurement program (Carl Zeiss, Oberkochen, Germany). Briefly, the stained sections were evaluated at 200× magnification and ten representative staining fields of each section, contains more than 95% tumor cells, were analyzed to verify the mean optical density (MOD), which represented the strength of staining signals as measured per positive pixel. The MOD data were statistically analyzed using the t-test to compare the average MOD difference between different groups of tissues; \( p < 0.05 \) was considered significant.

**Western blotting.** Western blotting was carried out according to standard methods as described previously (11), by using anti-GOLPH3, anti-p16\(^{\text{Ink4A}}\), anti-p21\(^{\text{Cip1}}\), anti-p27\(^{\text{Kip1}}\), anti-p57\(^{\text{kip2}}\), anti-cyclin A, anti-cyclin E, anti-cyclin D1, anti-p-Rb, anti-Rb, anti-p-AKT, anti-AKT, anti-p-GSK-3β, anti-GSK-3β, anti-p-FOXO1, anti-FOXO1, anti-Ki67, anti-p-FOXO3α, anti-FOXO3α, anti-p-FOXO4, anti-FOXO4, anti-P84, anti-EF-1α antibodies (Cell Signaling, Danvers, MA, USA). The membranes were stripped and re-probed with an anti-α-tubulin mouse monoclonal antibody (Sigma, Saint Louis, MI, USA) as a loading control.

**MTT assay.** Cells were seeded in 96-well plates at initial density of \(2 \times 10^3\)/well. At each time point, cells were stained with 100 μl sterile MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) dye (0.5 mg/ml, Sigma) for 4 hours at 37°C, followed by removal of the culture medium and addition of 100 μl of dimethyl sulphoxide (Sigma, Saint Louis, MI, USA). The absorbance was measured at 570 nm, with 655 nm as the reference wavelength. All experiments were carried out in triplicates.

**Anchorage-independent growth ability assay.** Five hundred cells were trypsinized and suspended in 2 ml complete medium plus 0.3% agar (Sigma, Saint Louis, MI, USA). The
agar-cell mixture was plated on top of a bottom layer with 1% agar completed medium mixture. About 10 days, viable colonies that were larger than 0.1 mm were counted. The experiment was carried out for each cell line in triplicates.

**Colony formation assays.** Cells were plated in 6-well plated (5×10² cells per plate) and cultured for 10 days. The colonies were stained with 1% crystal violet for 30 seconds after fixation with 10% formaldehyde for 5 minutes.

**Flow cytometry.** Cells were harvested, washed with cold PBS, and processed for cell cycle analysis by using flow cytometry. Briefly, the cells were fixed in 75% ethanol and stored at -20°C overnight for later analysis. The fixed cells were centrifuged at 1,000 rpm for 5 min and washed with cold PBS twice. RNase A (20 μg/ml final concentration) and propidium iodide staining solution (50 μg/ml final concentration) were added to the cells and incubated for 30 minutes at 37°C in the dark. Fifty thousand cells were analyzed by using a Cytomics™ FC 500 instrument (Beckman Coulter, USA) equipped with CXP software. Modifit LT 3.1 trial cell cycle analysis software was used to determine the percentage of cells in the different phases of the cell cycle.

**Bromodeoxyuridine labeling and immunofluorescence.** Cells were plated on coverslips (Fisher No.12-545-80, Fisher Scientific, Pittsburgh, PA). After 24 hours, cells were incubated with bromodeoxyridine (BrdU) for 1 hour and stained with anti-BrdU antibody (Upstate, Billerica, MA, USA) according to the manufacturer’s instruction. Gray level images were acquired under a laser scanning microscope (Axioskop 2 plus; Carl Zeiss Co. Ltd.).

**Xenografted tumor model.** The xenografted tumor model was performed as previously reported (15). Female Balb/c nude mice (4~5 weeks of age, 18~20 g) were purchased form the Slac-Jingda Laboratory Animal (Hunan, China), and were housed in barrier facilities on a 12-hour light/dark cycle. All experimental procedures were approved by the Institutional
Animal Care and Use Committee of Sun Yat-sen University. The Balb/c nude mice were randomly divided into 4 groups (n=5/group). A 0.72 mg E2 60-day release pellet (Innovative Research of America) was implanted subcutaneously on the dorsal side of each mouse 1 day before tumor cell implantation to support the growth of the estrogen-dependent MCF-7 cell derived tumors. For tumor cell implantation, MCF-7-GOLPH3 or MCF-7-GOLPH3-RNAi or their respective control cells (2×10⁶) in 0.25 ml of the mixture were injected into the mammary fat pads of female athymic mice. Tumors were examined twice weekly; length, width, and thickness measurements were obtained with calipers and tumor volumes were calculated. On day 23, animals were euthanized, and tumors were excised and weighed.

**Statistical analysis.** All statistical analyses were carried out by using the SPSS version 13.0 statistical software packages. Comparisons between groups for statistical significance were carried out with a 2-tailed paired Student’s t-test. A general linearized model was used to compare tumor volumes within groups at each time point. The relationship between GOLPH3 expression and clinicopathologic characteristics was analyzed by the χ² test. Survival curves were plotted by the Kaplan-Meier method and compared by using the log-rank test. Survival data were evaluated by using univariate and multivariate Cox regression analyses. A p-value of less than 0.05 was considered statistically significant in all cases.
Results

**GOLPH3 is upregulated in human breast cancer cells and tissues**

Real-time PCR and western blotting analysis showed that GOLPH3 mRNA and protein expression were significantly upregulated in all 7 tested breast cancer cell lines, compared to normal cells (HMECs; Fig. 1A and Supplemental Fig. S1A). Furthermore, comparative analysis revealed that the expression levels of GOLPH3 in six pairs of human breast cancer tissues were higher than those in matched adjacent non-tumor tissues (Figs. 1A-1B and Supplemental Fig. S1B). These results suggest that GOLPH3 expression is upregulated in breast cancer.

**GOLPH3 expression is associated with the clinical features of breast cancer**

In order to investigate the relationship between GOLPH3 expression and the clinical features of breast cancer, GOLPH3 expression was examined in 258 paraffin-embedded, archived breast cancer tissues using IHC staining. The samples included 25 cases of clinical stage I (9.7%), 123 cases of stage II (47.5%), 76 cases of stage III (29.3%) and 34 cases of stage IV (13.1%) breast cancer. Statistical analyses of the IHC staining are summarized in Supplemental Tables 2 and 3. As shown in Fig. 1C, IHC staining showed that GOLPH3 expression in breast cancer increased with advanced clinical stage and in the tumors with poor pathological differentiation (Supplemental Fig. S2). Statistical analysis revealed that GOLPH3 levels were strongly associated with the clinical stage \( p < 0.001 \), T classification \( p = 0.011 \), N classification \( p < 0.001 \) and M classification \( p < 0.001 \), but inverse correlated with expression of estrogen receptor (ER; \( p = 0.045 \)) (Supplemental Tables 2 and 3).

Moreover, Kaplan–Meier survival curves and the log-rank test Survival analysis showed that the overall survival of patients with high levels of GOLPH3 was significantly poorer than patients with low levels of GOLPH3 \( p<0.001 \) (Fig. 1D). Additionally, similar results were obtained in stage I-II subgroup patients and stage III-IV subgroup patients (Supplemental Fig. S3).
S3). Univariate and multivariate analyses indicated that clinical stage, T classification, M classification, and expression of GOLPH3 and Ki67 were independent prognostic factors (Supplemental Table 4), suggesting that GOLPH3 may be a prognostic factor for survival in breast cancer patients.

**GOLPH3 promotes the proliferation of breast cancer cells**

Striking, we found that the areas in breast cancer specimens that displayed high levels of GOLPH3 staining and also showed strong Ki67 staining signals, and areas with low GOLPH3 expression displayed weakly detectable Ki67 expression (Supplemental Fig. S4). Statistical analyses indicated that the expression of GOLPH3 was strongly associated with the expression level of Ki67 ($p<0.001$) (Supplemental Table 2 and 3), suggesting that GOLPH3 overexpression may contribute to the proliferation of breast cancer cells.

To further investigate the effect of GOLPH3 on proliferation, MCF-7 and MDA-MB-231 breast cancer cells stably overexpressing ectopic GOLPH3 were established (Fig. 2A). As shown in Fig.2B, the proliferation rate was significantly increased in GOLPH3 overexpressing MCF-7 and MDA-MB-231 cells, compared to control cells. These results were further confirmed by colony formation assay (Fig.2C). Furthermore, we found that silencing GOLPH3 also significantly reduced cell proliferation that analyzed by MTT and colony formation assays (Fig.2A, 2B and 2D). Collectively, our results suggest that overexpression of GOLPH3 plays important roles in proliferation of breast cancer cells *in vitro*.

**GOLPH3 promotes the tumorigenicity of breast cancer cells in vitro and in vivo**

The anchorage-independent growth assay revealed that overexpressing GOLPH3 significantly increased, but silencing GOLPH3 decreased the anchorage-independent growth ability of MCF-7 and MDA-MB-231 cells in soft agar (Fig. 3A and 3B), indicating that GOLPH3 plays a significant role in the tumorigenicity of breast cancer cells *in vitro*. We then
further examined whether GOLPH3 could promote the tumorigenicity of breast cancer cells \textit{in vivo}. As shown in Fig.3C, GOLPH3–transduced tumors grew significantly faster than the control tumors at each different time point ($p<0.001$), whereas the tumors formed by GOLPH3-silenced cells grew much slower than control tumors ($p<0.001$). The tumors formed by GOLPH3-transduced breast cancer cells were larger and had higher tumor weights than vector-control tumors. Conversely, tumors formed by GOLPH3-silenced cells were smaller, in both size and weight, than the tumors formed from shRNA-vector control cells (Fig. 3C, left and right panels). Importantly, IHC assay showed that areas of strong GOLPH3 signals displayed intense cyclin D1 and Ki67 staining, whereas areas with low levels of GOLPH3 expression exhibited lower cyclin D1 and Ki67 staining signals (Supplemental Fig. S5), further supporting the notion that GOLPH3 promotes the proliferation and tumorigenicity of breast cancer cells.

GOLPH3 regulates the G1-S phase transition in breast cancer cells

To further investigate the mechanism of GOLPH3 mediated-proliferation, the BrdU incorporation and flow-cytometry assays were performed. As shown in Fig.4A and 4C, overexpression of GOLPH3 in MCF-7 and MDA-MB-231 cells significantly increased the percentage of cells which incorporated BrdU, whereas knockdown of GOLPH3 significantly reduced the percentage of BrdU incorporated-cells. In parallel, flow-cytometry assay indicated that upregulation of GOLPH3 dramatically increased the percentage of S phase cells and decreased the percentage of cells in the G1/G0 phase. Conversely, silencing of GOLPH3 increased the percentage of cells in the G1/G0 phase and decreased the percentage of S phase cells (Fig. 4B and 4D). Taken together, these results suggest that GOLPH3 contributes to the G1-S phase transition in breast cancer cells.

Importantly, we found that the expression levels of $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{kip2}$ were drastically reduced in GOLPH3-transduced cells, accompanied by significantly increased
levels of cyclin D1 and phospho-Rb, compared to control cells (Fig. 5A and 5C).

Consistently, the expression of p21^{Cip1}, p27^{Kip1} and p57^{kip2} were significantly increased, whereas cyclin D1 and phospho-Rb decreased, in GOLPH3 silenced cells (Fig. 5B and 5C). However, GOLPH3 has no effects on the expression of p16^{Ink4A}, cyclin A and cyclin E. (Fig. 5C).

**GOLPH3 downregulates FOXO1 transactivity and activates AKT signaling pathway**

It has been reported that p21^{Cip1}, p27^{Kip1}, p57^{kip2} and cyclin D1 are regulated by the transcriptional factor FOXO1 (16, 17). We then examined the effect of GOLPH3 on the regulation of FOXO1 transactivity. As shown in Fig. 6A, FOXO1 reporter activity was significantly decreased in GOLPH3 overexpressing cells and significantly increased in GOLPH3 silenced cells. Importantly, we did not observed significant alterations of estrogen receptor activity and NF-κB reporter activity in GOLPH3 transduced- and GOLPH3 silenced-cells (Supplemental Fig.S6A), further suggesting the specific effect of GOLPH3 on the FOXO1 transactivity.

As FOXO1 is a downstream target of the PI3K/AKT signaling pathway (18, 19), the protein levels of phospho-AKT and phospho-FOXO1 were analyzed. As shown in Fig. 6B, the levels of phospho-AKT and phospho-FOXO1 were increased in GOLPH3 overexpressing cells and decreased in GOLPH3 silenced cells. However, the expression levels of phospho-FOXO3a and FOXO4 were only slightly increased in GOLPH3-transduced cells and decreased in GOLPH3-silenced cells (Supplemental Fig.S6B). As expected, the cellular fractionation experiment showed that FOXO1 expression in the nuclei was markedly decreased in GOLPH3-transduced cells and increased in GOLPH3-silenced cells (Supplemental Fig.S6C). Moreover, ChIP assay indicated no physical binding of GOLPH3 to FOXO1 promoter regions (Supplemental Fig.S6D). Taken together, these data suggest that GOLPH3 may promote the proliferation of breast cancer cells by downregulation of FOXO1 transcriptional activity via
activation of the AKT signaling pathway.

To further determine the role of FOXO1 in GOLPH3-mediated cell proliferation, we silenced the expression of FOXO1 in GOLPH3-silenced cells. As expected, the luciferase activity of the FOXO1 reporter was dramatically decreased in GOLPH3 silenced cells after FOXO1 siRNA(s) transfection (Supplemental Fig. S7A). Combined knockdown of FOXO1 in GOLPH3 silenced cells decreased the expression of p21<sup>Cip1</sup>, p27<sup>Kip1</sup>, and p57<sup>kip2</sup>, but increased the cyclin D1 expression (Supplemental Fig. S7B and S7C). Moreover, colony formation and MTT assays showed that silencing of FOXO1 restored the growth rate of GOLPH3-silenced MCF-7 and MDA-MB-231 cells (Fig. 6C and 6D). Collectively, our results suggest that FOXO1 plays an important role in the effect of GOLPH3 on proliferation in breast cancer cells.
Discussion

The key findings of this study are that GOLPH3 overexpression was significantly associated with progression of human breast cancer, and that ectopic expression of GOLPH3 increased the proliferation and tumorigenicity of breast cancer cells. We demonstrated that GOLPH3 promotes cell proliferation by accelerating the G1-S phase transition through downregulation of FOXO1 transcriptional activity via activation of AKT signaling pathway. This study provides new insights and strong evidences that GOLPH3 overexpression plays important roles in promoting the tumorigenicity and progression of human breast cancer.

Tumorigenesis is a complex multi-step process, characterized by uncontrolled cell growth and tumor formation, is largely associated with progressive accumulation of genetic and various epigenetic alterations in genes or proteins that regulate cell proliferation (20, 21). Therefore, identification of the genes and their products that lead to tumorigenesis is critical for providing new diagnostic and prognostic methods and potential therapeutic targets. Previously, it has been reported that GOLPH3 is amplified at the 5p13 region in several types of cancer, including breast cancer (9). The clinical significance and biological role of GOLPH3 in breast cancer progression, however, remains largely unknown. Herein, we found that GOLPH3 was significantly upregulated in a large cohort of human breast cancer tissues, and that GOLPH3 levels significantly correlated with the clinical characteristics of breast cancer, including the clinical stage, TNM classification, and expression of ER and Ki67. Importantly, survival analysis demonstrated that patients with low GOLPH3 expression have better overall survival compared to patients with high levels of GOLPH3, suggesting that GOLPH3 is a predictor for breast cancer patient survival.

Mounting evidences have established that the tumor suppressor FOXO1 plays an important role in tumorigenesis via the regulation of a variety of biological processes, such as
proliferation, differentiation, stress responses and apoptosis through transcriptional induction of the CDK inhibitors, including p21\textsuperscript{Cip1}, p27\textsuperscript{Kip1} and p57\textsuperscript{Kip2} (22-24). Indeed, deregulation of FOXO1 expression is observed in multiple cancers, including breast cancer, prostate cancer, chronic myelogenous leukemia, glioblastoma, rhabdomyosarcoma and leukemia (24-29). In the present study, we found that FOXO1 transactivity was decreased in GOLPH3 transduced cells and enhanced in GOLPH3 silenced cells, suggesting that GOLPH3-induced proliferation and tumorigenesis may be due to modulation of FOXO1 activity. Upregulation of GOLPH3 accelerated the G1-S phase transition; whereas downregulation of GOLPH3 induced G1-S phase arrest. Furthermore, we demonstrated that the mechanism of GOLPH3-mediated proliferation was linked to alternations in the expression of the cell cycle inhibitors p21\textsuperscript{Cip1}, p27\textsuperscript{Kip1} and p57\textsuperscript{Kip2} as well as the CDK regulator cyclin D1.

AKT is a serine/threonine protein kinase that plays key roles in multiple cellular processes, such as cell proliferation, apoptosis, transcription, cell migration and glucose metabolism. Activated AKT could stimulate the phosphorylation and impact various downstream targets, including GSK-3\(\beta\), BAD, IKK, p27, MDM2 and the FOXO family of transcription factors (30). It has been demonstrated that phosphorylation of FOXO at Ser256 by AKT leads to the nuclear/cytoplasmic translocation and subsequent degradation of FOXO1 via the ubiquitin-proteasome system (18, 19). Our study demonstrates that the levels of phospho-AKT and phospho-FOXO1 increased in GOLPH3 overexpressing cells and decreased in GOLPH3 silenced cells. The mechanism by which GOLPH3 activates the AKT signaling pathway is under investigation in our laboratory currently.

Metastases have already disseminated to adjacent lymph nodes or distant organs at the time of diagnosis in most breast cancer patients (31). It has been reported that GOLPH3 connects the Golgi membranes to actin cytoskeleton through binding to myosin MYO18A (32). MYO18A, a novel p21-activated kinase PAK2-binding partner, has been proven to play an
important role in the modulation of membrane ruffle formation, focal adhesion turnover, actin
filament reorganization and the migratory ability of epithelial cells (33). Thus, the role of
GOLPH3 in breast cancer cell migration and metastasis needs to be further explored.

In summary, our results suggest that GOLPH3 plays an important role in human breast
cancer progression. Full understanding of the precise role of GOLPH3 in human cancer may
provide the opportunity to develop a novel therapeutic strategy by suppressing expression of
GOLPH3 in breast cancer cells. Additionally, GOLPH3 has potential as a relevant clinical
indicator of disease progression and as a prognostic marker for patient survival in human breast
cancer. Translational research on the clinical use of GOLPH3 is required to generate a
methodology and evaluate the molecular diagnostic ability of GOLPH3 in breast cancer.
Reference


Figure Legends

Figure 1. GOLPH3 expression is upregulated in breast cancer. (A) Western blotting analysis of GOLPH3 expression in HMECs and seven breast cancer cell lines (left panel), and in matched primary breast cancer tissues (T) and adjacent noncancerous tissues (ANT) (right panel). α-Tubulin was used as a loading control. (B and C) IHC staining analysis of GOLPH3 protein expression (B) in matched primary breast cancer tissues (T) and adjacent noncancerous tissues (ANT), and (C) in normal breast tissues and breast cancer tissues of different clinical stages. (D) Kaplan-Meier curves with univariate analyses (log-rank) for breast cancer patients with low GOLPH3 expressing (n=125) versus high GOLPH3 expressing tumors (n=133).

Figure 2. GOLPH3 is essential for breast cancer cell proliferation and tumorigenicity. (A) Western blotting analysis of GOLPH3 expression in GOLPH3 transduced- and GOLPH3 silenced- cells. α-Tubulin was used as a loading control. (B-D) MTT assays (B) and colony formation assay (C and D) indicated that the growth rates increased in GOLPH3 overexpressing cells and decreased in GOLPH3 silenced cells. Values are mean ± SD of three independent experiments; * p<0.05.

Figure 3. GOLPH3 promotes the tumorigenicity of breast cancer cells in vitro and in vivo. (A and B) Anchorage-independent growth assay in GOLPH3 overexpressing cells (A) and GOLPH3 silenced cells (B). Soft agar colony formation (colonies larger than 0.1 mm diameter) was quantified after 10 days of culture. (C) Xenograft model in nude mice. Indicated cells were inoculated into the fat-pad of the nude mice (n = 5/group) (left panel). Representative images of tumor growth (left panel). Tumor volume growth curves (middle panel). Mean tumor weights (right panel) 23 days after inoculation. All data are as mean ± SD; p<0.05.

Figure 4. GOLPH3 regulates the G1-S-phase transition in breast cancer cells. (A) Representative micrographs (left panel) and quantification (right panel) of BrdU incorporated
cells in indicated cells. (B) Flow cytometric analysis of vector control and GOLPH3 overexpressing cells. (C) Representative micrographs (left panel) and quantification (right panel) of BrdU incorporation in vector and GOLPH3 shRNA infected cells. (D) Flow cytometric analysis of vector and GOLPH3 shRNA infected cells. All values are mean ± SD of three independent experiments; *p<0.05.

Figure 5. The effect of GOLPH3 on G1-S-phase regulators. (A and B) Real-time PCR analysis of p21Cip1, p27Kip1, p57kip2 and cyclin D1 expression in GOLPH3 transduced cells (A) or GOLPH3 shRNA infected cells (B). Expression levels were normalized to GAPDH. (C) Western blotting analysis of p16INK4A, p21Cip1, p27Kip1, p57kip2, cyclin A, cyclin E, cyclin D1, phosphorylated Rb (p-Rb) and total Rb proteins in GOLPH3 transduced cells or GOLPH3 shRNA infected cells. α-Tubulin was used as a loading control. Values are mean ± SD of three independent experiments; *p<0.05.

Figure 6. GOLPH3 downregulates FOXO1 transcriptional activity via activation of the AKT signaling pathway. (A) Related FOXO1 reporter activity in GOLPH3 transduced cells or GOLPH3 shRNA infected cells. (B) Western blotting analysis of p-AKT, total AKT, p-GSK-3β, total GSK-3β, phosphorylated FOXO1 and total FOXO1 in GOLPH3 transduced cells or GOLPH3 shRNA infected cells. (C) Quantification of colony formation assays in indicated cells. (D) MTT assays showed that silencing FOXO1 increased the proliferation of GOLPH3 shRNA(s)-infected cells. Values are mean ± SD of three independent experiments; *p<0.05.
Figure 1

A

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B

Patient 1

Patient 2

Patient 3

Patient 4

Patient 5

Patient 6

C

Normal

Clinical stage I

Clinical stage II

Clinical stage III

Clinical stage IV

200X

400X

D

Overall survival

Cumulative survival (%) vs. survival time (months)

Low GOLPH3 expression (n=125)

High GOLPH3 expression (n=133)

P<0.001
Overexpression of GOLPH3 Promotes Proliferation and Tumorigenicity in Breast Cancer via Suppression of the FOXO1 Transcription Factor

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