

## Original Article

# Rab5a suppresses autophagy to promote drug resistance in cancer cells

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**Abstract:** Cancers are huge problems that need to be investigated thoroughly. Rab5a plays an important part in the regulation of intracellular membrane trafficking. However, its role in cancer and autophagy has not been fully determined. In this study, we analyzed the correlation between Rab5a expression and patients' prognosis and then explored the effect of Rab5a knockdown on different cell lines using western blotting and fluorescence. Our results showed that up-regulated Rab5a positively correlated with the prognosis of gastric cancer patients. After knocking down Rab5a, mTOR activity was inhibited and autophagy flux increased. We also found that in our cisplatin-resistant cells, knockdown of Rab5a activated autophagy via mTOR pathway and could reverse drug resistance while over-expression of Rab5a in drug sensitive cells increased drug tolerance. In conclusion, our study demonstrates that Rab5a can suppress autophagy through mTOR and promote drug resistance in gastric cancer cells.

**Keywords:** Rab5a, mTOR, autophagy, chemoresistance, cancer

## Introduction

Cancers are serious diseases that endanger human health. The occurrence and development of cancers depend on the dynamic regulation of intracellular biological processes such as autophagy and apoptosis. Autophagy is a cell-based physiological process that involves the breakdown of cells with or without selectivity by lysosomes to generate energy or small biological molecules and is highly conserved in eukaryotes. Autophagy can effectively clean up the garbage such as protein aggregates or damaged organelles within the cells to maintain homeostasis or enable cellular survival under various stressed conditions such as nutrient starvation [1]. However, there are differences in the activity and role of autophagy in different stages of tumor development. As in some drug-resistant tumor cells, autophagy is somehow inhibited [2, 3].

As a sensor of energy and nutrition status, mammalian target of rapamycin (mTOR) can sensitively respond to many micro-environmen-

tal factors including amino acids, cellular energy levels, hormones, growth factors, nutritional status and other changes, thus playing a gating role in autophagy [4, 5]. mTOR signaling pathway is mainly regulated by three upstream pathways, PI3K/AKT, LKB1/AMPK, and Ras/AMPK.

As a member of the Rab family of small GTPases, Rab5a is an important rate-limiting component for the regulation of endocytosis and early endosome fusion [6]. Rab5a is not only involved in intracellular material transport and protein sorting, but also functions to regulate signal transduction, receptor downregulation and cytoskeleton reconstruction [7, 8]. Rab5a also takes part in the autophagy process. For example, vacuolin-1 can block the autophagosome-lysosome fusion by activating Rab5a [9]. Moreover, inactivation of Rab5a results in reduced mTOR activity and disordered intracellular localization of mTOR [10, 11]. However, whether Rab5a can regulate autophagy through mTOR pathway in human cancers requires further study.

## Suppression of autophagy by Rab5a

Here, we found that the expression of Rab5a was positively correlated with the overall survival of patients with gastric cancer. Knockdown of Rab5a in tumor cells inhibited mTOR activity and activated autophagy. In the cisplatin-resistant gastric cancer cells [12-14], both the expression and activity of Rab5a were significantly increased compared with sensitive cells. Knocking down Rab5a expression activated autophagy in drug-resistant cells and reversed drug resistance.

### Materials and methods

#### *Chemicals and cell lines*

Cisplatin, 3-methyladenine (3-MA), chloroquine (CQ), and rapamycin were bought from Selleck Chemicals (Shanghai, China). Human gastric cancer cell lines BGC823 and SGC7901 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI 1640 medium supplemented with 10% of fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 µg/ml of streptomycin (Life Technologies/Gibco, Grand Island, NY, USA). The cells were grown at 37°C in a humidified incubator with 5% CO<sub>2</sub>. The cisplatin-resistant cells were developed from the parental cells subjected to persistent gradient exposure to cisplatin for about 12 months, through increasing cisplatin concentration from 0.05 µg/ml until the cells acquired resistance to 1 µg/ml.

#### *Plasmid, siRNA and transfection*

The GFP-Rab5a plasmid was kindly provided by Professor Yuying Chen (Beijing University, China). The plasmid was transfected into cells with X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland). In brief, cells were seeded overnight in 6-well plates (3-5 × 10<sup>5</sup>/well). DNA was diluted to a final concentration of 1 µg plasmid DNA/100 µl medium before adding 2 µl DNA Transfection Reagent. The transfection reagent: DNA complex was added to the cells in a dropwise manner after incubating for 15 min. The transfected cells would be harvested for further analysis after incubation for 1-3 days at 37°C.

The siRNA was transfected into cells with Lipofectamine RNAiMAX Reagent (Life Technologies, CA, USA) according to manufacturer's instructions. In brief, cells were seeded over-

night in 6-well plates (3-5 × 10<sup>5</sup>/well). 9 µl Lipofectamine RNAiMAX Reagent or 30 pmol siRNA were diluted separately in 150 µl Opti-MEM Medium and mixed up for 5 min before adding to cells. The transfected cells were incubated for 1-3 days before harvesting for further analysis. Small interfering RNA (siRNA) specific for Rab5a (1#:5'-GUCCUAUGCAGAU-GACAAUTT-3'; 2#:5'-CAGCCAUAGUUGUAUAUG-ATT-3'), VPS34 (5'-GGUGAUGAAUCAUCUCCA-ATT-3') and control siRNA was synthesized in GenePharma (Shanghai, China).

#### *Western blotting*

In brief, cells were lysed in whole-cell lysate buffer containing 1% phosphatase inhibitor cocktail. Lysates containing 20-30 µg protein were loaded onto 8% or 12% sodium dodecyl sulfate-polyacrylamide gels for electrophoresis (SDS-PAGE) and the separated proteins transferred to poly vinylidene fluoride (PVDF) membranes (Pall, NY, USA). After blocking with 5% fat-free milk for 1 h in Tris-buffered saline (TBS), the membranes were incubated with the primary antibody overnight at 4°C and then the peroxidase conjugated secondary antibody (Agilent, CA, USA) for 1 h on the next day. WB bands were visualized with an enhanced chemiluminescence kit (EMD Millipore, MA, USA). The primary antibodies used were γH2AX, XRCC1, GFP (1:2000 dilution, abcam, Cambridge, UK), Rab5a (1:2000 dilution, proteintech, IL, USA), and SQSTM1, LC-3, p-mTOR, actin, cleaved-PARP1, tubulin (1:2000 dilution, Cell Signaling Technology, Danvers, MA, USA).

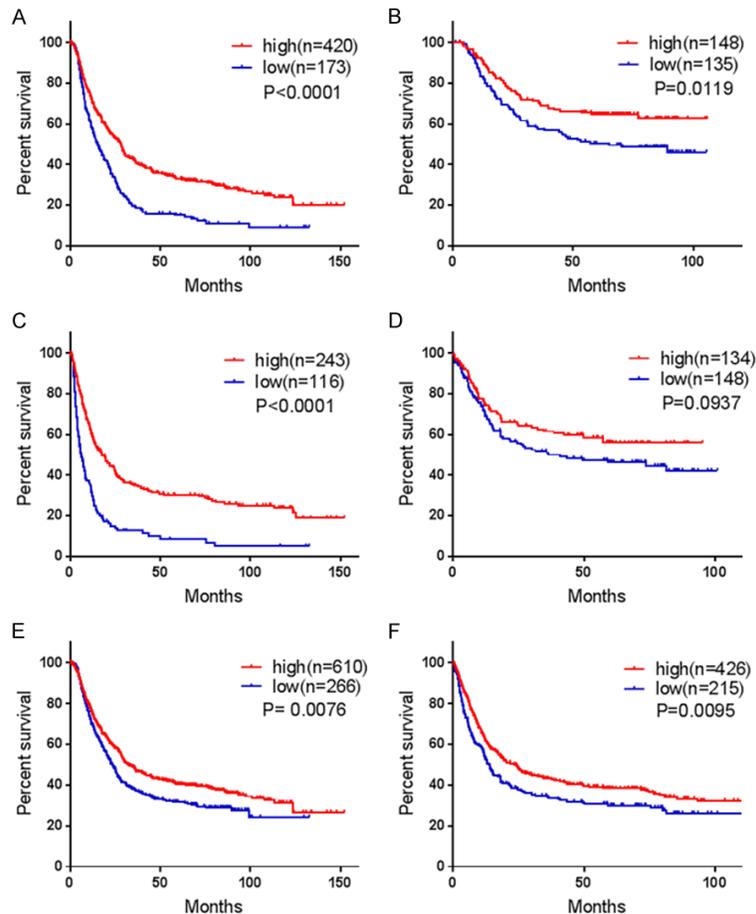
#### *Lyso-tracker Red staining*

Lyso-tracker Red probes (Invitrogen, USA) were used following the manufacturer's instruction. Briefly, cells were cultured in 6 well-plates and treated. Then changed the medium with fresh medium containing 50 nM probes and incubated the cells in 37°C for 30 minutes before harvest. Then the cells were washed with 1 × PBS and sent for confocal microscope analysis (Carl Zeiss, Germany).

#### *mRFP-GFP-LC3 analysis*

A tandem monomeric RFP-GFP-tagged LC3 plasmid was used to monitor autophagy flux as

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**Figure 1.** Expression of Rab5a is related with poor prognosis in gastric cancer patients. (A and B) Kaplan-Meier analyses of the correlation between Rab5a RNA levels and overall survival (A) in 593 gastric cancer patients and progression-free survival (B) in 283 gastric cancer patients. All of the data sets were included except the GSE62254 data set. (C and D) Kaplan-Meier analyses of the correlation between Rab5a RNA levels and overall survival (C) in 359 gastric cancer patients and progression-free survival (D) in 282 gastric cancer patients. Only GSE62254 data set was analyzed. (E and F) Kaplan-Meier analyses of the correlation between Rab5a RNA levels and overall survival (E) in 876 gastric cancer patients and progression-free survival (F) in 641 gastric cancer patients. All data sets were included. All data were acquired from Kaplan-Meier Plotter database and patients were split into high (red) and low (blue) expression by median.

previous reported [14]. Tandem mRFP-GFP-LC3 plasmid transfected in treated cells for 48 h, then cells were washed with  $1 \times$  PBS, and sent for confocal microscope analysis (Carl Zeiss, Germany).

### Statistical analysis

Data were expressed as the mean  $\pm$  SD. The statistical significance of the differences between the cell lines was determined by the parametric unpaired *Student's t* test.

Differences were considered significant when  $P < 0.05$ .

### Results

#### *Elevated Rab5a expression predicts better prognosis in gastric cancer patients*

To investigate the relevance of Rab5a to human cancers, we first analyzed the effect of Rab5a mRNA expression on prognosis of cancer patients [15]. We noted that in gastric cancer, patients with high expression of Rab5a had significantly longer overall survival and progression-free survival (**Figure 1A-C**). However, in one of the groups we analyzed, there was no significant difference between their progression-free survival (**Figure 1D**). This might result from longer survival and less patients in this group. Upon combining all of these groups, elevated Rab5a levels were significantly associated with better prognosis (**Figure 1E and 1F**).

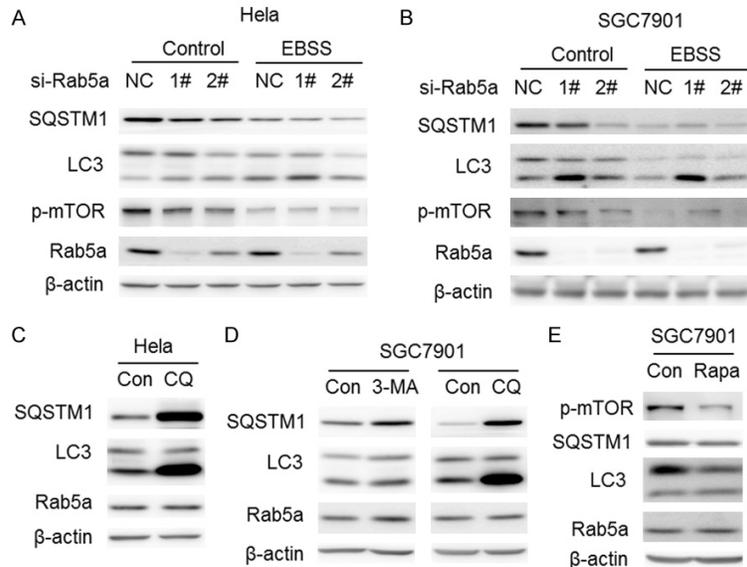
#### *Knockdown of Rab5a activates autophagy and inhibits mTOR activity*

Autophagy is a process mainly responsible for intracellular material degradation and recycling to enable cell survival. We found that knockdown of Rab5a by small interfering RNA in HeLa and SGC7901

cells led to accumulation of LC3-II or increase in LC3-II/I ratio and decrease in SQSTM1 expression under normal condition and nutrient starvation (**Figure 2A and 2B**). Besides, we found that phosphorylated mTOR level was decreased while knocking down Rab5a (**Figure 2A and 2B**). These results indicated that down-regulation of Rab5a could facilitate autophagy and inhibit mTOR activity.

To explore whether Rab5a could regulate autophagy through mTOR pathway, we used differ-

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**Figure 2.** Rab5a knockdown facilitates autophagy by inhibiting mTORC1 activity. A and B. HeLa and SGC7901 cells transfected with control siRNA or Rab5a siRNA for 48 h followed by incubation with normal medium or EBSS for 4 h. Phosphorylated mTOR and autophagy markers SQSTM1 and LC3 were immunoblotted with indicated antibodies. C-E. HeLa or SGC7901 cells were treated with or without autophagy inhibitors CQ or 3-MA (25  $\mu$ M CQ or 5 mM 3-MA for 24 h) or mTOR inhibitor rapamycin (100 nM rapamycin for 48 h) before harvest and then the cell extracts were immunoblotted with Rab5a antibody and autophagy markers.

ent inhibitors to treat cells individually. 3-methyladenine (3-MA) could nonspecifically inhibit class III PI3K and block autophagy at early stage whereas lysosomal inhibitor chloroquine (CQ) would interrupt the formation of autolysosomes to block autophagy. In contrast, rapamycin is a classical mTORC1 inhibitor which can induce autophagy. We observed that none of these treatments were able to change the Rab5a expression (Figure 2C-E), which suggested that Rab5a might work upstream of mTOR. Thus, knockdown of Rab5a induces autophagy under normal condition and stressed condition, possibly via inhibition of mTOR activity.

### *Rab5a is positively associated with drug-resistance in cancer cells*

We next explored whether Rab5a was involved in drug resistance. We found that Rab5a expression and mTOR level were elevated in drug-resistant cells (Figure 3A). Herein, as an important scaffold protein involved in DNA repair pathway, XRCC1 was used to confirm the drug-resistant characteristic of SGC7901/DDP and BGC823/DDP cells. Correspondingly,

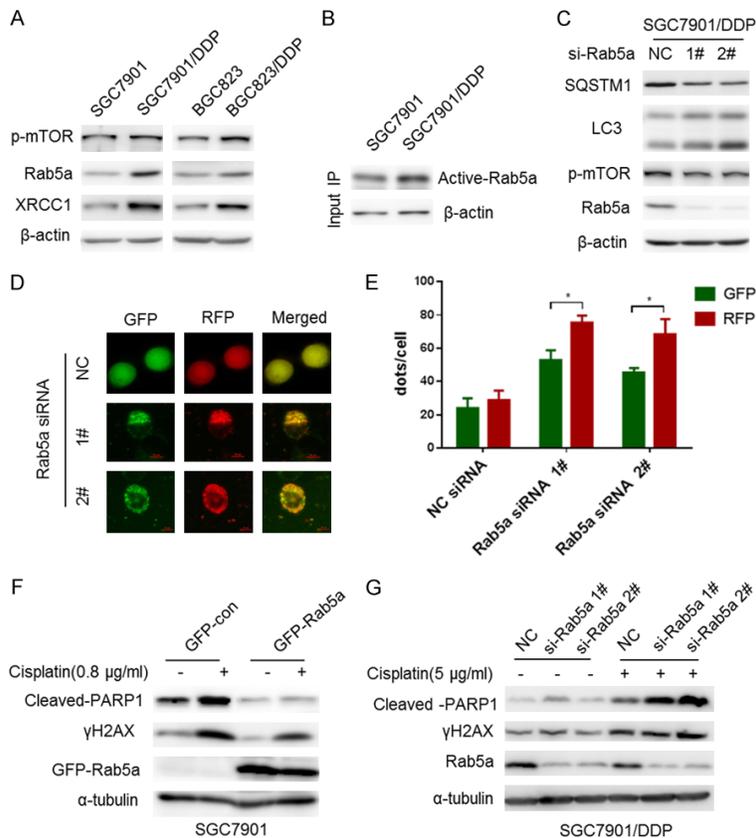
activated Rab5a form also increased in drug-resistant cells (Figure 3B). Knockdown of Rab5a in drug-resistant SGC7901/DDP cells led to decreased mTOR activity and increased autophagy flux (Figure 3C). To further determine the role of Rab5a in autophagy in drug-resistant cells, we used tandem mRFP-GFP-LC3 reporter. When autophagy flux is increased and LC3 is finally located in autolysosomes, GFP will undergo fluorescence quenching in acidic condition since RFP is more stable than GFP and only red fluorescence can be observed. Therefore, increasing red spots represented activated autophagy after Rab5a knockdown (Figure 3D and 3E).

We then exogenously overexpressed Rab5a in drug-sensitive SGC7901 cells and treated them with 0.8  $\mu$ g/ml cisplatin. We indeed found that Rab5a overexpressed SGC7901 cells showed decreased  $\gamma$ H2AX and cleaved-PARP1 levels, which indicated reduced cisplatin-induced DNA double strand break and cell death (Figure 3F). In contrast, in SGC7901/DDP cells with Rab5a knockdown,  $\gamma$ H2AX and cleaved-PARP1 levels were increased after treating with 5  $\mu$ g/ml cisplatin (Figure 3G). These results suggest that Rab5a level is positively associated with drug resistance in gastric cancer cells.

### *Rab5a regulates mTOR in cisplatin-resistant cells*

Next, we tried to further investigate the mechanism of Rab5a inhibiting autophagy. Rapamycin could not change Rab5a expression while nutrient starvation reduced Rab5a expression (Figure 4A and 4B). Knockdown of VPS34, a subunit of PI3K complex essential for the initiation and maturation of autophagosomes, blocked Rab5a downregulation induced autophagy (Figure 4C and 4D), indicating that Rab5a worked upstream of PI3K complex. We also examined the lysosomes patterns by LysoTracker staining and found that the number

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**Figure 3.** Rab5a knockdown activates autophagy and reserves drug resistance. A. The Rab5a expression and mTOR level in SGC7901, BGC823 and their corresponding drug-resistant cells were determined by western blotting. B. Activated Rab5a in SGC7901 and SGC7901/DDP cells was tested by Rab5 Activation Assay Kit. Cells were harvested and immunoprecipitated by anti-active Rab5 antibody and then immunoblotted with Rab5a antibody. C. SGC7901/DDP cells transfected with control siRNA or Rab5a siRNA for 48 h before harvest and were analyzed by immunoblotting with indicated antibodies to determine the mTOR activity and autophagy level. D and E. SGC7901/DDP cells co-transfected with mRFP-GFP-LC3 plasmids and control siRNA or Rab5a siRNA for 48 h. The distribution were analyzed by confocal microscopy. The number of dots was calculated and compared. *P* values were calculated using parametric unpaired *Student's t* test. \**P* < 0.05. F. SGC7901 cells transfected with Rab5a-GFP for 48 h followed by exposure to 0.8 μg/ml cisplatin for 24 h before collection. Expression of DNA double strand break marker γH2AX and cell death marker cleaved-PARP1 levels were determined by western blotting. G. SGC7901/DDP cells transfected with control siRNA or Rab5a siRNA for 48 h followed by exposure to 5 μg/ml cisplatin for 24 h before collection. Expression of DNA double strand break marker γH2AX and cell death marker cleaved-PARP1 levels were determined by western blotting.

of lysosomes was not significantly influenced following Rab5a knockdown (Figure 4E). Our results suggest that Rab5a act upstream of mTOR in cisplatin-resistant cells.

### Discussion

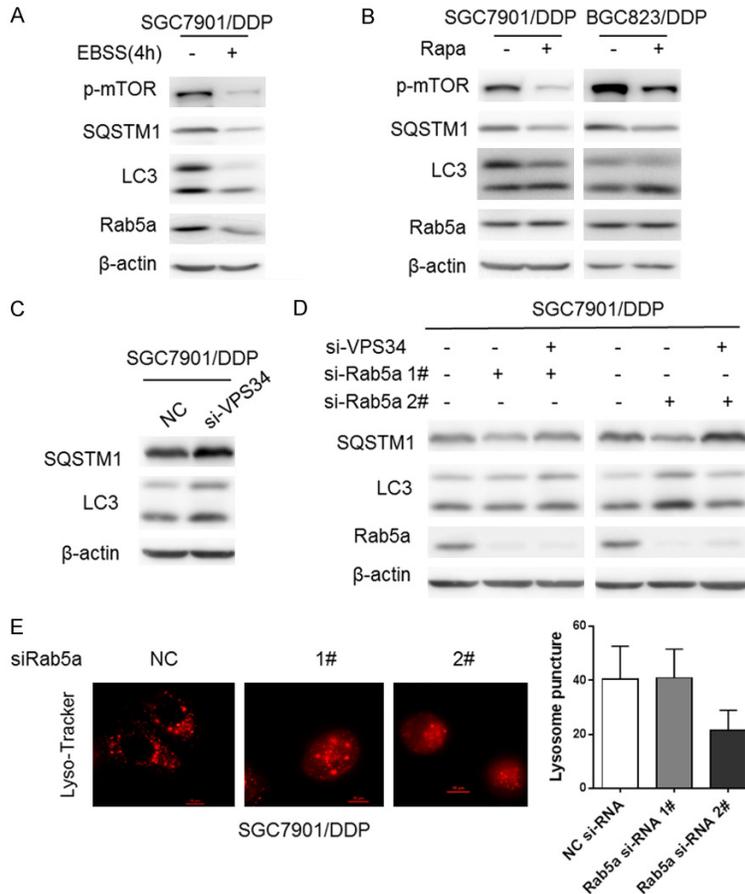
Autophagy plays a dual regulatory role in tumor cells in inhibiting or promoting tumor occur-

rence and development [16-18]. The protective effect of autophagy on tumorigenesis is mainly manifested as that autophagy can provide the basis for the growth and reproduction of tumor cells by mediating the intracellular energy and the recycling of metabolites [19]. Interestingly, the high expression of autophagy promoters in tumors predicts poorer prognosis while the high expression of suppressor genes often indicates better prognosis [20, 21]. We found that the overall survival of gastric cancer patients with high Rab5a expression was significantly longer. Knockdown of Rab5a in gastric cancer cells activated autophagy. This suggests that poor prognosis in patients with low expression of Rab5a may be due to the protection of tumor caused by autophagy.

In tumors resistant to chemotherapeutic drugs, the role of autophagy is also double-sided. Many studies have found that autophagy can help tumor cells resist drug-induced death [22-24]. Some studies also find that inhibition of autophagy can lead to drug tolerance [2, 3]. We found that knockdown of Rab5a in drug-resistant gastric cancer cells could activate autophagy and increase drug sensitivity, indicating the negative regulatory function of autophagy on tumor resistance. Certainly, the mechanism needs further exploration.

Rab5a is an important player in the endocytosis pathway responsible for the regulation of endosomal biological synthesis [25]. The extreme loss of Rab5a leads to a significant reduction in early endosomes, late endosomes and lysosomes [6]. Lysosomes are important organelles for the completion of autophagy [26, 27]. Our results showed that knocking down Rab5a did not significantly affect the amount of

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**Figure 4.** Knocking down Rab5a in drug-resistance cells activates autophagy through inhibiting mTOR pathway. **A.** SGC7901/DDP cells were cultured in normal medium or EBSS for 4 h before harvest and then the expression of Rab5a and autophagy markers were determined by western blotting. **B.** SGC7901/DDP and BGC823/DDP cells were treated with 100 nM rapamycin for 48 h before harvest and then the expression of Rab5a and autophagy markers were determined by western blotting. **C.** SGC7901/DDP cells transfected with normal siRNA or VPS34 siRNA for 48 h before harvest and the expression of autophagy markers SQSTM1 and LC3 were determined by western blotting. **D.** SGC7901/DDP cells co-transfected with VPS34 siRNA or Rab5a siRNA for 48 h were harvested and the expression of autophagy markers were determined by western blotting. **E.** SGC7901/DDP cells transfected with control siRNA or Rab5a siRNA for 48 h followed by Lyso Tracker Red to stain lysosome. The distribution were analyzed by confocal microscopy. *P* values were calculated using parametric unpaired *Student's t* test.

intracellular lysosomes. This indicates that regulation of autophagy by Rab5a is not completely dependent on its function in lysosomes. In fact, Rab5a plays an important role in regulating the activity of mTOR, which is a key regulator of autophagy [28]. Depletion of Rab5a leads to the inactivation and disordered localization of mTOR. Inactivation of mTOR results in the formation of a protein complex consisting of ATG1, ATG7, and ATG13, which is the initial step in autophagy, followed by the activation of Vps34 to form the cystic nucleus. We found that EBSS

induced the activation of autophagy and downregulation of Rab5a, but the use of 3-MA and CQ and other autophagy inhibitors did not change Rab5a expression. Furthermore, Rab5a expression did not change during autophagy induced with mTOR inhibitor rapamycin. However, knockdown of VPS34 blocked the activation of autophagy induced by the lack of Rab5a. These results suggest that Rab5a is an upstream regulator of mTOR and inhibits autophagy by activating mTOR. The detailed molecular mechanism needs further studies.

In summary, our findings provide a new perspective for understanding the effect of Rab5a on autophagy and tumor function.

### Acknowledgements

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### Disclosure of conflict of interest

None.

### Abbreviations

mTOR, mammalian target of rapamycin; PARP1, poly (ADP-ribose) polymerase 1; Atg, autophagy related;  $\gamma$ H2AX, phosphorylated histone H2AX; XRCC1, X-ray repair cross complementing group1; SQSTM1, Sequestosome-1; LC3, Microtubule-associated proteins 1A/1B light chain 3B; 3-MA, 3-methyladenine; CQ, chloroquine; EBSS, Earle's balanced salt solution.

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