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ORIGINAL ARTICLE

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# RNF135 promotes cell proliferation and autophagy in lung adenocarcinoma by promoting the phosphorylation of ULK1

**Lichun Zhuang[a#], Guanhui Shi[b#], Yuejun Sun[c*]**

[a]*Department of Pathology, Jiangyin Fifth People's Hospital, Jiangyin, Jiangsu Province, China*
[b]*Department of Pathology, Hongze Hospital, Huaian, Jiangsu Province, China*
[c]*Department of Pathology, Affiliated Jiangyin Hospital of Nantong University, Jiangyin, Jiangsu Province, China*

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**Abstract**
*Objective*: To detect the expression of RING finger protein 135 (RNF135) in lung adenocarcinoma tissues and explore its role in the progression of lung adenocarcinoma.
*Methods*: Bioinformation analysis, quantitative polymerase chain reaction, and immunoblotting technique discovered the expression of RNF135 in lung adenocarcinoma tissues. Cell counting kit-8 and colony formation, immunostaining, and immunoblot assays examined the effects of RNF135 on cell growth and autophagy. Co-immunoprecipitation (Co-IP), immunostaining, and immuoblotting were conducted to confirm the mechanism.
*Results*: RNF135 was highly expressed in lung adenocarcinoma. In addition, RNF135 promoted lung adenocarcinoma cell growth. Further, data confirmed that RNF135 promoted autophagy in lung adenocarcinoma cells. Mechanically, RNF135 directly interacted with Unc-51-like autophagy activating kinase 1 (ULK1) to promote its phosphorylation level.
*Conclusion*: RNF135 promoted cell growth and autophagy in lung adenocarcinoma by promoting the phosphorylation of ULK1.


## Introduction

Lung cancer is the fastest growing cancer type with high mortality. In the past 50 years, many countries have reported that the incidence and mortality of lung cancer has increased significantly, with the highest mortality rate accounting for 28% of all cancer types.[1] Lung adenocarcinoma is the most common type of lung cancer, accounting for 30–35% of primary lung cancers. There is an urgent need to explore effective treatments for this cancer type.

Autophagy is a catabolic process in normal cells that provides energy and macromolecular precursors (amino

[#]These authors contributed equally to the work and should be considered first co-authors.

***Corresponding author:** Yuejun Sun, Department of Pathology, Affiliated Jiangyin Hospital of Nantong University, No. 163 Shoushan Road, Jiangyin City, Wuxi City, Jiangsu Province 214400, China. *Email address*: sunyj@jyrmyy.com

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acids, nucleic acids, sugars, and fatty acids). Recently, autophagy has been found to contribute to the growth and survival of various tumors, including lung cancer, by maintaining tumor metabolism and eliminating damaged organelles.[2] Several studies confirmed that autophagy affects the progression of lung adenocarcinoma.[2]

In order to explore more efficient treatment options for lung cancer, especially lung adenocarcinoma, it is necessary to conduct in-depth studies on its molecular and metastasis mechanisms and screen out potential therapeutic targets.[3]

RING finger protein 135 (RNF135) is an E3 ubiquitin ligase consisting of an N-terminal ring finger domain, two helical domains, and a C-terminal PRY-SPRY/B30.2 domain, with a size of approximately 200 amino acid residues.[4] Studies have shown that RNF135 has multiple functions and affect the progression of multiple tumors.[5] For example, overexpression of RNF 135 regulates the viability, proliferation, and invasion of tongue cancer cells.[6] Knockdown of RNF135 inhibits the proliferation and migration of glioma cells and leads to cell cycle stagnation in the G0/G1 phase *in vivo*.[7] Mutations in RNF135 lead to phenotypic abnormalities that promote the overgrowth of neurofibroma.[8] In addition, inhibition of RNF135 expression also inhibits its malignant phenotypes, such as glioma cell migration.[5] However, RNF135 is rarely reported in lung cancer and the mechanism is unclear.

This study aimed to investigate the role of RNF135 in lung adenocarcinoma and explore its mechanism. We found that RNF135 is highly expressed in lung adenocarcinoma, and the highly expressed RNF135 promotes tumor cell proliferation and autophagy. Further studies showed that RNF135 could directly interact with Unc-51-like kinase 1 (ULK1) to promote its phosphorylation level, thus playing a cancer-promoting effect.

## Materials and methods

### *Bioinformatics*

Transcriptome data were obtained from The Cancer Genome Atlas (TCGA) genomics program databases (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga). The transcript per million of RNF135 in lung adenocarcinoma was analyzed using TCGA database.

### *Cell culture and transfection*

Human normal lung epithelial cell line BEAS-2B and lung adenocarcinoma cell lines, including H1299, PC-9, A549, and SPC-A1 cells, were purchased from American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). After 12 h of culture, lipofectamine™ 3000 transfection reagent (Invitrogen, Carlsbad, CA, USA) was used to transfect sh-NC, sh-RNF135, pcDNA3.1-vector, and pcDNA3.1-RNF135. Plasmids were transfected into cells according to the manufacturers' instructions, and 100 $\mu$L of the mixture was slowly added to six-well plate.

### *Immunoblotting*

Cells were lysed in a buffer containing 1% Triton X-100, 150-mM NaCl, and 50-mM Tris (trisaminomethane; pH 7.5). Proteins were transferred onto polyvinylidene difluoride membranes (Millipore Sigma, MA, USA), which were blocked at room temperature for 2 h in tris-buffered saline containing 0.2% Tween 20 (TBST) and 5% nonfat milk. Primary antibodies, including RNF135 (H00084282-B01; 1:500; Novus, Colorado, USA), LC3 (ab192890; 1:1000; Abcam, Cambridge, UK), p62 (ab109012; 1:1000; Abcam), Beclin-1 (ab207612, 1:1000; Abcam), ULK1 (ab177472, 1:1000; Abcam), p-ULK1 (Ser-317; #37762; 1:500; Cell Signaling, Danvers, MA, USA), p-ULK1 (Ser-555, ab229537; 1:500; Abcam), and beta-actin (ab8226; 1:3000; Abcam) were incubated with membranes overnight at 4°C; secondary antibodies were incubated for 1 h and photographed after chemiluminescence.

### *Cell counting kit-8 (CCK-8) assay*

Lung adenocarcinoma cells were plated onto a 96-well plate and maintained for 24, 48, and 72 h. Cells were subsequently incubated with CCK-8 (Beyotime, Beijing, China) for 4 h. Then the OD 450 value was measured.

### *Colony formation assay*

Lung adenocarcinoma cells were plated on a six-well plate (1000 cells per well) and maintained for 14 days. The cells were fixed with methanol and stained with 0.1% crystal violet for 15 min, and the photographs were captured.

### *Immunofluorescent staining*

lung adenocarcinoma cells were fixed with 4% paraformaldehyde (PFA) in 5% bovine serum albumin (BSA) and incubated with primary antibodies against LC3 (ab192890; 1:200; Abcam), RNF135 (mouse, H00084282-B01; 1:200; Novus, Colorado, USA), and p-ULK1 (rabbit, Ser-317; #37762; 1:200; Cell Signaling). Secondary antibodies conjugated with Alexa 488 (Invitrogen, CA) were added. Subsequently, cells were stained with 4′,6-diamidino-2-phenylindole (DAPI; 1:3000) for 3 min. Coverslips were mounted with 90% glycerol in phosphate-buffered saline (PBS) solution, and examined. Images were captured using fluorescent microscope and analyzed using the Image J 9.0 software.

### *Statistics*

The GraphPad 5.0 software was used for analysis. Each experiment was repeated thrice. The unpaired Student's *t*-test was used to determine statistical significance between two groups. One-way ANOVA, followed by Tukey's post hoc test, was used for multiple comparisons.

## Results

### *RNF135 is highly expressed in lung adenocarcinoma*

We first analyzed RNF135 expression levels in TCGA database through bioinformation analysis. The high transcript per million (TPM) of RNF135 was found in lung adenocarcinoma tissues (Figure 1A). We then discovered the expression of RNF135 in lung adenocarcinoma cell lines. Through immunoblot assay, we observed high RNF135 expression in lung adenocarcinoma cell lines, including H1299, PC-9, A549, and SPC-A1, compared to normal lung epithelial cell line BEAS-2B (Figure 1B). Therefore, RNF135 is highly expressed in lung adenocarcinoma tissues.

### *RNF135 promotes the growth of lung adenocarcinoma cells*

The RNF135 short hairpin RNA (shRNA) and its overexpression plasmid plasmid cloning DNA (pcDNA)3.1-RNF135 were transfected into lung adenocarcinoma cell lines, including H1299 and PC-9. Through immunoblot assay, the transfection of its shRNA obviously decreased its expression, and the transfection of pcDNA3.1-RNF135 significantly increased RNF135 expression in two types of lung adenocarcinoma cells (Figure 2A). Through CCK-8 assays, the downregulation of RNF135 suppressed cell growth with decreased OD450 value at 24-, 48- and 72-h time points, and overexpression of RNF135 promoted lung adenocarcinoma cell growth (Figure 2B). In addition, results of colony formation assays showed that downregulation of RNF135 decreased the colony numbers of H1299 and PC-9 cells, but its overexpression increased colony numbers (Figure 2C). We therefore believed that RNF135 promotes the growth of lung adenocarcinoma cells.

### *RNF135 promotes autophagy in lung adenocarcinoma cells*

We then discovered whether RNF135 promotes autophagy of lung adenocarcinoma cells. Through Immunoblot assay, RNF135 overexpression increased LC3II-LC3I ratio, and its downregulation decreased this ratio, suggesting autophagy promotion by RNF135 in lung adenocarcinoma cells (Figure 3A). Consistently, the overexpression of RNF135 increased the levels of Beclin-1 and decreased the expression of p62, and knockdown of RNF135 decreased Beclin-1 levels and increased p62 expression in H1299 and PC-9 cell lines (Figure 3A). We performed immunostaining assay and discovered that RNF135 overexpression increased the expression of LC3 whereas its knockdown decreased LC3 expression in H1299 and PC-9 cell lines (Figure 3B). Therefore, RNF135 promotes autophagy in lung adenocarcinoma cells.

### *RNF135 interacts with ULK1 to promote its phosphorylation level in lung adenocarcinoma cells*

The mechanism underlying RNF135 promoting progression of lung adenocarcinoma was investigated. Through co-immunoprecipitation (Co-IP) assays, we noticed that RNF135 could interact with ULK1 in H1299 and PC-9 cells (Figure 4A). We then observed the phosphorylation levels of ULK1 in H1299 and PC-9 cells after the overexpression or knockdown of RNF135. Immunoblot assay results showed that the overexpression of RNF135 increased the phosphorylation levels of ULK1 at the point of both Ser-317 and Ser-555 in H1299 and PC-9 cells (Figure 4B). However,

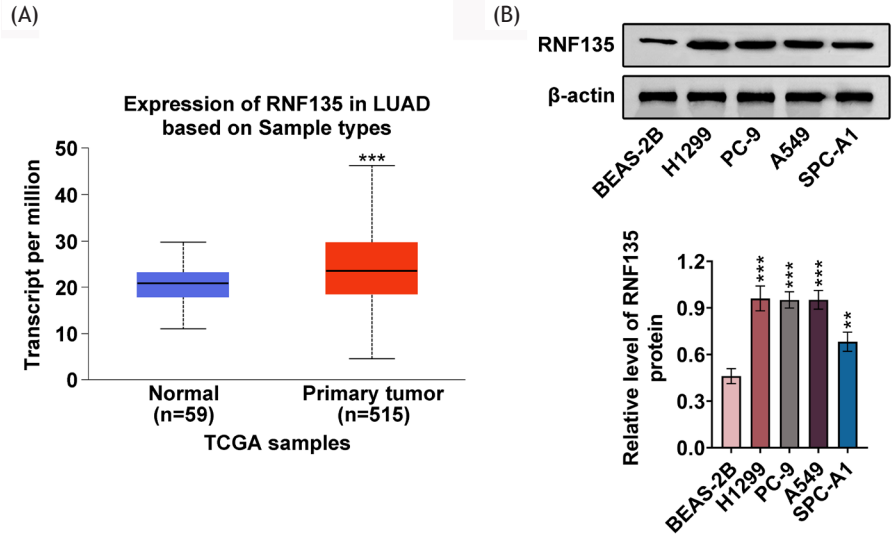


**Figure 1** RNF135 is highly expressed in lung adenocarcinoma. (A) TCGA database showed the transcripts per million of RNF135 in 515 lung adenocarcinoma tissues and 59 normal tissues. (B) Immunoblot assay showed the expression of RNF135 in human normal lung epithelial cell line BEAS-2B and lung adenocarcinoma cell lines, including H1299, PC-9, A549, and SPC-A1. **P < 0.01, ***P < 0.001.

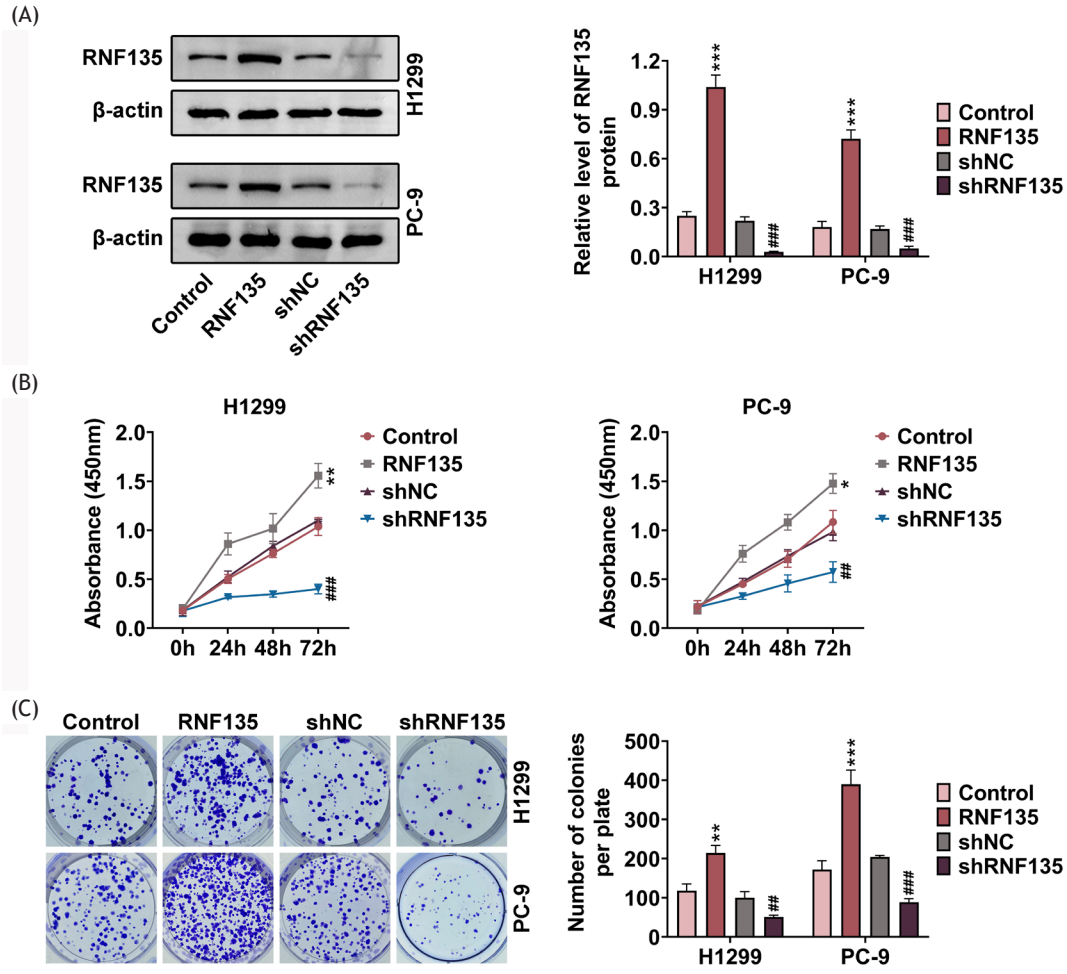


**Figure 2** RNF135 promotes the growth of lung adenocarcinoma cells. (A) Immunoblot assay showed the expression of RNF135 in H1299 and PC-9 cells upon the indicated transfection. (B) CCK-8 assay showed the growth of H1299 and PC-9 cells upon the indicated transfection for 24, 48 and 72 h. The OD450 value was measured. (C) Colony formation assay showed the growth of H1299 and PC-9 cells upon the indicated transfection. The colony numbers were counted and compared. RNF135 vs. control, *P < 0.05, **P < 0.01, ***P < 0.001. shRNF135 vs. shNC, ##P < 0.01, ###P < 0.001. NC: negative control.

knockdown of RNF135 decreased the phosphorylation of ULK1 (Ser-317 and Ser-555) in H1299 and PC-9 cells (Figure 4B). Further, through immunostaining assay, we discovered that overexpression of RNF135 increased the phosphorylation levels of ULK1 (Ser-317) in H1299 and PC-9 cells (Figure 4C). Therefore, RNF135 interacts with ULK1 to promote its phosphorylation level in lung adenocarcinoma cells.

## Discussion

Lung adenocarcinoma is a type of non-small cell lung cancer (NSCLC), most of which originate from bronchial mucosal epithelium.[9] The disease progresses slowly; hence, the initial symptoms are generally not evident. Most of the patients have already contacted the advanced stage at the time of diagnosis, and it is difficult to have good results through traditional treatment methods, such as surgery and chemoradiotherapy (CRT, CRTx, CT-RT).[10] Targeted therapy has broad prospects for treating lung adenocarcinoma. The effectiveness of targeted therapy varies with individual lung adenocarcinoma patient.[11] Targeted drug therapy is the latest treatment method, especially for lung adenocarcinoma patients, most of whom have epidermal growth factor receptor (EGFR) mutations through genetic testing.[12] Targeted drug therapy provides precise treatment for mutation points. Herein, we found that RNF135 was highly expressed in lung adenocarcinoma tissues and contributed to the progress of cancer. Therefore, we believed that it could act as a target of lung adenocarcinoma.

Autophagy is an important mechanism for maintaining the stability of intracellular environment.[13] It is the process by which cells maintain homeostasis by self-digesting proteins and recycling energy through organelles in the face of nutrient deficiency. Dysfunction of autophagy is considered as one of the important mechanisms of cancer development, as the loss of function mutations of autophagy-related genes leads to spontaneous tumors.[14] Increasing evidence suggested that autophagy-related genes regulate

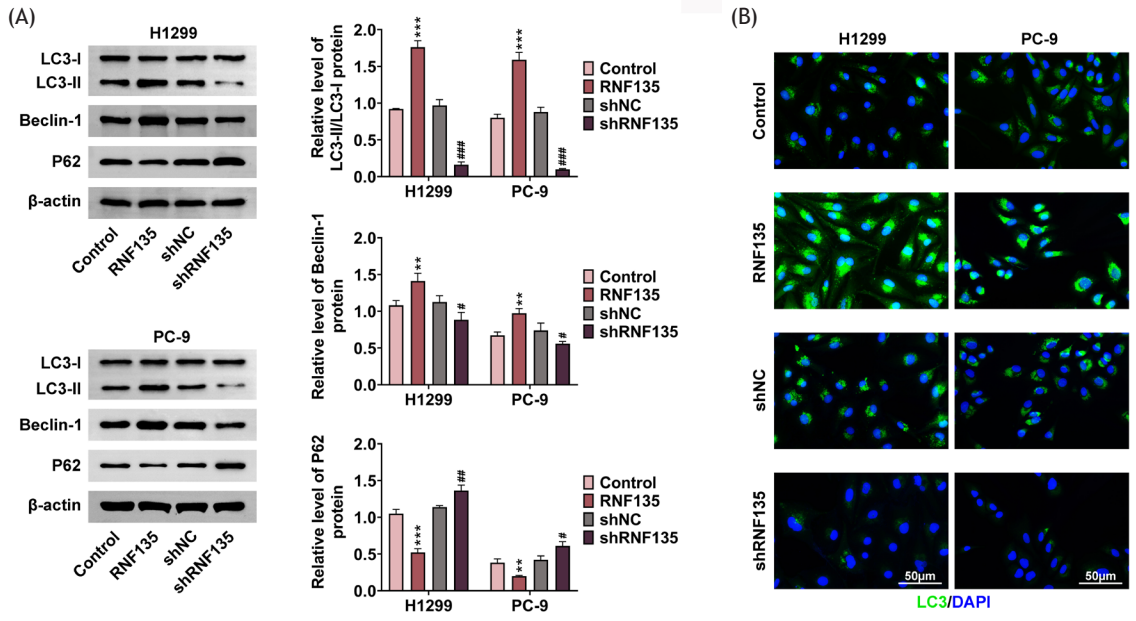


**Figure 3** RNF135 promotes autophagy in lung adenocarcinoma cells. (A) Immunoblot assay showed the expression of LC3II, LC3I, Beclin1, and p62 in H1299 and PC-9 cells upon the indicated transfection. (B) Immunostaining assay showed the expression of LC3 in H1299 and PC-9 cells upon the indicated transfection. Green panel indicates LC3. Scale bar, 50 um. RNF135 vs. control. **P < 0.01, ***P < 0.001. shRNF135 vs. shNC, #P < 0.05, ##P < 0.01, ###P < 0.001. NC: negative control.

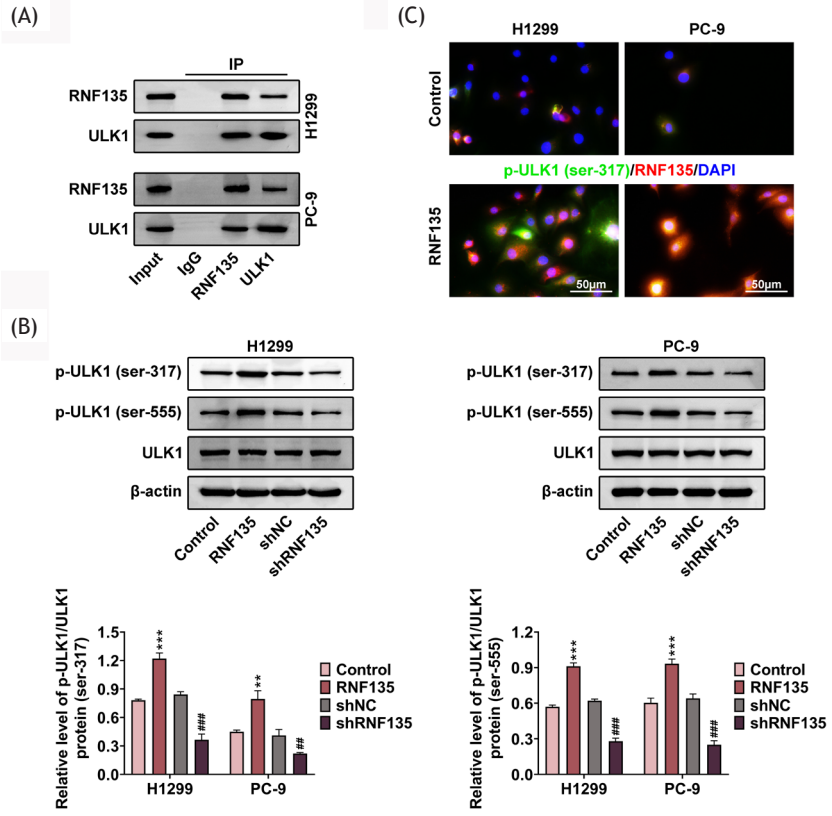


**Figure 4** RNF135 interacts with ULK1 to promote its phosphorylation level in lung adenocarcinoma cells. (A) Co-IP assay showed interaction between RNF135 and ULK1 in H1299 and PC-9 cells. (B) Immunoblot assay showed the expression and phosphorylation (ser-317 and ser-555) levels of ULK1 in H1299 and PC-9 cells upon the indicated transfection. (C) Immunostaining assay showed the expression of RNF135 and p-ULK1 in H1299 and PC-9 cells upon the indicated transfection. Red panel indicates RNF135; green panel indicates p-ULK1. Scale bar, 50 um. RNF135 vs. control, **P < 0.01, ***P < 0.001. shRNF135 vs. shNC, ##P < 0.01, ###P < 0.001. NC: negative control.

immune cell infiltration and are associated with the prognosis of lung adenocarcinoma. For example, in lung adenocarcinoma, activated EGFR promotes tumor progression by inhibiting autophagy through tyrosine-phosphorylated BECN1.[15] Tyrosine kinase inhibitors, such as erlotinib or gefitinib, inhibit the kinase activity of EGFR and induce autophagy.[16] In this study, we revealed that RNF135 promotes autophagy in lung adenocarcinoma cells. We proposed that RNF135 could suppress the progression of lung adenocarcinoma via mediating autophagy.

Through a series of *in vitro* assays, such as CCK-8 and colony formation assay, we found that RNF135 suppressed the growth of lung adenocarcinoma cells. Further, through immunoblot and immunostaining assays, we revealed that RNF135 mediated the autophagy of lung adenocarcinoma cells. These results confirmed the effects of RNF135 on the progression of lung adenocarcinoma *in vitro*. RNF135 is an E3 ubiquitin ligase with RING finger domains that plays a crucial role in the development of various cancer types.[8,17] It is highly expressed in various cancer types, such as breast cancer and hepatocellular carcinoma.[5,18,19] In addition, RNF135 contributes to the growth and metastasis of multiple cancer types. Here we also revealed its role in lung cancer, and confirmed that it could act as a target for treatment of lung cancer. However, the precise mechanism needs further study.

Mechanistic studies have demonstrated that RNF135 directly interacts with ULK1 to promote its phosphorylation level in lung adenocarcinoma. ULK1 is a key protein kinase in the process of autophagy that promotes the formation and progression of autophagy.[20] ULK1 is an autophagy activating enzyme whose action is regulated by various post-translational modifications, including phosphorylation. Phosphorylation of ULK1 significantly affects autophagy.[21] Extensive studies have demonstrated that inhibition of ULK1 expression can inhibit lung cancer progression and metastasis.[22] Here, our results confirmed that RNF135 contributed to the progression of lung cancer via mediating ULK1 phosphorylation.

This study discovered the expression of RNF135 in lung adenocarcinoma tissues, and confirmed the effect of RNF135 on lung adenocarcinoma cell proliferation and autophagy. Interestingly, we revealed that RNF135 affected lung adenocarcinoma progression via mediating ULK2, an accepted regulator of autophagy.

## Conclusion

We revealed that RNF135 is highly expressed in lung adenocarcinoma, and highly expressed RNF135 promotes tumor cell proliferation and autophagy. Mechanistic studies established that RNF135 directly interacts with ULK1 to promote its phosphorylation level, thus playing a cancer-promoting role.

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## Competing interests

The authors declared no potential conflict of interest with respect to research, authorship, and/or publication of this article.

## Ethics approval

This research did not contain any studies with human participants or animals performed by any of the authors.

## Data availability

The authors declare that all data supporting the findings of this study are available in the paper; any raw data can be obtained from the corresponding author upon request.

## Contribution of authors

Lichun Zhuang, Guanhui Shi, and Yuejun Sun designed and completed the study. All authors supervised, analyzed, and interpreted the data. All authors prepared and reviewed draft of the manuscript for publication. They read and approved the final manuscript.

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