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TRIM67 interacts with ENAH to regulate the apoptosis and autophagy of lung cancer cells

Rui Liu[1,2], Kun Miao[1,2], Ling Gao[1,2] and Ling He[1,2]

[1] *Department of Oncology, The First People's Hospital of Yunnan Province, Kunming 650032, Yunnan, P.R. China*
[2] *The Affiliated Hospital of Kunming University of Science and Technology, Kunming, Yunnan, P.R. China*

Abstract. The aim of this study was to further clarify the functional mechanism of the triangular 67 (TRIM67) gene in lung cancer cells. We detected the expression of TRIM67 in lung cancer cells by RT-qPCR and Western blot, transfected si-NC, si-TRIM67, and pcDNA-ENAH into the cells. The expression of TRIM67 and ENAH was detected by Western blot and immunofluorescence localization, and CO-IP and GST pull-down experiments verified the interaction. Flow cytometry, Western blot, and transmission electron microscopy (TEM) evaluated the apoptosis and autophagy levels. TRIM67 was highly expressed in lung cancer cell lines. Knockdown of TRIM67 promoted apoptosis and autophagy of A549 and NCI-H1299 cells. TRIM67 interacted with the ENAH protein. ENAH restored the effect of knocking down TRIM67 and further inhibited apoptosis and autophagy of A549 and NCI-H1299 cells. TRIM67 inhibits apoptosis and autophagy of lung cancer cells by interacting with ENAH.

Key words: TRIM67 — Lung cancer — ENAH — Cell apoptosis — Autophagy

Introduction

Lung cancer has become one of the cancers with the highest morbidity and mortality, which places a heavy burden on cancer patients worldwide (Sung et al. 2021). The survival rate of lung cancer in the recent five years has been low because the parts of routine treatment, such as radiotherapy and chemotherapy, have side effects like serious drug resistance (Siegel et al. 2020). Therefore, exploring new biological mechanisms of lung cancer cells for potential targets and effective treatment is pivotal.

Autophagy is a catabolism process that transports cellular proteins and organelles through the lysosome pathway, separating cytoplasmic substances into autophagosomes and fusing with lysosomes to form autolysosomes (Yu L et al. 2018). Autophagy is initially considered to perform cell steady-state functions or recover cell components to meet the energy needs of cell survival, and it occurs in almost all cells (Towers and Thorburn 2016). Several studies have confirmed that autophagy plays a crucial role in tumorigenesis and cancer progression, and it is usually activated during anticancer therapy, such as radiotherapy, chemotherapy, and targeted therapy (Levy et al. 2017). Autophagy plays a dual role in tumor progression. This means that the opposite function of autophagy, for example, situational dependence and stimulus dependence, makes it a tumor inhibitory factor and a protective factor for cancer cell survival (Maheswari and Sadras 2018).

Apoptosis, known as type I programmed death, is a process in which multiple genes regulate the homeostasis of the cell's internal environment, which in turn promotes cell death (Wong 2011). In cancer treatment, apoptosis is usually mediated by chemotherapeutic drugs, which is significant in the clinical treatment of human cancer, and cancer cell apoptosis is regulated by many apoptosis-related genes and signal pathways (Morana et al. 2022). It was proven that apoptosis was closely related to autophagy. Autophagy destroys

Correspondence to: Ling He, Department of Oncology, The First People's Hospital of Yunnan Province, 157 Xishan District, Kunming City, Yunnan Province, 650032, P.R. China
E-mail: heling701111@163.com

most of the cytoplasm and organelles, which can result in apoptosis or necrosis, ultimately leading to cell death (Maiuri et al. 2007). It was illustrated that autophagy mediates apoptosis by regulating Atg5 and Atg12, and autophagy further promotes apoptosis by mediating a variety of stress signals, which is a critical factor for cancer treatment (Yousefi et al. 2006; Rubinstein et al. 2011). As a result, it is imperative to discuss autophagy and apoptosis in the malignant progression of lung cancer cells.

Trigonal motif (TRIM) protein is a shared domain composed of one or two B-box motifs and helical domains, followed by ring fingers. It involves various biological processes, such as innate immunity, viral infection, cancer, etc. (Yaguchi et al. 2012). TRIM family proteins have been further found to be essential regulators of protein-protein interaction and enzyme activation in intracellular signal transduction, apoptosis, and autophagy (Hatakeyama 2017). TRIM67, a member of the TRIM protein family, has been reported to inhibit the malignant progression of cancer cells (Li and Li 2022). Activated homolog (ENAH), a member of the phosphoprotein (VASP) family stimulated by Ena/vasodilator, is a critical regulatory protein in cell movement, morphology, adhesion, invasion, and metastasis (Philippar et al. 2008; Gurzu et al. 2012). ENAH has been linked to the progression of many cancers, with disorders in solid tumours such as colorectal cancer (Toyoda et al. 2009), cervical cancer (Gurzu et al. 2009), and pancreatic cancer (Pino et al. 2008). However, the potential role of TRIM67 and ENAH in developing lung cancer has not been fully elucidated. In this study, we explored the interaction between TRIM67 and ENAH in lung cancer cells. Further, we clarified the potential mechanism of TRIM67 and ENAH in lung cancer cell apoptosis and autophagy, to provide the scientific basis for whether TRIM67 and ENAH can be used as prognostic markers or therapeutic targets for lung cancer treatment.

Materials and Methods

Cell culture

Human normal pulmonary epithelial cells (BEAS2B), human non-small cell lung cancer cell lines (A549, NCI-H1299, and NCI-H1650), and human lung cancer cells (NCI-H460) were purchased from Wuhan Procell Life Science&Technology Co., Ltd. The cells were cultured in RPMI-1640 or DMEM medium (Sigma-Aldrich, MO, USA) containing 10% fetal bovine serum (FBS; Gibco, CA, USA) and 1% Penicillin/Streptomycin (Sigma-Aldrich), respectively, and cultured in a 5% CO_2 cell incubator at 37°C. The cell culture medium was changed every 3 days and subcultured when the cell density reached 80–90%.

Table 1. PCR primer sequence

Gene	Primer	Sequence (5'-3')
TRIM67	F	5'- GAAAGTGTCTGCGGAGTTTGA-3'
	R	5'- TGACTCGGGCGTTGTAGGT-3'
GAPDH	F	5'-TGACCACAGTCCATGCCATCAC-3'
	R	5'-CGCCTGCTTCACCACCTTCTT-3'

F, forward; R, reverse.

Cell transfection

The cells were cultured overnight in a 24-well plate. When the cell density reached about 60–70%, si-NC, si-TRIM67, and pcDNA-ENAH were transfected into the cells according to the instructions of Lipofectamine 3000 reagent (Invitrogen, Grand Island, NY, USA). The cells were cultured at 37°C and 5% CO_2 incubator for 48 h, and the transfection efficiency was detected.

Real-time fluorescence quantitative PCR (RT-qPCR)

The total RNA of cells of each group was extracted with TRIzol reagent (Invitrogen, 15596026) and reverse transcribed into single-strand complementary DNA (cDNA) by One Step Prime Script miRNA cDNA Synthesis Kit (Takara, Kyoto, Japan). Using SYBR Green PCR Master Mix (Life Technologies, CA, USA), follow the manufacturer's procedures for RT-qPCR. The sequence of RT-qPCR primers was shown in Table 1. Taking GAPDH as the internal reference, the value was calculated by the $2^{-\Delta\Delta Ct}$ method.

Western blotting

The total proteins of each group were extracted with RIPA buffer (Sigma-Aldrich, USA) containing 1% protease inhibitor and phosphatase inhibitor, separated with 10% SDS-PAGE gel, and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA). At room temperature, the film was sealed with 5% skim milk for 2 h, the first antibody was added and spent the night at 4°C, and then the second antibody with HRP conjugated (1:2000, ab205718, Abcam, UK) was incubated at room temperature for 1 h. The protein was observed by chemiluminescence. Anti-GAPDH antibody (1:1000, ab181602, Abcam, UK) was used as control. ECL chemiluminescence solution development (BD Biosciences) was used for exposure and observation, and ImageJ was used for protein band analysis. The following primary antibodies were used: Anti-TRIM67 (1:1000, ab312832, Abcam, UK), Anti-Bcl-2 (1:10000, ab182858, Abcam, UK), Anti-Bax (1:2000, ab32503, Abcam, UK), Anti-Caspase-3 (1:5000, ab32351, Abcam, UK), Anti- ATG5 (1:10000, ab108327, Abcam, UK), Anti-P62 (1:10000, ab109012, Abcam, UK), Anti-ENAH (1:1000, ab124685, Abcam, UK).

Detection of apoptosis by flow cytometry

The cells were collected and washed twice with PBS and resuscitated with 200 μl PBS. The apoptosis rate was detected by Annexin-V-FITC/PI apoptosis kit (Absin, China). According to the manufacturer's instructions, 5 μl Annexin V-FITC and 5 μl PI were added to each well and incubated for 15 min in a dark room, and then apoptosis was detected by FACScan flow cytometry.

Transmission electron microscopy (TEM)

The cells were plated into 6-well plates to grow overnight. After trypsin digestion and collection by centrifugation, cells were fixed in ice-cold 2.5% glutaraldehyde at 4°C overnight and further fixed in 1% osmium tetroxide (OsO_4) at 4°C for 2 h. Then, cells were dehydrated in a series of ethanol solutions from 50% to 100% and subsequently embedded in Epon812 epoxy resin. Ultrathin sections (60–70 nm) were cut from the blocks used a microtome, and stained with uranyl acetate and lead citrate. Finally, sections were reviewed under a TEM (JEM-2000EX, JEOL, Japan).

Immunofluorescence colocalization experiment

Cells were inoculated on a 24-well plate (2×10^4 cells/well). After 24 h, the cells were washed twice with phosphate-buffered saline (PBS). The cells fixed by 4% paraformaldehyde were infiltrated for 10 min and sealed with bovine serum albumin for 1 h. Subsequently, the cells were double stained with one antibody TRIM67 and ENAH and incubated overnight at 4°C. The next day, the cells were incubated with corresponding secondary antibodies for 1 h DAPI staining. Finally, the stained cells were observed and photographed under a fluorescence microscope (400857, Nikon, Japan).

Co-immunoprecipitation (Co-IP)

The cells were harvested and lysed with IP cleavage buffer, and the supernatant was extracted by centrifugation. The protein AhamhamG Sepharose (Santa Cruz Biotechnology) was incubated with Anti-TRIM67 and Anti-ENAH at 4°C for 60 min and then washed twice. All IPs were shaken and incubated overnight at 4°C. The beads were collected by centrifugation and washed three times with lytic buffer. Immunoprecipitate was analyzed by immunoblotting.

Glutathione S-transferase (GST) pull-down assay

Whole-cell lysates were prepared from NCI-H1299 cells in modified RIPA buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP40, 10% glycerol, 25 mM NaF) supplemented with protease and phosphatase inhibitors. An equal amount of protein (1 mg) from the clarified lysate was added to GST, GST-RPS3, or GST-RPS3 deleted for the KH domain (GST-RPS3-ΔKH; aa 41–111) immobilized onto glutathione-agarose beads and incubated on a shaker for 4 h at 4°C. Protein complexes bound to agarose beads were then collected, washed 4 times in modified RIPA buffer, and eluted into Laemmli sample buffer. After the separation of proteins by SDS-PAGE, gels were either Coomassie stained or electrotransferred for subsequent immunoblotting analysis using TRIM67 and ENAH antibodies.

Statistical analysis

All the experimental data in this paper were expressed as mean ± standard deviation (mean ± SD). The data were analyzed and plotted with GraphPad Prism 7. T-test (t-tests) was used for comparison between the two groups, one-way analysis of variance (One-way ANOVA) was used

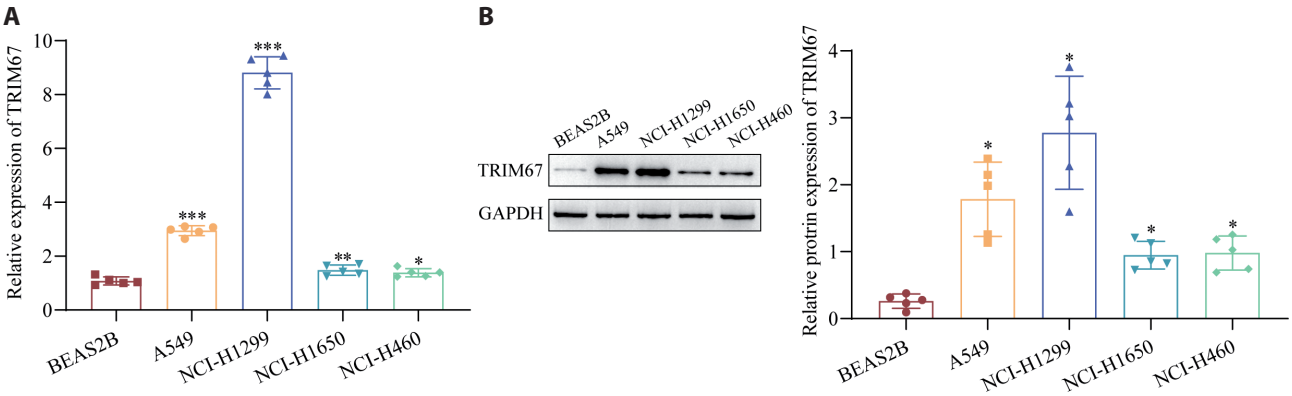
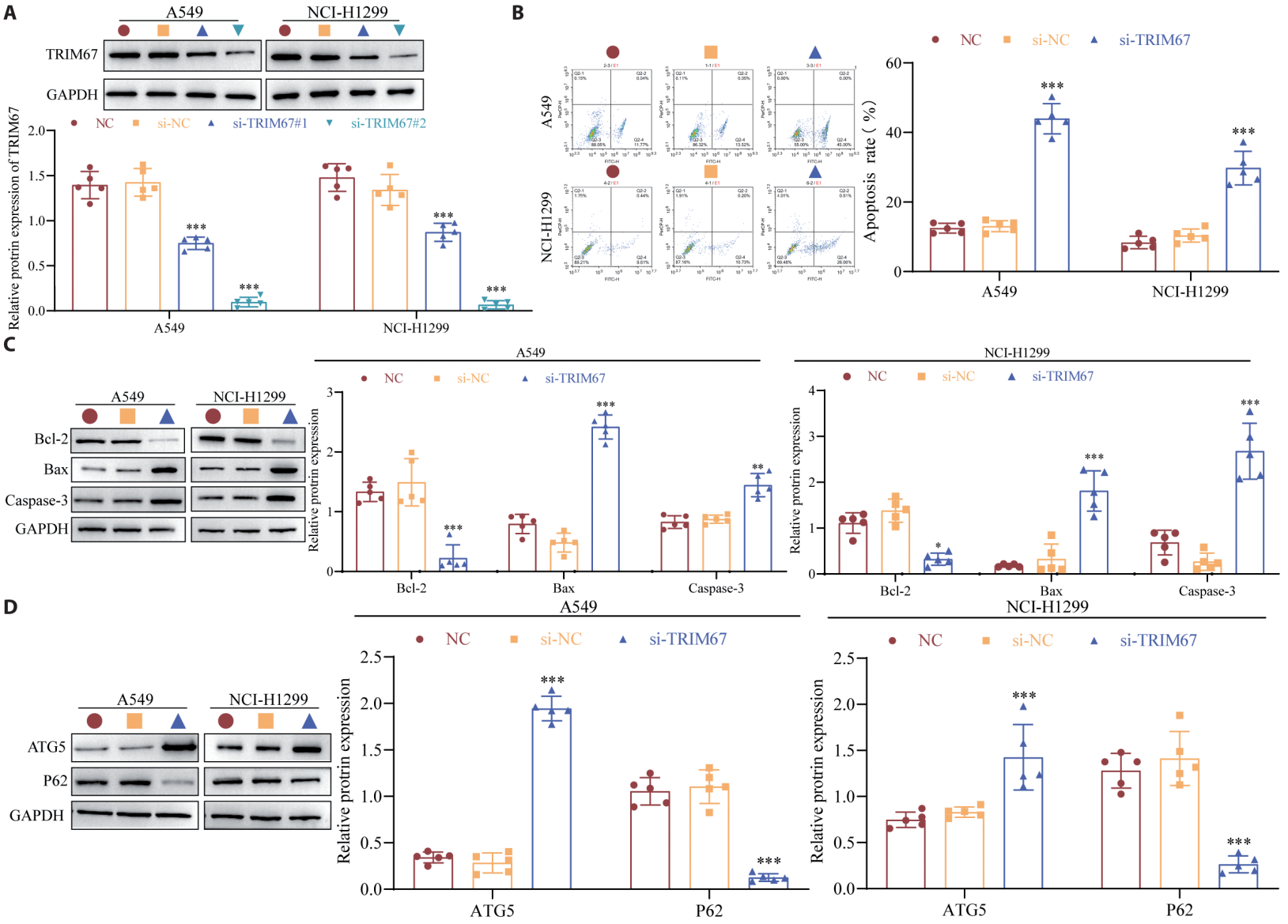


Figure 1. High expression of TRIM67 in lung cancer cells. **A.** RT-qPCR detection of TRIM67 expression levels in A549, NCI-H1299, NCI-H1650, and NCI-H460 cells. **B.** Western blot detection of TRIM67 expression levels in A549, NCI-H1299, NCI-H1650, and NCI-H460 cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* control (BEAS2B) group ($n = 5$).


A
A549
NCI-H1299
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NC
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NCI-H1299
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Apoptosis rate (%)
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NCI-H1299
C
A549
NCI-H1299
Bcl-2
Bax
Caspase-3
GAPDH
Relative protrin expression
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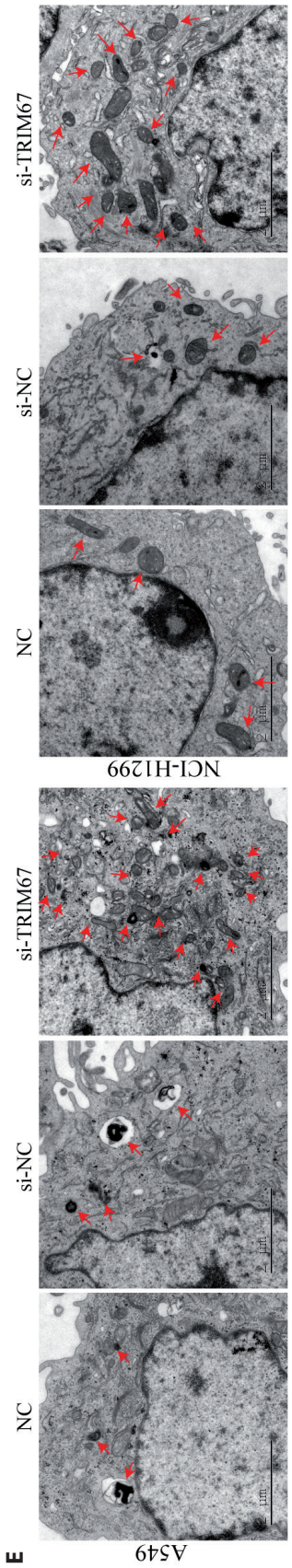


Figure 2. Knockdown of TRIM67 promotes apoptosis and autophagy of lung cancer cells in experimental groups. **A.** Western blot detection of transfection efficiency. **B.** Detection of cell apoptosis rate by flow cytometry. **C.** Western blot was used to detect apoptosis-related proteins Bcl-2, Bax, and Caspase-3 expression. **D.** Western blot detection of autophagy-related biomarkers ATG5 and P62 expression. **E.** Electron microscopic observation of autophagosomes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* NC group ($n = 5$).

for comparison between groups, and two-way analysis of variance (Two-way ANOVA) was used for pairwise comparison between groups. $p < 0.05$ indicates that the difference is statistically significant.

Results

High expression of TRIM67 in lung cancer cells

First of all, using human normal lung epithelial cells (BEAS2B) as the control group, we detected the expression level of TRIM67 in lung cancer cells by RT-qPCR and Western blot and found that the expression level of TRIM67 was significantly increased in human lung cancer cell lines (Fig. 1), especially in A549 and NCI-H1299 cells. Thus, in the follow-up experiment, we will select A549 and NCI-H1299 cells for related research.

Knockdown of TRIM67 promotes apoptosis and autophagy of lung cancer cells

To further clarify the mechanism of TRIM67 in lung cancer cells, we transfected si-NC and si-TRIM67 into lung cancer cells. The results of Western blot detection showed that there was no obvious difference between NC group and si-NC group in A549 and NCI-H1299 cells, but TRIM67 expression decreased significantly in si-TRIM67#1 group and si-TRIM67#2 group (Fig. 2A), particularly in si-TRIM67#2 group. So, si-TRIM67#2 transfection was selected for the follow-up experiment. The results of the apoptosis rate detected by flow cytometry showed that the si-TRIM67 group promoted the apoptosis of A549 and NCI-H1299 cells (Fig. 2B), which was consistent with the results of Western blot detection. Si-TRIM67 group notably down-regulated the protein expression of Bcl-2 and up-regulated the expression level of Bax, Caspase-3 (Fig. 2C). Then, the expression of ATG5 in A549 and NCI-H1299 cells was increased in the si-TRIM67 group, while the expression of P62 was significantly reduced in A549 and NCI-H1299 cells (Fig. 2D). The results of transmission electron microscopy (TEM) showed that the number of autophagosomes increased in A549 and NCI-H1299 cells transfected with si-TRIM67 (Fig. 2E). These results suggested that knocking down TRIM67 promoted apoptosis and autophagy of A549 and NCI-H1299 cells.

Interaction between TRIM67 and ENAH protein

It has been reported that the interaction between TRIM67 and other proteins was important in regulating the biological process of cells (Urbina et al. 2021). Many studies have confirmed that ENAH was a key factor in the proliferation, invasion, and migration of cancer cells (Deng et al. 2022).

Menon et al. (2021) found that the Enah/Vasp family protein ENAH interacts with TRIM67 through mass spectrometric analysis. According to these findings, we first performed Western blot and immunofluorescence co-localization assay and found that after knocking down TRIM67, the protein expression of TRIM67 and ENAH in A549 and NCI-H1299 cells decreased (Fig. 3A), and the fluorescence expression decreased (Fig. 3B). Secondly, we carried out Co-IP and GST pull-down measurements to confirm the interaction between TRIM67 and ENAH. As the results, in A549 and NCI-H1299 cells, TRIM67 protein interacts with ENAH protein in cells (Fig. 3C), and GST-labeled TRIM67 protein could pull down GST-labeled ENAH protein (Fig. 3D). This indicated that there is a close interaction between TRIM67 and ENAH proteins.

TRIM67 regulates apoptosis and autophagy of lung cancer through ENAH

Based on the above experiments, we further co-transfected pcDNA-ENAH into cells to explore the interaction between TRIM67 and ENAH in lung cancer cells. The results of the Western blot showed that the expression of ENAH in the si-TRIM67 group was lower than that in the si-NC group, and the expression of ENAH in the si-TRIM67+pcDNA-ENAH group was significantly increased (Fig. 4A). While Co-transfection of pcDNA-ENAH further restored the effect of si-TRIM67, inhibited the apoptosis of A549 and NCI-H1299 cells, up-regulated the expression of Bcl-2 and down-regulated the expression of Bax and Caspase-3 (Fig. 4B,C). Then pcDNA-ENAH reversed the promoting effect of si-TRIM67 on the expression of ATG5 and the inhibition of P62 expression in A549 and NCI-H1299 cells (Fig. 4D), and pcDNA-ENAH reduced the autophagosome in lung cancer cells (Fig. 4E). Our results show that the interaction between TRIM67 and ENAH inhibits apoptosis and autophagy of lung cancer cells.

Discussion

TRIM gene family was a superfamily of highly conserved E3 ubiquitin ligase proteins that participate in the regulation of cardiovascular, neurological, and cancer diseases by forming ubiquitin-proteasome complexes (Gushchina et al. 2018). For example, TRIM32 was abnormally expressed in breast cancer, which regulates cell growth and apoptosis through the NF-κB signal pathway (Zhao et al. 2018). TRIM31 was associated with invasive phenotype and poor prognosis in patients with pancreatic cancer, and it was a promising strategy to enhance gemcitabine response during pancreatic cancer chemo-resistance (Yu C et al. 2018). TRIM67 was a member of the TRIM protein family. It has been confirmed that TRIM67 inhibits cell proliferation, migration, and invasion by mediating MAPK11 in colorectal cancer (Liu et al. 2020). However, the mechanism of TRIM67 in lung cancer cells was not completely clear. In this study, we found that the expression of TRIM67 was significantly downregulated in lung cancer cell lines, and knocking down TRIM67 promoted apoptosis and autophagy in A549 and NCI-H1299 cells.

TRIM protein was widely involved in transcriptional regulation, cell proliferation, apoptosis, DNA repair, and metastasis of cancer cells. TRIM15 promoted the invasion and metastasis of pancreatic cancer cells by mediating lipid metabolism through the APOA1-LDLR axis (Sun et al. 2021). Previous studies found that TRIM67 inhibits colorectal cancer occurrence and tumor growth *in vivo*, and it is an inhibitor in various biological functions, such as proliferation, colony formation, apoptosis, cell cycle, migration, and invasion *in vitro* (Liu et al. 2020). Secondly, autophagy plays a crucial role in the initiation and progression of tumors. Growing evidence showed that TRIM family proteins are essential in regulating cancer cell proliferation, apoptosis, scorching death, innate immunity, and autophagy (Wan et al. 2021). TRIM proteins regulate the formation of autophagy receptors and autophagosomes, while diverse TRIM proteins may have different functions in autophagy. Hatakeyama et al. (2017) found that the knockout of 21 types of TRIM proteins reduces the formation of LC3B bodies. TRIM13 is located in the endoplasmic reticulum, induces autophagy through its coil-coil domain during endoplasmic reticulum stress, and interacts with p62 (Tomar et al. 2012). In colorectal cancer, the knockdown of TRIM39 causes the accumulation of autophagosomes (Hu et al. 2021). In our study, we also found that TRIM67 is not only an inhibitor of apoptosis of lung cancer cells, but also TRIM67 was related to autophagy of lung cancer cells. Knockout of TRIM67 increased the formation of autophagosomes and the expression of ATG5 while down-regulated the expression of P62. More and more research has proven that autophagy plays a vital function in the regulation of cell death or survival. Guo et al. (2021) have reported that quercetin triggers autophagy and encourages apoptosis in lung cancer cells as a more effective treatment strategy for lung cancer. This was consistent with our results.

Multiple interactions between TRIM proteins and proteins have been reported. In inflammatory regulation, TRIM67 competitively bound β-transducer repeat protein (β-TrCP) and IκBα to inhibit β-TrCP-mediated IκBα degradation, resulting in the inhibition of NF-κB activation triggered by TNF-α (Fan et al. 2022). The interaction between TRIM67 and MAPK11 inhibits the proliferation, apoptosis, colony formation, migration, and invasion of tumour cells (Liu et al. 2020). Menon et al. (2021) demonstrated that TRIM9 and TRIM67 interact with proteins to regulate the morphology and function of neurons, and ENAH was one of the multiple interaction partners of TRIM67. Consistent

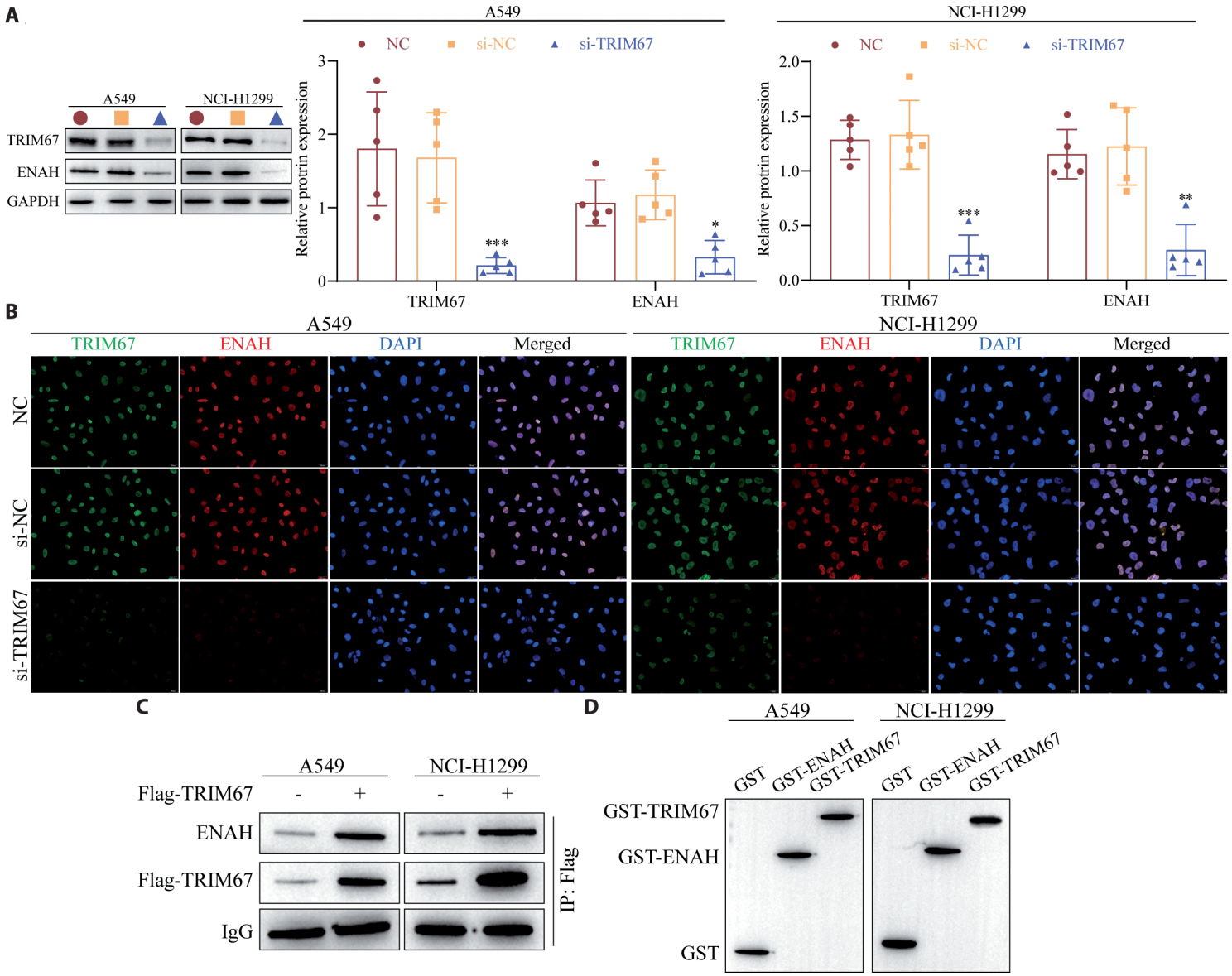
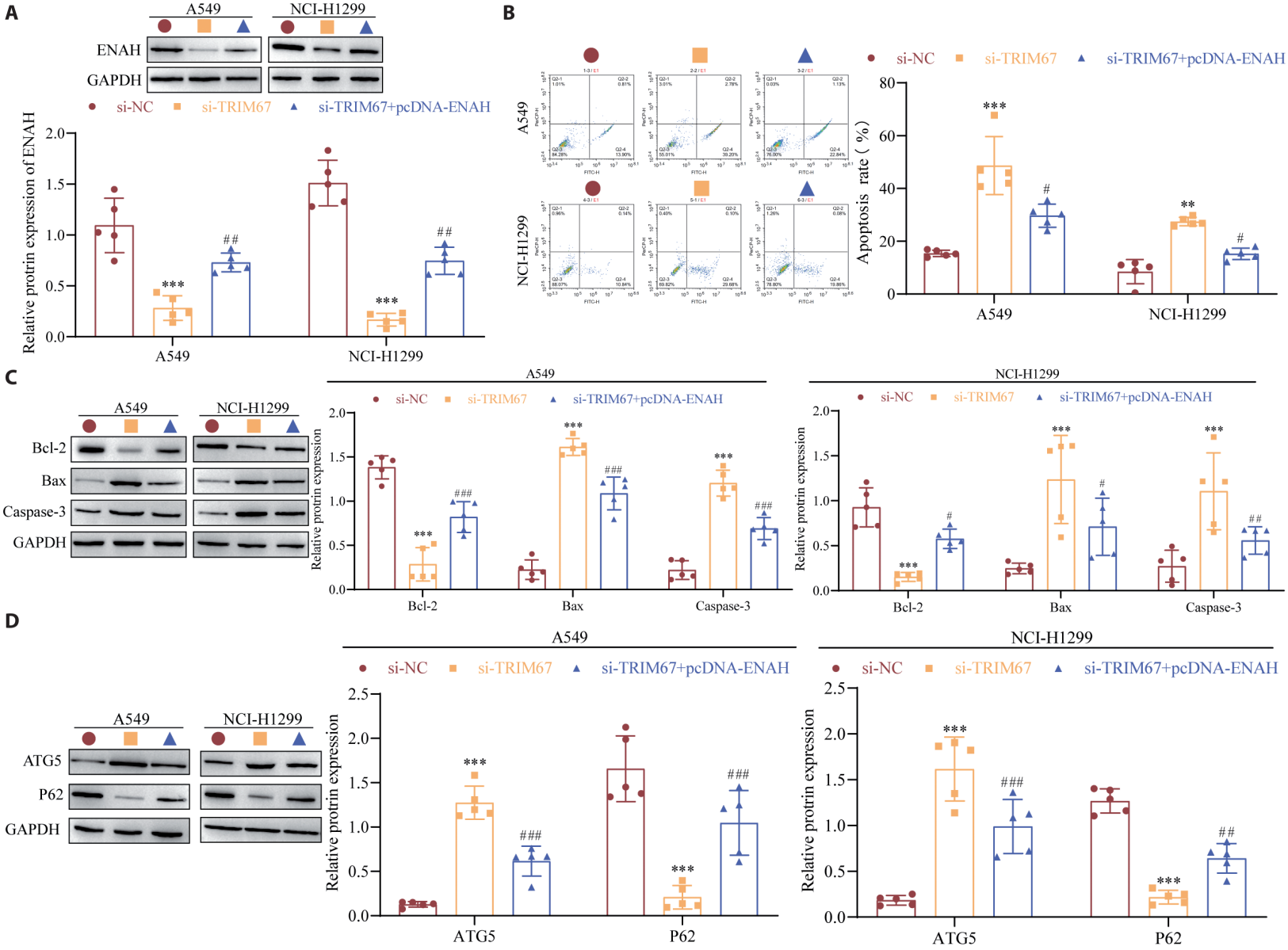


Figure 3. Interaction between TRIM67 and ENAH protein. **A.** Western blot detection of TRIM67 and ENAH expression in cancer cells. **B.** Immunofluorescence localization of TRIM67 and ENAH. **C.** Co-IP to verify the interaction between TRIM67 and ENAH. **D.** GST pull-down verifies the interaction between TRIM67 and ENAH. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* NC group ($n = 5$).


A
A549
NCI-H1299
ENAH
GAPDH
si-NC
si-TRIM67
si-TRIM67+pcDNA-ENAH
Relative protrin expression of ENAH
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A549
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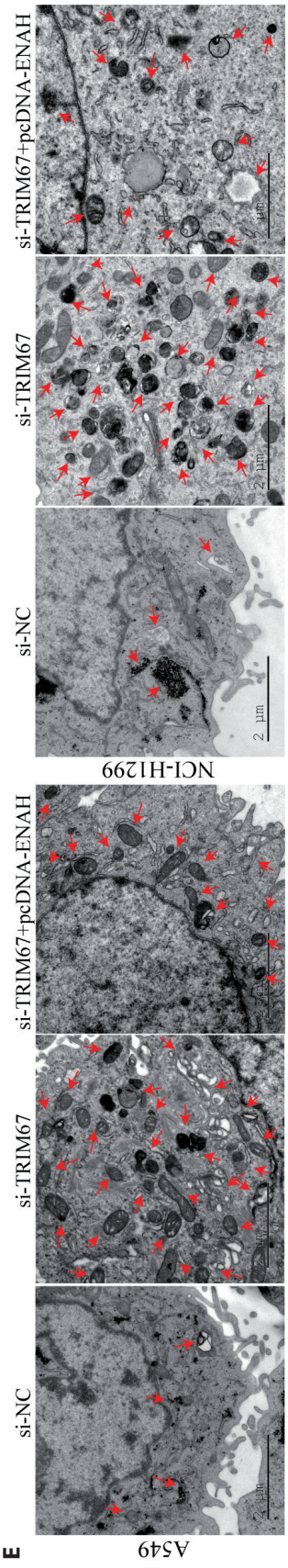

Figure 4. TRIM67 regulates apoptosis and autophagy of lung cancer through ENAH. **A.** Western blot detection of ENAH protein expression. **B.** Detection of cell apoptosis rate by flow cytometry. **C.** Western blot was used to detect the expression of apoptosis-related proteins Bcl-2, Bax, and Caspase-3. **D.** Western blot detection of autophagy-related biomarkers ATG5 and P62 expression. **E.** Electron microscopic observation of autophagosomes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* si-NC group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs.* si-TRIM67 group ($n = 5$).

with this, our results further demonstrated the interaction between TRIM67 and ENAH proteins in lung cancer cells. The Ena/VASP family consists of three proteins: Ena-VASP (Evl), Mena (ENAH), and VASP. Because they all have similar trigonal domains, they can combine with other proteins to form complexes to regulate cell migration and movement (Boëda et al. 2007). Previous studies have reported that the disorder of ENAH is associated with tumour cell invasion, migration, and poor prognosis (Di Modugno et al. 2006). Peroxisome proliferator-activated receptor gamma (PPARγ) suppresses the proliferation and migration of gastric cancer (GC) cells by inhibiting the expression of TERT and ENAH (Guo et al. 2016). It has been found that hMENA (11a) maintains the proliferation and survival of breast cancer cells after PI3K inhibitor treatment, while hMENA (11a) silencing increases the molecules involved in cancer cell apoptosis (Trono et al. 2016). Interestingly, our study found that ENAH restored the effect of knocking down TRIM67, inhibiting apoptosis and autophagy of lung cancer cells.

To sum up, our data provide prominent insights into the tumor-inhibitory effect of TRIM67 in lung cancer. We confirmed that the expression of TRIM67 is closely related to apoptosis and autophagy of lung cancer cells. Then, knocking down TRIM67 promotes apoptosis and autophagy of lung cancer cells through interaction with ENAH. However, further studies are needed to identify the direct binding sites of the interaction between TRIM67 and ENAH, as well as other potential mechanisms of their tumor-inhibitory function. In addition, this study only explored the mechanism of TRIM67 and ENAH in lung cancer cells at the cellular level. In the future, more *in vivo* studies and clinical analyses are needed to further confirm the function of TRIM67 as a potential therapeutic target for lung cancer.

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Conflict of interest. All authors declare that they have no conflict of interest.

Data availability statement. Data is applicable after the approval of co-authors.

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