

RETRACTED ARTICLE: The LINC01260 Functions as a Tumor Suppressor via the miR-562/CYLD/NF- κ B Pathway in Non-Small Cell Lung Cancer

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Purpose: Recently, long noncoding RNAs (lncRNAs) have been identified as novel and potential therapeutic targets in various cancer types. Nonetheless, the levels and biological effects of lncRNAs in non-small cell lung cancer (NSCLC) remain largely unknown. In this study, we aimed to identify the effects of lncRNA-LINC01260 throughout the progression of NSCLC and explore the underlying mechanism.

Methods: Quantitative real-time PCR (qRT-PCR) and Western blot were performed to measure LINC01260, miR-562, and CYLD expression and protein levels. Luciferase reporter assay was employed to investigate the relationship between LINC01260 and miR-562, and miR-562 and CYLD, respectively. The viability and migration of cells were evaluated using CCK-8, colony formation, and transwell assays. The effects of LINC01260 were identified through tumorigenesis *in vivo*. ELISA was performed to detect the activity of NF- κ B and p65 expression.

Results: In NSCLC tissues and cell lines, LINC01260 expression was downregulated, which corresponded to a lower survival rate of patients with NSCLC. Knockdown of LINC01260 accelerated the proliferation, colony formation, and migration of NSCLC cells. Moreover, downregulation of LINC01260 inhibited apoptosis of NSCLC cells by regulating the expression of Bcl-2 and Bax proteins *in vitro*. *In vivo*, the downregulation of LINC01260 promoted tumor growth. miR-562 was identified as the target gene of LINC01260, which was downregulated in NSCLC tumors. Furthermore, CYLD was identified as the target gene of miR-562. The effects of LINC01260 were exerted by regulating CYLD via sponging miR-562. ELISA confirmed that the upregulation of CYLD inhibited NF- κ B activity; however, the co-transfection of sh-LINC01260 partly reversed the inhibition. Additionally, CYLD reduced p65 expression; however, downregulation of LINC01260 slightly increased the expression level.

Conclusion: This study revealed a novel LINC01260/miR-562/CYLD/NF- κ B pathway in the pathogenesis of NSCLC and suggested a potential therapeutic target for NSCLC.

Keywords: non-small cell lung cancer, long noncoding RNA, miR-562, CYLD

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Introduction

Worldwide, and especially in China, lung cancer is the leading cause of cancer-related death.¹ Nearly 85% of patients with lung cancer suffer from non-small cell lung cancer (NSCLC). Interestingly, over the past 20 years, a decrease in the rate of small cell lung cancer has been reported across the globe.² Despite the effort of scientists and continued improvements in the general treatment of lung cancer, the

5-year survival rate of patients has yet to be increased.³ Approximately 90% of patients with NSCLC lose their lives due to the effects of distant metastasis, rather than those of the primary tumor. Therefore, metastasis is regarded as a key element for both the prognosis and treatment of lung cancer.⁴ The complex molecular and cellular mechanisms related to lung cancer metastasis are still unknown. Investigating possible treatment targets to improve the prognosis of patients with NSCLC can therefore have a significant impact.

Long noncoding RNAs (lncRNAs) are non-protein coding transcripts longer than 200 bp.⁵ lncRNAs are involved in regulating basic biological activities by targeting gene expression at nearly all levels. It has been revealed that various lncRNAs can regulate the malignancy of cells, such as the transformation, drug sensitivity, viability, and migration by regulating various molecular mechanisms of malignant tumor cells.⁶ Furthermore, it has been reported that lncRNAs also affect tumor initiation, development, and metastasis by the regulation of gene expression at both transcriptional and post-transcriptional levels as well as at the epigenetic level. Therefore, lncRNAs have been investigated as prognostic biomarkers and potential treatment targets for tumors.⁷ For instance, lncRNA-TPTEP1 inhibited the proliferation of NSCLC cells by downregulating miR-328-5p expression, whilst lncRNA-00312 attenuated cell proliferation and migration of renal cell carcinoma cells through miR-34a-5p/SS1 axis.^{8,9} Additionally, lncRNA-AOC4B suppressed the viability, invasion capability, and induced the apoptosis of NSCLC cells by inhibiting the Wnt/ β -catenin pathway.¹⁰ LINC00473 is the most highly induced gene of LKB-inactivated human primary NSCLC samples and has been integrated as a biomarker in clinical treatment.¹¹ As reported before, lncRNA LINC01260 was lowered in spinal cord glioma and co-expressed with CARD11 in multiple cancers, such as breast invasive carcinoma and prostate adenocarcinoma.¹²⁻¹³ In this study, we have demonstrated that LINC01260 was low-expressed in NSCLC patients. However, the role of LINC01260 played in the procedure of NSCLC and the underlying mechanism is still unknown.

lncRNAs have been known to exert many functions, such as acting as scaffolds or as enhancers to modulate transcription of their target genes.^{14,15} Interestingly, more and more studies have suggested that lncRNAs could function as a sponge of miRNAs and contribute to the carcinogenesis of many tumors.^{16,17} Herein, the subsequent

mechanistic experiments indicated that LINC01260 might exert its function by competitively sponging miR-551 to regulate CYLD expression, which inhibits the activation of nuclear factor-kappa B (NF- κ B) signaling. The NF- κ B family consists of five master transcription factors, RelA/p65, RelB, c-Rel, p50/p105 and p100/p52 which are coordinating regulators of inflammatory and immune responses and playing an important role in oncogenesis disorders.¹⁸ For instance, NF- κ B signaling promotes cancer development and progression by regulating various target genes controlling the cell proliferation, angiogenesis and metastasis.¹⁹ Therefore, the aberrant NF- κ B signaling was associated with a lot of tumors, including colorectal, breast, lung, pancreatic and ovarian carcinoma.^{20,21}

Combined with these findings, LINC01260 might also play a critical role in the progression of NSCLC. Therefore, this study was performed to explore the role of LINC01260 in the proliferation and migration of NSCLC cells by sponging miR-551 and raising CYLD expression and then regulating NF- κ B pathway.

Materials and Methods

Patients and Specimens

We obtained NSCLC and NSCLC-adjacent tissue (≤ 3 cm) samples from 32 patients with NSCLC who had undergone surgery at Shengli Clinical College of Fujian Medical University, Fujian Provincial Hospital from January 2016 to June 2018. The patients had no secondary tumors and did not receive any other therapy. Samples were snap-frozen in liquid nitrogen and then stored in freezing storage tubes at -80°C . Then, we performed the storage and transportation process without the use of and potential inference from any enzymes. We obtained written informed consent from all participants and the approval for this research from the Ethics Committee of the Fujian Provincial Hospital.

Cell Culture

We purchased normal bronchial epithelial cell lines (HBE) as well as NSCLC cell lines (SPC-A1, H1650, H1650, and A549) from ATCC (Rockville, MD, USA). We used Dulbecco's Modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Hyclone, USA), 100 IU/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin (Invitrogen, USA) or Roswell Park Memorial Institute (RPMI) or 1640 medium (GIBCO-BRL; Thermo Fisher Scientific,

Waltham, MA, USA) to culture the cells. Cells were maintained in a humidified incubator at 37°C with 5% CO₂.

Cell Transfection

Genesee Biotech (Guangzhou, China) designed and synthesized Lentivector-mediated sh-LINC01260 and non-targeting plasmids (sh-control). Lentivirus infection of NSCLC cells was carried out using Polybrene (8 ng/mL). Following PCR amplification of cDNA encoding LINC01260 and CYLD (cylindromatosis), the cDNA was subcloned into the pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA, USA). We used an empty pcDNA3.1 plasmid as a negative control. hsa-miR-562 mimics were obtained from Genesee (Shanghai, China) and used as negative control mimics. Lipofectamine 2000 reagent (Invitrogen, CA, USA) was applied for transfection experiments in accordance to the instructions. All cells were cultured to approximately 50–60% view of culture dish and transfected with serum-free media for 24 h.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from NSCLC tissues and cultured cells using TRIzol reagent (Invitrogen, USA). A reverse transcription kit (Takara, Tokyo, Japan) was used for the reverse transcription of mRNA and miRNA into cDNA. Next, the miRNA Extraction Kit (T802, Hs Gene, China) was used to extract total miRNA from tissues and cells. QRT-PCR was carried out using Roche Lightcycler 480 (Roche, USA). Relative gene expression, normalized by GAPDH and U6 references, was calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows:

5'-CGCGCCAAAGAGCTCACC-3' (forward) and 5'-ACCGCACCGGTCCGAGGTATT-3' (reverse) for miR-562;
5'-CTCTCTTCGGCAGCAGCACATATA-3' (forward) and 5'-AAATATCGAACGCTTCACGA-3' (reverse) for U6;
5'-CCACACACACCAGAGAGGAC-3' (forward) and 5'-ATTATCCACACAGCAGCCA-3' (reverse) for LINC01260;
5'-GAAGAGAGAGACCCTCACGCTG-3' (forward) and 5'-ACTGTGAGGAGGGGAGATTCAGT-3' (reverse) for GAPDH;
5'-CACCAAGATGCCCAATACCA-3' (forward) and

5'-CTTCAGCCAATGAGCCCACT-3' (reverse) for CYLD;

5'-CCCGAGAGGTCTTTTCCGAG-3' (forward) and 5'-CCAGCCCATGATGGTTCTGAT-3' (reverse) for Bax;

5'-GGTGGGGTCATGTGTGTGG-3' (forward) and 5'-CGGTTTCAGGTAAGTCAATCC-3' (reverse) for Bcl-2.

Luciferase Reporter Assay

The 3'-UTR sequence (200 bp pre and post the binding sequence) of the CYLD gene transcript was included in the pGL3 vector containing luciferase reporter genes via whole gene synthesis technology, creating a wild-type (WT) 3'-UTR group. We used a site-directed mutagenesis kit to mutate the core region of miRNA binding with the 3'-UTR into invalid binding sequences, forming a control plasmid with a mutant (Mut) 3'-UTR group. pGL3-LINC01260 WT group and pGL3-LINC01260 Mut group were also constructed. Each group was transfected with marine kidney luciferase internal reference plasmids and miR-562 mimics. We then removed all of the cell culture solution after transfecting for 24 h. Following the manufacturer's instructions, we lysed the cells by adding appropriate amount of lysate. After centrifugation, an aliquot of 100 µL of the supernatant was taken for testing. The RLU value measured by firefly luciferase was divided by the RLU value measured by sea kidney luciferase as an internal reference. We then compared the activation level of different sample target reporter genes using the obtained ratio. This procedure was repeated three times.

Cell Migration

Transwell chambers were placed above a 24-well plate. We then digested and suspended the transfected NSCLC cells. Next, cells were diluted to 1×10^5 and added into the upper chamber, along with 100–200 µL of cell suspension. Five hundred microliters of complete culture medium containing FBS was added to the lower chamber. The culture medium was removed 24–48 h later, and cells were washed with PBS. The cells in the lower chamber were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet (Beyotime, Nantong, China) for 10–15 min, and then washed with PBS. Eventually, the cells were harvested with 10 fields in each well (200× magnification). Image-pro Plus 6.0 (Media Cybernetics, USA) was utilized for counting migrated cells. Each experiment was conducted in triplicate.

Colony Formation Assay

1×10^3 NSCLC cells were seeded into agar (1.5 mL) and placed on the bottom of the well in triplicate. Cells were replenished with 2 mL of complete medium twice a week. Two weeks later, colonies were dyed with 0.5 mL of 0.1% crystal violet for one hour, and then colonies ≥ 0.5 mm were quantified using a TE2000-U dissection microscope (Nikon, Tokyo, Japan).

Cell Proliferation

We seeded NSCLC cells at 1×10^4 , and then cultured for 0, 24, 48, and 72 h in 96-well plates. Then, we used Cell Counting Kit-8 (CCK-8; Dojindo, Japan) to test cell proliferation. We replaced the culture medium with 110 μ L of mixed culture medium containing 10 μ L of CCK-8 and incubated for 1 h at 37°C in the dark. Finally, we examined the OD value at 450 nm using TECAN infinite M200 Multimode microplate reader (Tecan, Mechelen, Belgium). We set 6 replicate wells in each group. This procedure was repeated three times.

Western Blotting

Cell lysis was performed in RIPA buffer (Beyotime, Nantong, China) containing protease and phosphatase inhibitors (Beyotime, Nantong, China). Following manufacturer guidelines, a standard bicinchoninic acid (BCA) test was performed to determine the protein concentration in the cell lysate. The samples (80 μ g protein per lane) were separated with 10% SDS-PAGE gel by electrophoresis. The proteins were electrotransferred to PVDF membranes (ThermoFisher, USA). Membranes were then blocked with 5% skim milk TBST at 4°C for 1 h, prior to overnight incubation with anti-GAPDH (1:1000, ab8226, abcam, USA), anti-CYLD (1:1000, ab200476, abcam, USA), anti-Bcl-2 (1:1000, ab32124, abcam, USA), anti-Bax (1:1000, ab32503, abcam, USA), and anti-p53 (1:1000, ab16592, abcam, USA) at 4°C. The membranes were then washed three times with TBST and incubated with the corresponding secondary antibodies for one hour at 20–25°C. Lastly, Phototope-HRP Western Blot Detection System (Cell Signaling Technology, Danvers, MA, USA) was used to detect the expression of proteins. The protein expression levels were calculated as relative expression to GAPDH. This procedure was repeated three times.

ELISA

Cell supernatant was collected 24 hours after culture and washed 3 times with PBS. Then, the Nuclear Extraction Kit purchased from Cayman (Item No. 10,009,277) was

applied for nuclear extracts. NF- κ B p65 activity was determined using the Transcription Factor Assay Kit (10,007,889, Cayman Chemical, USA). The 96-well plate supplied with this kit is ready to use. A total of 100 μ L reagents was added to the designated wells and the provided 96-well cover sheet was used to seal the plate overnight at 4°C. Empty the wells and wash five times with 200 μ L of Wash Buffer (1 \times). After the final wash, tap the plate on a paper towel to remove any residual Wash Buffer. Dilute the Transcription Factor NF- κ B (p65) Primary Antibody 1:100 in Transcription Factor Antibody Binding Buffer (ABB, Cayman). Add 10 μ L to each well except the Blk wells and seal the plate with the cover sheet. Then, the plate was incubated at 20–25°C for one hour. Add 100 μ L Transcription Factor Goat Anti-Rabbit HRP Conjugate (1:100) to each well and incubate for one hour at 20–25°C. After washing, 100 μ L Transcription Factor Developing Solution was added to each well and the plate was incubated for 30 min at 20–25°C in the dark. Finally, stop solution was added into each well. The absorbance at 450 nm was measured using TECAN infinite M200 Multimode microplate reader (Tecan, Mechelen, Belgium). All procedures were carried out in triplicate.

Tumor Xenograft Model

BALB/c male nude mice (Shanghai SLAC Laboratory Animal Co. Ltd., Shanghai, China), 4 to 5 weeks old (18–20 g), were randomly separated into two groups ($n = 3$ /group). The mice were cultured under standard conditions (24°C \pm 2°C, 50 \pm 10% relative humidity, 12 h light/dark cycles) and with unlimited access to standard rodent maintenance feed (KEAO XIELI FEED Co., Ltd.) and water. Hygienic conditions were maintained by weekly cage changes. Animal health and behavior were monitored every day and body weights were assessed weekly over the course of the study. 1×10^6 of A549 cells, transfected with sh-LINC01260 or sh-control, were subcutaneously injected into the right flank of the mice. Tumor volumes were calculated after observation with the following formula: volume = (length \times width²)/2. After three weeks, mice were sacrificed, and tumor tissues were obtained. Xenografts then went through immunohistochemical staining for Ki-67 and PCNA. We obtained the approval for the animal experiments from the Animal Care and Use Committee of Shengli Clinical College of Fujian Medical University.

Immunohistochemical Staining

Anti-Ki-67 (1:200) and anti-PCNA (1:200) primary monoclonal antibody (Cell Signaling Technology) were used to incubate tumor tissue sections, and further incubation was performed with the secondary antibody. The sections were then counterstained with hematoxylin for 30 s, followed by incubation with 3,3'-DAB (Maxim, Fuzhou, China) for 5 min. Finally, we imaged the samples using a TE2000 microscope (Nikon).

Statistical Methods

Statistical analysis was carried out using GraphPad Prism 7.0 software. Kaplan–Meier and Log rank tests were used to calculate overall survival rates. The statistically significant differences between the two groups were determined using two-tailed Student's *t*-test. The comparisons among different groups (>2 groups) were analyzed by one-way analysis of variance (one-way ANOVA) with a Tukey's post hoc test. Pearson correlation analysis was applied to analyze the correlation between LINC01260 and miR-562, and CYLD and miR-562 expressions. All data were presented as the mean \pm SEM. P values less than 0.05 were considered as statistically significant.

Results

LINC01260 Was Significantly Downregulated in NSCLC Tissues and NSCLC Cell Lines

First, we measured the expression level of LINC01260 in NSCLC tumor tissues as well as in adjacent non-cancerous tissues. It was found that LINC01260 was significantly downregulated in NSCLC tissues (Figure 1A). Next, patients with lung cancer were divided into a metastasis group and a non-metastasis group and I+II and III+IV groups. The data suggested that LINC01260 was expressed at significantly lower levels in the tissues of patients with cancer metastasis and III+IV group (Figure 1B and C). ROC curve analysis showed that LINC01260 might be a viable diagnostic marker for patients with NSCLC (AUC=0.8667, Cut off value=0.8122), while survival analysis showed that a low expression of LINC01260 predicts a poor prognosis (HR 0.4097, $P=0.0085$) (Figure 1D and E). In addition, we also measured the expression level of LINC01260 in NSCLC cell lines. LINC01260 expression level was found to be lower in lung cancer cell lines than in HBE, the normal bronchial epithelial cell lines (Figure 1F). All data indicated that LINC01260 is involved in the development of NSCLC.

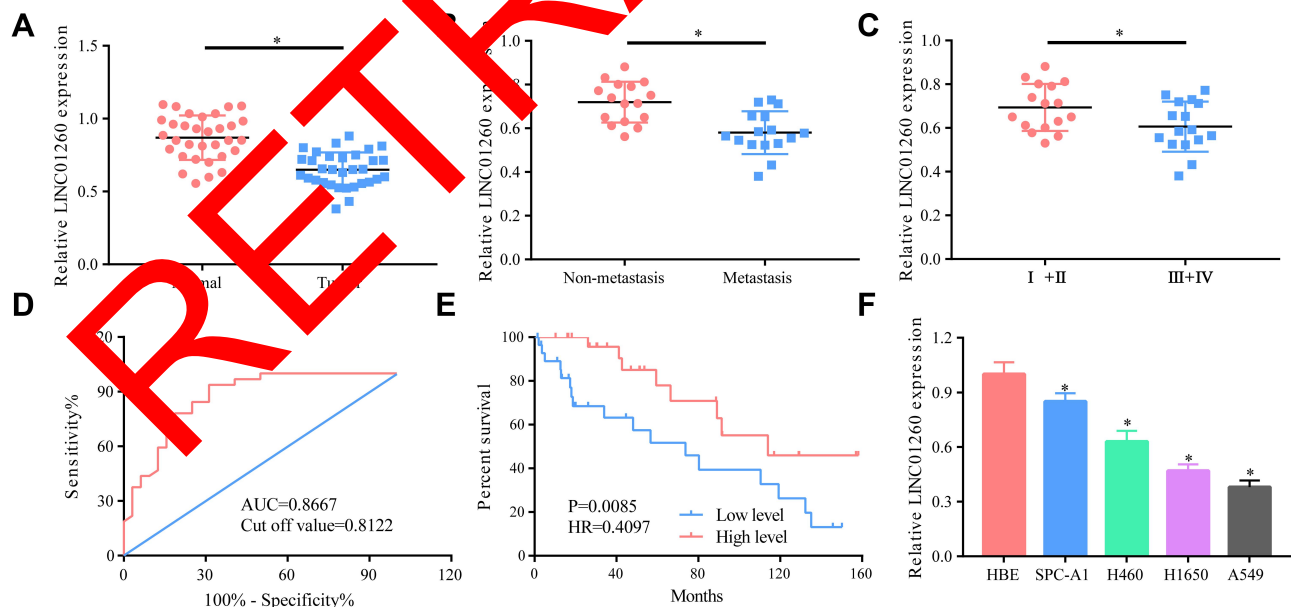


Figure 1 LINC01260 was significantly downregulated in NSCLC tissues and NSCLC cell lines. (A) The level of LINC01260 was distinctly lower in lung cancer tissues than that in normal tissues. (B) The level of LINC01260 in metastasis group was decreased than that in non-metastasis group. (C) The level of LINC01260 in the tissues of patients with lung cancer in stage III+IV was significantly lower than that in patients with lung cancer in stage I+II. (D) ROC curve analysis showed that AUC = 0.8667 and Cutoff value = 0.8122. (E) Survival analysis showed that a low expression of LINC01260 was a predictor of a worse prognosis, $R^2=0.4097$, $P=0.0085$. (F) The LINC01260 expression was generally lower in lung cancer cell lines than that in normal bronchial epithelial cells. * $p<0.05$.

LINC01260 Knockdown Facilitated Cell Proliferation and Migration

We explored the potential effect of LINC01260 on the occurrence and development of lung cancer using A549 and H1650 cell lines. The level of LINC01260 was significantly reduced after cells were transfected with sh-LINC01260 (Figure 2A). CCK-8 results showed that the proliferation of lung cancer cells was markedly enhanced after downregulation of LINC01260 expression (Figure 2B and C). Additionally, we performed a plate cloning experiment, and the results clearly indicated that the knockdown of LINC01260 significantly

upregulated the number of proliferating clones within the cells (Figure 2D). Finally, we confirmed using transwell assays that the downregulating of LINC01260 significantly promoted lung cancer cell migration (Figure 2E).

Downregulation of LINC01260 Inhibited Apoptosis in NSCLC Cells

In order to further investigate whether LINC01260 has an effect on the apoptosis of lung cancer cells, we examined the level of related apoptotic proteins. As indicated in Figure 3A and B, downregulation of LINC01260 led to increased Bcl-2

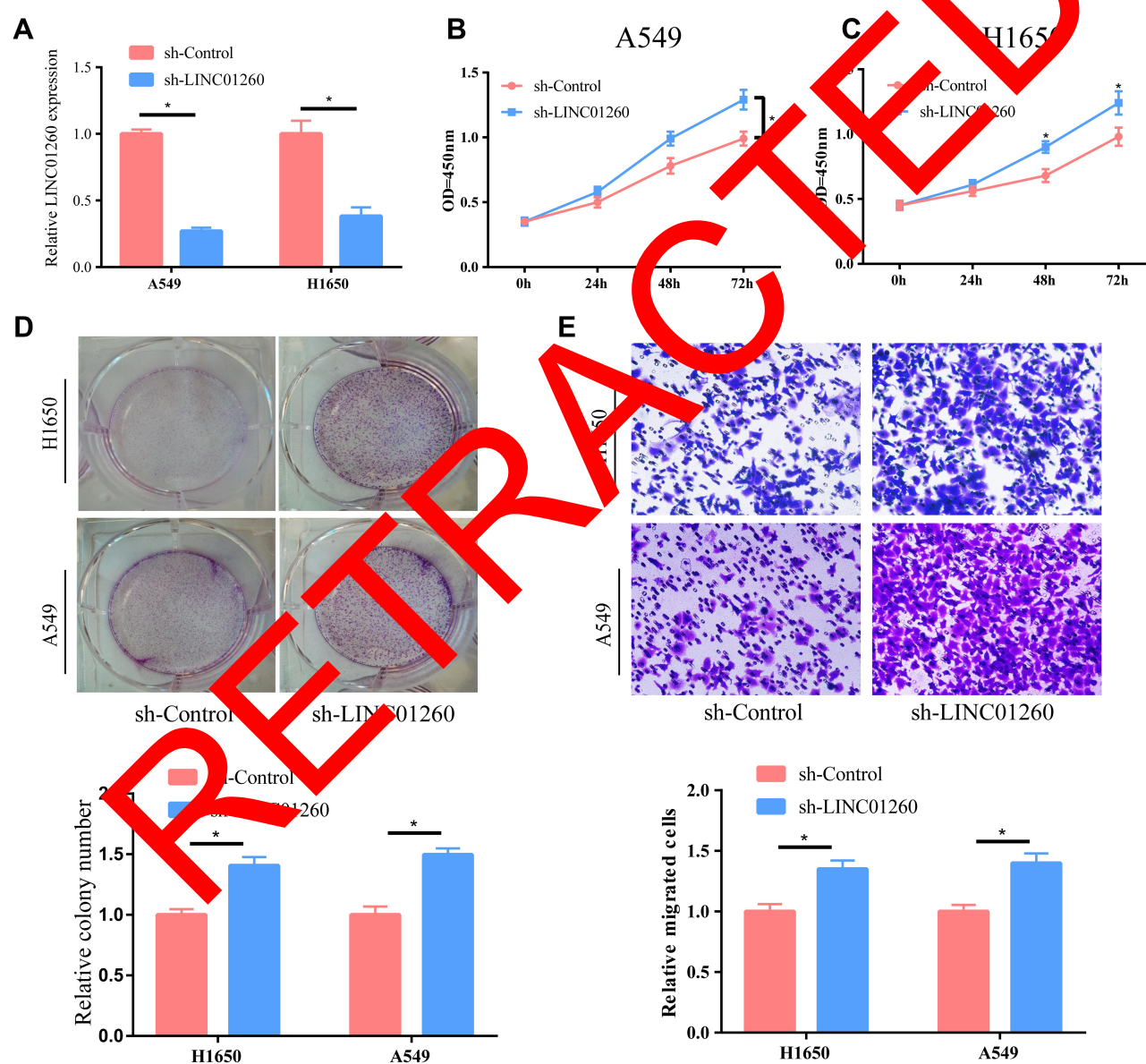


Figure 2 LINC01260 knockdown facilitated cell proliferation and migration. (A) After sh-LINC01260 transfected lung cancer cell lines A549 and H1650, the level of LINC01260 was significantly decreased. (B and C) After down-regulating the LINC01260 expression in A549 and H1650 cells, their proliferation ability was clearly strengthened. (D) Cell colony number was detected after LINC01260 down-regulated. (E) The ability of cell metastasis was significantly advanced after the level of LINC01260 in A549 and H1650 cells was down-regulated. * $p < 0.05$.

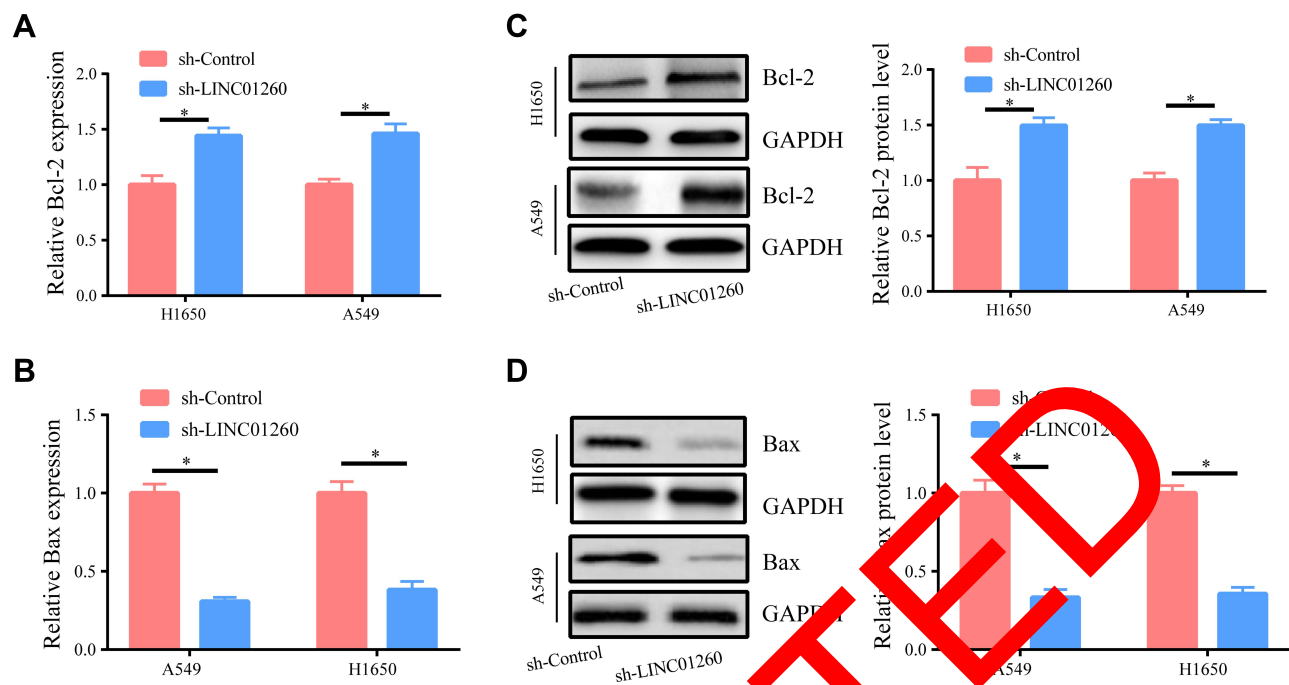


Figure 3 Downregulation of LINC01260 Inhibited Apoptosis in NSCLC Cells. (A) The expression of anti-apoptotic protein Bcl-2 mRNA was improved when LINC01260 down-regulated. (B) The pro-apoptosis protein Bax mRNA expression was reduced when LINC01260 down-regulated. (C and D) The protein level of Bcl-2 and Bax was determined when LINC01260 down-regulated. * $p < 0.05$.

and reduced Bax mRNA expression. Western blot was carried out to evaluate the protein levels of Bcl-2 and Bax. We found that downregulation of LINC01260 increased the anti-apoptotic protein Bcl-2 and decreased the protein expression of the proapoptotic protein Bax (Figure 3C and D). The above results concretely demonstrate that LINC01260 affects the apoptosis of lung cancer cells.

LINC01260 Knockdown Promoted Tumorigenesis of NSCLC in vivo

We further investigated the biological effects of LINC01260 on the development of NSCLC tumor in vivo by implanting A549 cells with sh-LINC01260 into nude mice by subcutaneous injection. Evidently, LINC01260 knockdown accelerated tumor growth in mice and LINC01260 was confirmed to be downregulated in harvested tumor tissues by analyzing the data of tumor growth curves and tumor weight (Figure 4A–D). It was discovered that the number of metastatic nodules was upregulated in the sh-LINC01260 group compared to that of the control group (Figure 4E). The subcutaneous tumors formed by LINC01260 knockdown in A549 cells had substantially more stained Ki-67 and PCNA proteins compared to the control group (Figure 4F and G), indicating that the downregulation of LINC01260 promoted tumor cell growth. In summary, these results revealed that

LINC01260 knockdown accelerated the development of NSCLC tumor in vivo.

miR-562 Was a Target Gene of LINC01260

We found that LINC01260 targeted miR-562 and a potential binding site between the two may exist, as determined using bioinformatics analysis via DIANA tools (LncBase Predicted v.2) (Figure 5A). In fact, we verified the binding relationship between the two by luciferase reporter assay (Figure 5B and C). The effect of LINC01260 on the expression of miR-562 was verified by downregulating the expression of LINC01260 in A549 and H1650 cells and examining the expression level of miR-562. It was found that the level of miR-562 was significantly elevated after the expression of LINC01260 was down-regulated (Figure 5D). On account of this regulatory relationship, we then examined the expression of miR-562 in lung cancer, finding a significantly raised expression of miR-562 in lung cancer and a negative correlation with LINC01260 ($R^2 = 0.2511$, $P = 0.0035$) (Figure 5E and F). The results confirmed that miR-562 was a target gene of LINC01260.

CYLD Was a Potential Target Gene of miR-562

We found that CYLD was a possible target gene of miR-562 (Figure 6A), and the binding relationship between them was

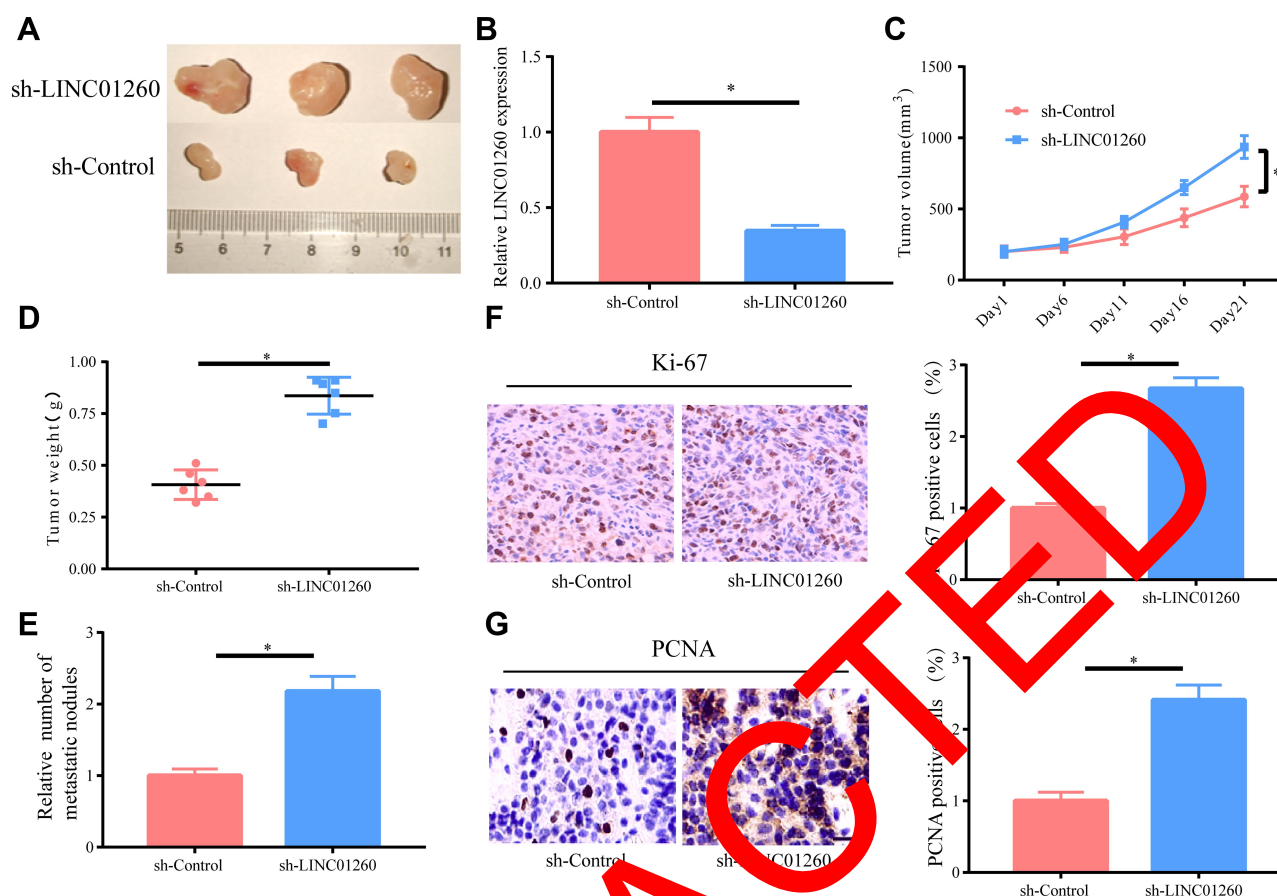


Figure 4 LINC01260 Knockdown Promoted Tumorigenesis of NSCLC in vivo. (A) Photographs of resected tumors of mice in sh-LINC01260 and sh-control groups and the level of LINC01260 in tissues of resected tumors. (B) Bar graph of relative LINC01260 expression in tissues of resected tumors. (C) Line graph of tumor volumes of mice in sh-LINC01260 and sh-control groups. (D) Scatter plot of tumor weights of mice in sh-LINC01260 and sh-control groups. (E) Bar graph of relative number of metastatic nodules in two groups. (F and G) Representative images of HE staining and immunohistochemical staining of Ki-67 and PCNA in mouse tumor tissues. * $p < 0.05$.

further verified by dual-luciferase reporter assay, prognosis and analysis through data mining analysis of miRDB (Figure 6B). We also detected the expression of other potential target genes of miR-562 in NSCLC tissues; however, no significant difference was found (Supplementary Figure 1). Therefore, CYLD was selected for the further study. After elevating the expression of miR-562 in A549 and H1650 cells, we measured CYLD expression and found that both mRNA and protein levels of CYLD were significantly reduced (Figure 7C and D). Further tests confirmed that the expression of CYLD in tumor tissues of patients with lung cancer was markedly lower than that in the control group, and was negatively correlated with miR-562 ($R^2=0.2454$, $P=0.0039$) (Figure 6E and F).

LINC01260 Exerted Its Effects by Regulating CYLD

Based on the above results, we speculated that LINC01260 regulated the function of CYLD by sponging miR-562. In

order to verify this, we showed that both the mRNA and protein levels of CYLD were significantly reduced after transfection of sh-LINC01260 in A549 and H1650 cells, and by upregulating CYLD, we could partially restore its expression (Figure 7A–D). CCK-8 and transwell assay experiments also showed that downregulating LINC01260 in lung cancer cells significantly promoted cell proliferation and migration, while upregulating CYLD could partially reverse this effect (Figure 7E and F).

LINC01260 Functions by Regulating the NF- κ B Signaling Pathway

As known, CYLD is a negative regulator of NF- κ B;²² therefore, we speculated that LINC01260 might ultimately affect the activation of NF- κ B through CYLD. First, we upregulated the expression of CYLD in A549 and H1650 cells and found that the activity of NF- κ B was significantly inhibited (Figure 8A). At the same time, we discovered that the expression of p65 was also significantly reduced compared

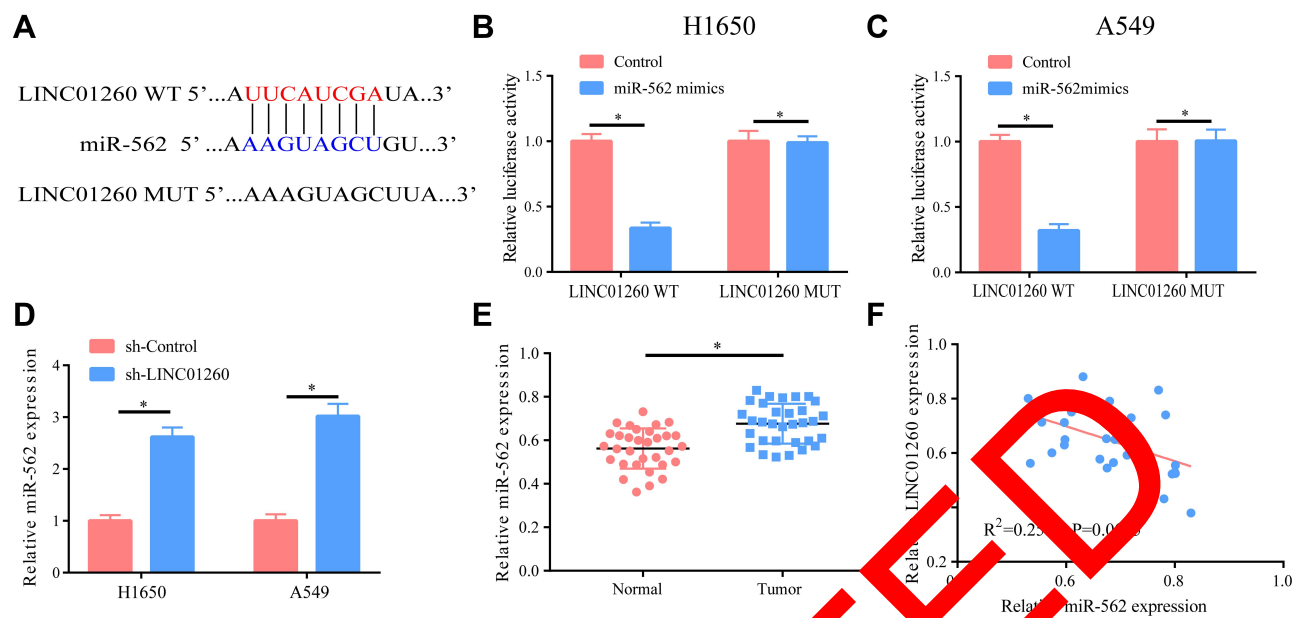


Figure 5 MiR-562 was a target gene of LINC01260. (A) Bioinformatics data revealed that there were potential binding sites between the two. (B and C) Dual-luciferase reporter gene indicated that there was a binding relationship between them. (D) After the level of LINC01260 in A549/H1650 cells was down-regulated, the expression of miR-562 was markedly elevated. (E) MiR-562 had a markedly higher level in lung cancer tissues than that in normal tissues. (F) The LINC01260 and miR-562 expression level has a negative correlation. * $p < 0.05$.

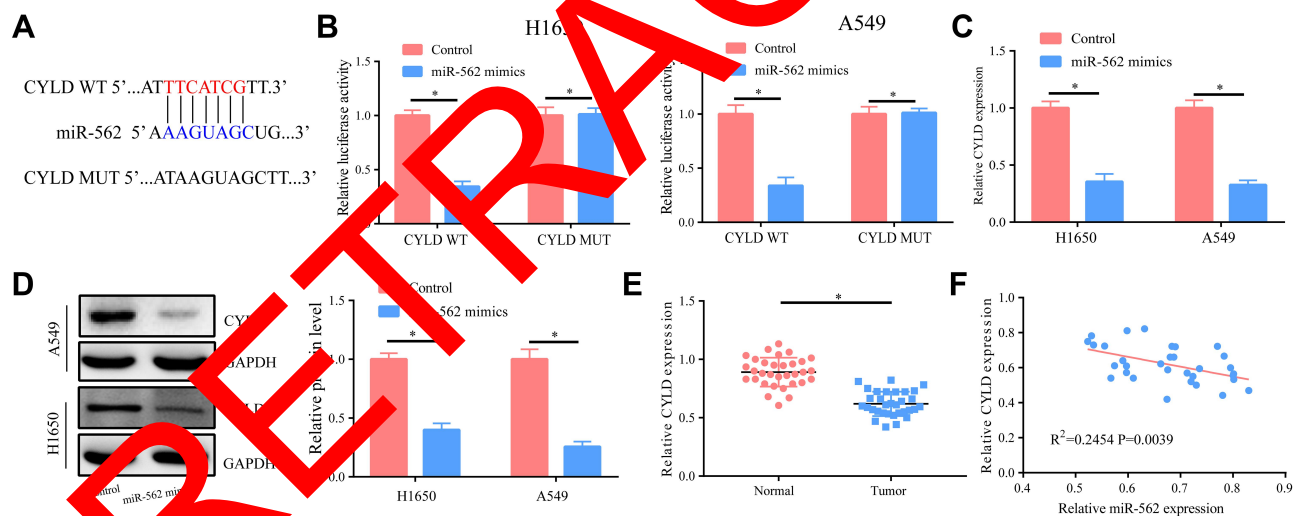


Figure 6 CYLD was a potential target gene of miR-562. (A) Bioinformatics analysis suggested that there exist potential binding sites between the two. (B) According to the dual-luciferase reporter gene assay, they have a binding relationship. (C and D) After up-regulation of the miR-562 level in A549/H1650 cells, the mRNA and protein levels of CYLD significantly lowered. (E) The level of CYLD decreased in lung cancer tissues. (F) The level of CYLD and miR-562 was inversely related. * $p < 0.05$.

to the control (Figure 8B). Next, we transfected cells with sh-LINC01260 and found that the downregulation of LINC01260 partially reversed the inhibitory effect of CYLD on the NF- κ B signaling pathway (Figure 8C). Furthermore, we found that the expression of p65 was partially reversed by sh-LINC01260 (Figure 8D).

Therefore, LINC01260 ultimately is capable of regulating the NF- κ B signaling pathway via CYLD.

Discussion

In recent studies, it has been found that metastasis and tumor progression are related to lncRNAs, which could be

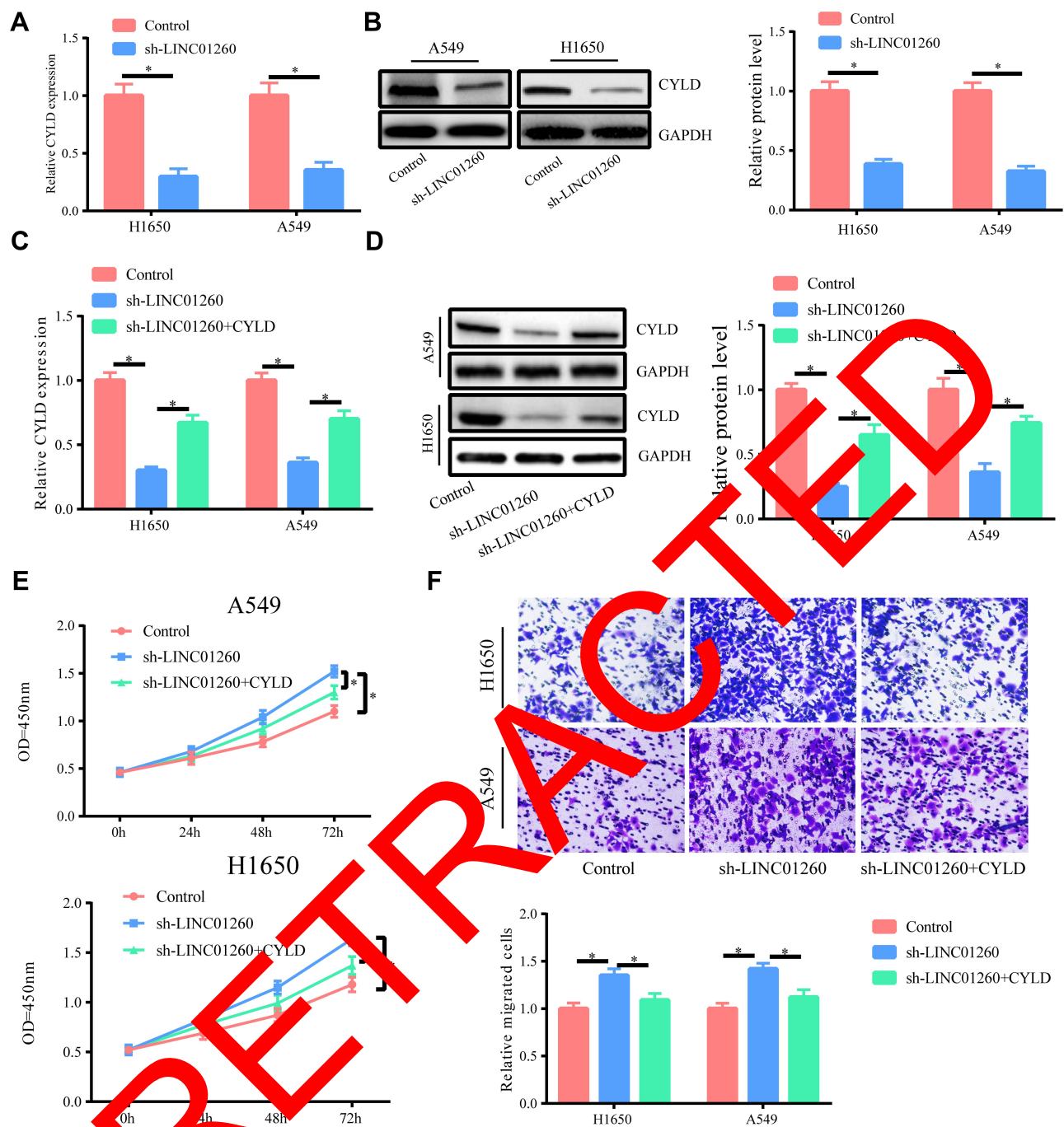


Figure 7 LINC01260 exerts its effects by regulating CYLD. (A and B) The CYLD mRNA and protein levels were distinctly down-regulated after A549/H1650 cells were transfected with sh-LINC01260. (C and D) Co-transfection of sh-LINC01260 and CYLD overexpression plasmid could increase the expression of CYLD compared to sh-LINC01260 transfection alone. (E and F) The elevated CYLD expression in A549/H1650 cells could partly inhibit the cell proliferation and migration ability enhanced by LINC01260 down-regulation. * $p < 0.05$.

used in diagnosis and prognosis.^{23,24} In this study, we discovered that LINC01260 was significantly reduced in NSCLC tissue samples and NSCLC cell lines. Meanwhile, we found that the expression of LINC01260 was significantly related to tumor stage, metastasis, and patient prognosis. Furthermore, the decrease in LINC01260 levels in

A549 and H1650 cells led to cell proliferation and migration, as confirmed using CCK-8, colony formation, and transwell assays. In vivo, we found that the knockdown of LINC01260 promoted tumor growth in mice; we also conducted immunohistological examinations in mice. However, the use of TNM classification and clinical data

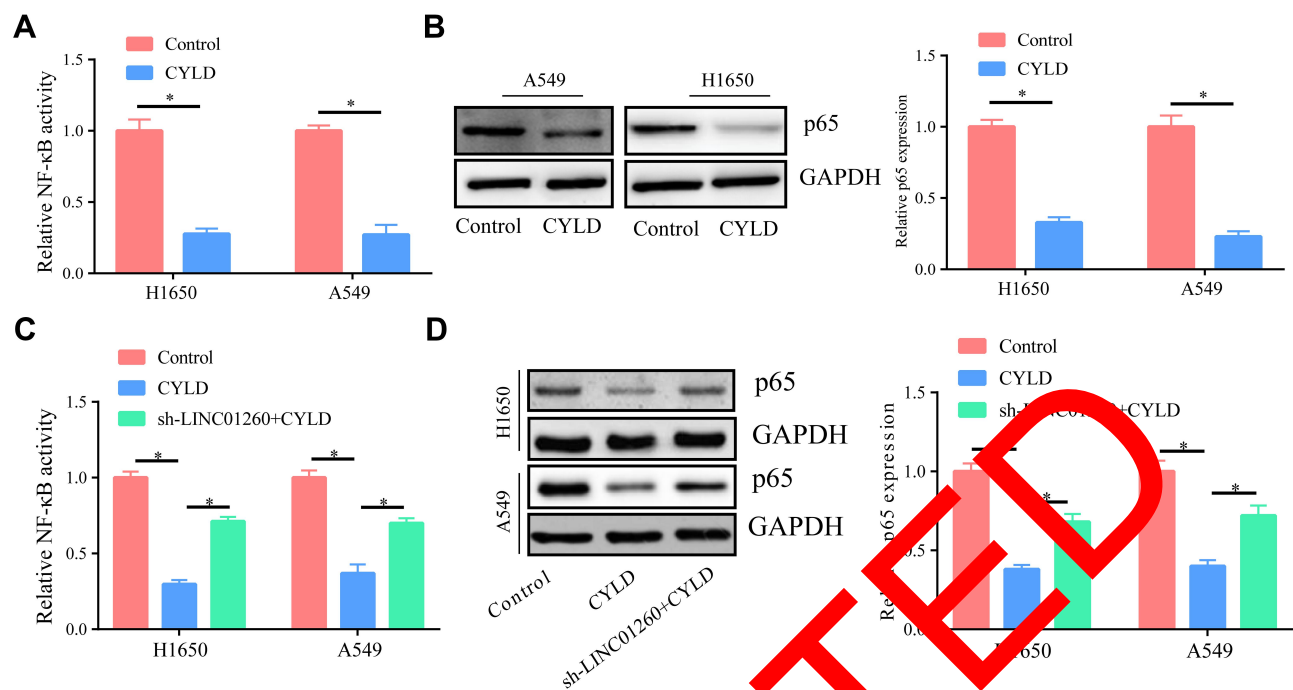


Figure 8 LINC01260 functions by regulating NF-κB signaling pathway. **(A)** Overexpressing CYLD in A549/H1650 cells significantly inhibited NF-κB activity. **(B)** After over-expressing CYLD in A549/H1650 cells, it significantly inhibited the expression of p65. **(C and D)** Simultaneously down-regulating the expression of LINC01260 can partially reverse the inhibitory effect of CYLD's increasing expression on NF-κB activation. * $p < 0.05$.

analysis of patients with NSCLC would allow for more convincing results. Additionally, a larger sample size of NSCLC tissues is needed for further study.

We found that LINC01260 knockdown induced greater expression of Bcl-2 and the reduction of Bax, indicating the anti-apoptotic effects of LINC01260 downregulation. Though there have been some studies investigating LINC01260, the role of LINC01260 in NSCLC was still unclear. Our study revealed the downregulation of LINC01260 in NSCLC, and more importantly, we described the functions it exerted in NSCLC, enriching the study of the underlying mechanisms of action in NSCLC.

Recently, a novel mechanism of RNA reciprocity has been revealed by competitive endogenous RNA (ceRNA). Noncoding RNAs regulate the expression of miRNA targets by functioning competitively against ceRNAs to provide the sponge effect of miRNAs.²⁵ The lncRNA takes part in the regulation of gene expression and a variety of biological processes, as does ceRNA. It has been proved that lncRNAs participate in the development of the tumor.^{26,27} This study, we determined the mechanism of action of LINC01260 by bioinformatics analysis and observed that miR-562 was a target gene. Furthermore, we confirmed a binding relationship between LINC01260

and miR-562 via a dual-luciferase reporter assay, and thus it can be speculated that LINC01260 functioned as a ceRNA. In the literature, there are few reports on the role of miR-562 in lung cancer. Therefore, we investigated the expression of miR-562 in lung cancer and found that miR-562 was highly expressed in lung cancer tissues. MiR-562 even further expressed after LINC01260 expression was downregulated, suggesting a negative correlation.

In this study, it was revealed that miR-562 targeted CYLD and regulated its expression. CYLD was originally identified as a tumor suppressor in benign tumors of skin appendages, and its function as a tumor suppressor has been reported in different types of cancer.^{28,29} This study detected the expression of CYLD in NSCLC tissues and revealed that the expression of CYLD was significantly lower in NSCLC than in control tissues, indicating that CYLD may play a role as a tumor suppressor gene in lung cancer. It was reported that lncRNA could serve as a ceRNA and regulate downstream target genes by sponging miRNA.³⁰ In this study, we confirmed that the expression of CYLD was reduced after LINC01260 downregulation. The results of further functional experiments showed that the upregulation of CYLD partially reversed the effect of sh-LINC01260 on cell proliferation and migration, and thus we confirmed that LINC01260 regulated CYLD expression by sponging miR-562.

The NF- κ B transcription factor family greatly influences numerous inflammatory and immune reactions, such as the reaction to cancer.^{31,32} Though the detailed mechanism of lncRNAs is well known, most lncRNAs execute their biological function through reciprocity with other molecules, such as microRNAs, other lncRNAs, genomic DNA, and especially proteins that participate in significant pathways such as NF- κ B signaling.³³ Through research in cell development, CYLD has been found to be a major regulator that turns off NF- κ B, prevents tissue damage and necrosis, and inhibits even the malignant proliferation of some cells.^{34,35} Thus, we predicted that LINC01260 was able to influence the activation of the NF- κ B signaling pathway via CYLD. In order to verify this, we first tested the effect of CYLD upregulation on the NF- κ B pathway in NSCLC cells. The results confirmed that CYLD significantly inhibited NF- κ B activity in both A549 and H1650 cells, and reduced the expression of p65. Subsequently, we downregulated the expression of LINC01260 and found that the inhibitory effect of CYLD on the NF- κ B pathway was partially reversed, further confirming our prediction.

Conclusion

In summary, we identified a novel LINC01260-miR-562-CYLD-NF- κ B regulatory axis in NSCLC pathogenesis. LINC01260, which has the potential to serve as a biomarker, acted as a tumor suppressor that inhibited NSCLC tumorigenesis via acting as a competing endogenous RNA and regulated the expression of CYLD through sponging miR-562 directly, which ultimately inhibited the activation of the NF- κ B pathway. Overall, we uncovered the roles of LINC01260 in NSCLC and the underlying mechanism. These findings not only provide a new direction for the study of pathogenesis of NSCLC but also suggest a new target for early intervention and treatment of patients with NSCLC.

Data Sharing Statement

The datasets supporting the conclusions of the current study are available from the corresponding author on reasonable request. Please contact the corresponding author, if you want to request the dataset.

Ethics Approval

This study was approved by the Ethics Committee for Animal Experimentation of the Fujian Provincial Hospital and conducted according to the National Institutes of Health guidelines for the care and use of laboratory animals.

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Disclosure

The authors declare that they have no competing interests in this work.

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