Silencing of *LINC01279* is activated apoptosis and autophagy in lung cancer by regulating FAK/ERK via SIN3A

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Abstract

Background: In human lung adenocarcinoma (LUAD) tissues, Long noncoding RNA *LINC01279* is significantly upregulated. However, the functions of *LINC01279* in LUAD is yet to be clarified.

Methods: In situ hybridization was employed to investigate the difference between expression of *LINC01279* in LUAD and in normal tissues. The result of in situ hybridization is verified by qRT-PCR. Cytoplasmic and nuclear experiments showed that *LINC01279* was mainly located in the cytoplasm of lung cancer cells. The loss of function experiment showed that *LINC01279* could inhibit the proliferation, colony formation, invasion and migration of lung cancer cells. The interaction between SIN3A and *LINC01279* was confirmed by RIP test. At the same time, through western bolt, we found that *LINC01279* plays a key role in the regulation of apoptosis and autophagy in lung adenocarcinoma.

Results: Our study confirmed that *LINC01279* was upregulated in LUAD tissues, the knocking-down of which significantly inhibited the growth of LUAD cancer cells both in vitro and in vivo. Mechanistic investigations revealed that *LINC01279* could directly interact with SIN3A and modulate the FAK and ERK protein expression in the cytoplasm. Moreover, the proteins of PARP and LC3B, P62, Beclin-1, respectively related with apoptosis and autophagy, were changed after *LINC01279* siRNA.

Conclusions: Taken together, our research found that *LINC01279* which is significantly up-regulated in LUAD tissues and cell lines, and promotes the changes of FAK and ERK proteins in downstream pathways by combining with SIN3A, promotes the proliferation of LUAD cells, and inhibits apoptosis and autophagy. The results of this work illustrated how *LINC01279* is part of a regulatory network that contributes to the oncogenesis of LUAD and proposed *LINC01279* could be a potential target for LUAD diagnosis and treatment.

Background

On a global basis, lung cancer is the leading cause of cancer deaths as greater than 1.6 million deaths from lung cancer occur each year (Bray et al.,2018). Overall survival (OS) of advanced lung cancer was only 10-12 months in the absence of targeted agents. The median OS of patients with advanced lung cancer who had driver genes mutations and received corresponding targeted therapy extended to more than 3 years. Currently, prolonging the survival of patient via targeted therapy and improve their quality of life is regarded as the trend of development in the treatment of cancer. And the development of new targeted therapies relies on the discovery of new targets.

Evidences has been accumulating that outline the important role of posttranscriptional events in the pathogenesis and development of cancer. It’s been found in the recent genome-wide studies that pathogenic gene mutations and chromosomal rearrangements often encompasses regions in the genome that don’t contain any known protein-encoding gene (Chen et al.,2016; Sun et al.,2016). This is hardly surprising, because for the vast majority of human genomes, only a small fraction (less than 2%) encodes proteins. Among the various transcribed RNAs, long

non-encoding RNAs (lncRNAs) are fairly promising biomarkers and targets for therapies, because they exhibit strong specificity in expression for different types of cancer and for different stages of the same cancer.

In this study, we studied the potential molecular mechanism of LncRNA *LINC01279* in the progression of non-small cell lung cancer. We found that the expression of *LINC01279* was significantly up-regulated in NSCLC tumor tissues and cell lines, and was associated with poor prognosis in patients with NSCLC, suggesting that *LINC01279* plays a critical role in the pathogenesis and development of lung cancer. The effects of *LINC01279* on LUAD were investigated through in vitro and in vivo assays (i.e., WST1 assay, colony formation assay, flow cytometry assay, xenograft model, and western blot). Subcellular fractionation assays were used to detect the subcellular location of *LINC01279*. Finally, the mechanism of *LINC01279* regulating SIN3A activate apoptosis and autophagy was explored by RIP and western blot assays. In summary, these findings may provide new insights into the key role of *LINC01279* in human NSCLC tumorigenesis, and *LINC01279* is expected to become a brand new biomarker for cancer diagnosis and prognosis.

Materials And Methods Cell Lines And Transfection

H838, H1299 and PC-9 cells (human NSCLC cell lines with different metastatic potentials were established at the Affiliated Hospital of Guangdong Medical University, Zhanjiang, China); H838, H1299 and PC-9 cells were purchased from Kobio Biology Co., Ltd. (Nanjing, China). H838, H1299 and PC-9 cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and incubated at 37°C with 5% CO2 in a humidified atmosphere. Cells were transfected with Lipofectamine® RNAiMAX Reagent (Invitrogen) according to the manufacture's instructions.

Clinical tissues collection

Clinical tissues taken from tumors and areas adjacent to the margin of tumors were collected from 100 consecutive patients with NSCLC who underwent curative resection between September 2014 to September 2016 in the Department of Thoracic surgery of the Affiliated Hospital of Guangdong Medical University (Zhanjiang, China). Tissue blocks were selected from live lung cancer tissue specimens and complete clinicopathological and follow-up data of the patients. The histopathological diagnosis was based on the (WHO) standard of the World Health Organization. Obtain the ethical approval of the Research Ethics Committee of Affiliated Hospital of Guangdong Medical University, and obtain the written informed consent of each patient.

Cell Proliferation, transwell migration and invasion assay

Cell proliferation test was performed using WST-1 kit (Beyotime Biotechnology, Shanghai,China). 1000 cells were inoculated into each hole in the 96-well plate. WST- 1 solution (10 μl) was added to 100 μl medium, and the optical density was measured at 450nm. Three independent experiments were carried out. Cell migration and invasion were determined by cross-hole migration test and Matrigel invasion test. In the cross-hole migration test, 5 × 104 cells were suspended in 200 μl serum-free DMEM and placed in the cell culture insert with plate (BD Falcon, SanJose, CA) (pore diameter 8 μ m; BD Falcon, SanJose, CA). The well contained 10% fetal bovine serum preheating medium. After incubated in 37 °C and 5%CO2 for 12 h, the cells were fixed in PBS with 4% paraformaldehyde. In Matrigel invasion assay, 1 × 10 5 cells were suspended in 200 μl DMEM without serum and placed in a cell culture insert pre-coated with 1 μl / μl Matrigel (BD Biosciences, SanJose,CA). A preheated medium containing 10% fetal bovine serum was added to the well. After incubated in 37°C and 5%CO2 for 24 hours, the cells were fixed in PBS with 4% paraformaldehyde. Gently remove the unmigrated or invaded cells at the top of the membrane with a cotton swab. The cells were stained with 0.1% crystal violet , and 8 regions were randomly selected for cell count under an optical microscope (100x magnification) to determine cell migration or invasion.

Cellular nucleus/cytoplasm fractionation

Cells were collected, resuspended in a 120ul complete lysis buffer in each tube, and incubated on ice for 10 minutes;(The tissue was incubated for 20 minutes). The cell lysates were centrifuged at 14,000 RPM at 4°C for 5 minutes, and the supernatant was collected as the cytoplasmic part. The remaining part contained the nuclear components. Each tube was washed with 150ul 70% alcohol and then centrifuged at 14,000rpm at 4°C for 5 minutes. The supernatant was discarded and the remaining part was used as the nucleus. A 250 ul or 350 UL complete buffer G, oscillating for 30s, with 350 UL 70% alcohol in each EP tube containing cytoplasmic and nuclear components, was mixed. RNA was purified using a separation and purification column, and then the sample was stored in a -80°C refrigerator.

QRT-PCR

Total RNA was extracted from cells or tissues using TRIZOL reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions; Cytoplasmic and nuclear RNA extraction as described above.

Total RNA (500 ng) was reverse transcribed in a final volume of 10 μl using random primers under standard conditions for the PrimeScript RT reagent Kit (TaKaRa, Dalian, China). The expression level of *LINC01279* was then determined by SYBR Premix Ex Taq (TaKaRa, Dalian, China). Results were normalized to the expression of GAPDH (cytoplasm) and U1-snRNA (nucleus). qRT-PCR and data collection were performed on ROCHE 480. The primers were listed in Additional file: Table S1.

Colony formation assay

After digestion by trypsin, the cells were re-seeded in 6-well plates with a density of 200 cells/well and Cultured in a warm box. After 10 days, the culture medium in the six holes was sucked out, and the cells were washed twice with PBS, then fixed with methanol for 20 minutes and dried. The fixed colonies were dyed by crystal purple for

20 min, then the crystal purple was sucked out, washed twice with PBS, dried, photographed and counted.

Flow cytometric assays of apoptosis

Fluorescein Isothiocyanate (FITC) Annexin V Apoptosis Detection Kit(BD Biosciences, Franklin Lakes, NJ, USA) was used to detected Cell apoptosis.After 48 hours of transfection, the cells were collected, washed with PBS, and then re- suspended in the binding buffer.5ul Annexin V-FITC and propidium iodide were added and dyed in dark for 15 min.Finally, flow cytometry (FCM) SOFTWARE BDFACSDiva6.1 was used for cell analysis.

RNA fluorescence in situ hybridization (FISH)

FISH assays were performed using Fluorescent In Situ Hybridization Kit (RiboBio, China) according to the protocol. Cy3-labeled *LINC01279* antisense probe 1 and cy3- labeled *LINC01279* sense probe were designed and synthesized by RiboBio (China). Cy3-labeled *LINC01279* antisense probe 2 was designed and synthesized by GenePharma (China). Cells were first fixed in 4% formaldehyde for 15 min. Then the cells were permeabilized in PBS containing 0.5% Triton X-100 at 4 °C for 30 min and pre-hybridizated at 37 °C for 30 min in pre-hybridization solution. After that, probes were added in the hybridization solution and incubated with the cells at 37 °C overnight in the dark. The next day, the cells were counterstained with DAPI and imaged.

Western Blot

The cell lysates were extracted from the RIPA buffer with a protease inhibitor mixture, and the proteins were separated by electrophoresis in 8-12% SDS-PAGE and then transferred to the polyvinylidene fluoride membrane.The membrane was sealed in 5% skim milk for 1h, and then incubated with primary antibody at 4°C overnight.On the second day, TBST was used to wash the membrane, incubate the secondary antibody for two hours, and then wash the membrane with TBST.Finally, enhanced chemiluminescence (EMDMillipore, Billerica, MA, USA) and Tanon 5200 chemiluminescence imaging systems (Shanghai, China) were used to detect protein bands.

RNA immunoprecipitation(RIP) assay

Complete lysis solution (100ulRIP lysis buffer, 0.5ul protease inhibitor cocktail and 0.25ul DNase inhibitor) was prepared according to the kit instructions.Cells werelysed in 100ul of complete lysis solution, and immunoprecipitated overnight with sin 3A antibody or IgG antibody and magnetic beads at 4°C, followed by 6 times of washes in Washing Buffer and protein digestion at 55 °C with protease k.At last, total RNA was isolated and analyzed by qRT-PCR.

Tandem mRFP-GFP-LC3 fluorescence

We used LC3 (tfLC3), a monomer labeled rFP-GFP, to monitor the autophagy flux.Plasmid with MRFP-GFP-LC3 was transfected into H1299 and PC9 cells with iMAX. After 48h, the cells were washed with PBS and incubated with EBSS (E2888, Sigma) for a specified duration, and then the distribution of MRFP-GFP-LC3 in the cells was analyzed by confocal microscopy. All experiments were repeated three times. **Tumor xenografts in animals**

Five-week-old male BALB/c nude mice were maintained under specific pathogen- free conditions and manipulated according to protocols approved by the Animal Care Committee of Nanjing Medical College. Stably transfected H1299 cells (5 × 106/0.2 ml PBS) were implanted into two sides of the same nude mouse in the armpit. Xenografts were examined every 3 days with digital calipers and tumor volumes were calculated using the following equation: volume = 1/2 (length × width2). Twenty days later, the mice were sacrificed, and tumors volumes were measured. The samples were embedded in paraffin for hematoxylin and eosin (HE) staining and immunohistochemistry staining.

Statistical analysis

All data were expressed as mean ± SD. Unless specified, the Student's t-test was performed for statistical significance analysis. P value < 0.05 was considered as statistically significant. All statistical analysis was done with the software Graphpad Prism (GraphPad Software, La Jolla, CA).

Results

LINC01279 is high expression in patients with NSCLC

Due to the roles lncRNA can play in early diagnosis and targeted therapy, they're selected to be the main focus of this study. There are some reports show that *LINC01279* is closely related to oncogenesis (Ferraro et al.,2013; Grill et al.,2014; Leone et al.,2015). Therefore, it has been functionally characterized, but the role in lung adenocarcinoma tumorigenesis is yet to be fully clarified. We are hence inspired to investigate the role of *LINC01279* in LUAD. Comparing 90 patients with noncancerous lung tissues, significant (P<0.05) upregulation of *LINC01279* was observed in LUAD tissues (Fig. 1A). Overexpression of *LINC01279* in LUAD specimens was also confirmed by in situ hybridization (Fig. 1B-C). Because of the

significantly increased expression of *LINC01279* in tumors, we further analyzed whether the increased expression of *LINC01279* is related to the prognosis of NSCLC.

Kaplan–Meier survival analysis indicates the difference in level of expression of *LINC01279* and overall survival rates is not statistically significant (P = 0.499; Fig. 1D). Overall, it indicated that although *LINC01279* was upregulated in LUAD tissues, it may not be a prognostic factor. .

Knockdown of *LINC01279* inhibits tumor progression, invasion and migration and promotes in-vitro apoptosis

In order to investigate the role of *LINC01279* in LUAD, we examined its level of expression in several LUAD cell lines. It was found that *LINC01279* is significantly upregulated in LUAD cell lines H1299, H838 and PC-9 as compared to in normal bronchial epithelial (HBE) cells (Fig. 2A). To further confirm the function of *LINC01279* and prevent off-target effects, small interfering RNA (siRNA) named si-1 and si-2 were selected to transfect H1299, H838 and PC-9 cells respectively to silence *LINC01279*, and the silencing efficiency was measured with QRT-PCR (Fig. 2B), which showed the knockdown was relatively successful. Comparing to the control cells, downregulating *LINC01279* expression significantly inhibited cell proliferation (WST assays, Fig. 2C), and significantly decreases clonogenic survival (colony- formation assay, Supplementary Fig. S1A-B). Additionally, transwell assays revealed that H1299, H838 and PC-9 cells with *LINC01279* silenced have their invasion and migration capabilities significantly compromised as compared to the controls (Fig. 2D-F). Last but not least, knockdown of *LINC01279* significantly promoted apoptosis in H1299, H838 and PC-9 cells (Fig. 2G-I).

To uncover the cellular localization of *LINC01279*, we measured *LINC01279* expression in both nuclear and cytoplasmic fractions from H838, PC-9 and H1299 cells by qRT-PCR. The differential enrichment of GAPDH for cytoplasmic and U1 for nuclear expressions were used as fractionation indicators. We observed that *LINC01279* level was primarily in cytoplasm (55%-75%), and less in nuclear fractions (25%-45%) (Supplementary Fig. S2A-C). This suggested that *LINC01279* may play a regulatory function mainly occurring in the cytoplasm.

In-vivo regulation of LUAD cell proliferation by *LINC01279*

To further investigate the in-vivo effect of *LINC01279* on the proliferation of LUAD cells, sh-*LINC01279* and control vector were stably transfected into H1299 cells and were then inoculated into nude mice. The control group was inoculated with unmodified H1299 cells. Fifteen days after the inoculation, the dimensions of the tumors formed in the sh-*LINC01279* group were substantially less than

those formed in the control group (Fig. 3A). The mean volume of the tumors by the end of the experiment was significantly smaller in the sh-*LINC01279* group than in the control vector group (Fig. 3B). At the same time, sh-*LINC01279* was transfected into H1299 cells, and it was found that its knockdown efficiency was significantly inhibited compared with the control group (Fig. 3C). Moreover, H1299 cells with and without *LINC01279* knockdown were tested by IHC for the proliferation marker Ki-67, and the former exhibited decreased positivity (Fig. 3D). All these results pointed at that knockdown of *LINC01279* in LUAD cells inhibits their proliferation in vivo. The in vivo results presented above, therefore, complemented the in vitro studies presented in last section.

LINC01279 exerts carcinogenic effects through FAK/ERK protein

To clarify the mechanism of *LINC01279*-driven oncogenesis of LUAD and to explore the changes in gene expression as a consequence of *LINC01279* upregulation, we performed a protein screening experiment to evaluate the effects of *LINC01279* knockdown. It was found that the protein expression level of ERK, P-ERK and FAK were significantly reduced after silencing *LINC01279* (Fig. 4A, Supplementary Fig. S3E-G), yet no changes of ERK and FAK mRNA were observed (Fig. 4B-C). While the expression levels of MET, AKT and P-AKT were not significantly different from those of the control group (Supplementary Fig. S3A-D) , which implies that the expression level of FAK, ERK and P-ERK may be related to the expression of *LINC01279*. Aberrant activation of FAK, ERK and P-ERK in tumor cells causes the dysregulation of an assortment of genes, most of which play parts in regulating proliferation, migration, and tumorigenesis (Wang et al.,2020). Through the database

http://kmplot.com/analysis/, we found that high expression of ERK is associated with poor survival prognosis of patients with lung adenocarcinoma, which is statistically significant (Fig. 4D). This indicates that ERK is an oncogene, and it has the same concept as *LINC01279* in this article through the downstream pathway of FAK, which then affects the expression of ERK, thereby promoting the proliferation of lung cancer. Next, in order to study the functional effects of FAK in NSCLC H1299 and H838 cells, we designed two targeted siRNAs named si-FAK-1 and si-FAK-2 respectively. First we used western blot showed that FAK and ERK protein was significantly decreased when knockdown FAK compared with the control group in NSCLC cells (Fig. 4E, Supplementary Fig. S3H-I). And then verified the knockdown efficiency of FAK by QRT-PCR, which confirmed that both types of siRNA can reduce the expression of FAK transcription (Fig. 4F). When silencing FAK, the RNA level of ERK is significantly reduced (Fig. 4G). This means that FAK regulates the expression of ERK before transcription. WST detection showed that NSCLC cells with FAK knockdown have their proliferation significantly inhibited (Fig. 4H). At the same time, migration and invasion assay was carried out to determine whether FAK influenced the migration and invasion of tumor cells. Knocking down FAK can also significantly

inhibit the invasion and migration of H1299 and H838 cells (Fig. 4L-M).

***LINC01279* affects the apoptosis of lung adenocarcinoma through FAK** Intrinsically different from necrosis, apoptosis is an active process, involving the expression and regulation of a series of genes. Abnormal apoptosis is related to malignant transformation of cells, tumour metastasis and resistance to anticancer drugs (Wong,2011). A lot of proof suggested that p53 played a tumor suppressor gene, which over expression can induce apoptosis and inhibit tumor growth (Paek et al.,2016; Lin et al.,2018; He et al.,2019). p21 was reported frequently to be bound up with cell cycle and apoptosis. Generally, p21 as the target of p53, it can cause apoptosis through p53/p21 pathway (Pothuraju et al.,2020).However, Wu et al. showed that upregulation of p21 reduced TRAIL mediated extrinsic apoptosis, relating with the resistance of histone deacetylase inhibitors in Acute Myeloid Leukemia (Wu et al.,2014). It meant p21 may regard as a inhibitor of apoptosis in some cancers.

It has been described that *LINC01279* affects the apoptotic function of lung adenocarcinoma cells. In order to further understand the mechanism of apoptosis in lung adenocarcinoma cells, we analysed the apoptosis-related proteins, and found that p53 protein increased, p21 protein decreased, and the c-PARP was increased after knockdown of *LINC01279* with siRNA at 72 h (Fig. 5A-D). These suggested that *LINC01279* could regulate both cell proliferation and programs cell death in lung adenocarcinoma cells. Flow cytometry showed that apoptosis of NSCLC cells increased (Fig. 5E), and the trend of proteins expression of p53, p21 and PARP were similar to *LINC01279* knockdown after silencing FAK (Fig. 5F-H). The above results indicated that FAK enhances the apoptosis ability of NSCLC cells, which is the same as the trend of knocking out *LINC01279*. It can be seen that *LINC01279* affects the apoptosis of lung adenocarcinoma through FAK.

LINC01279 interacts with SIN3A protein and increases its stability.

To explore the mechanism of *LINC01279*, firstly, subcellular fractionation location assays found that the expression level of *LINC01279* is considerably higher in the cytosol than in the nucleus (**Supplementary Fig. S2A-C**), thus suggesting that *LINC01279* may play a major regulatory function at the posttranscriptional level. Then, we searched the website GeneCards (https://www.genecards.org/) and found that there are many proteins that may bind to *LINC01279*. Among them, SIN3A was particularly notable due to its established role in tumorigenesis. Additionally, this protein had been known as a transcription factor (Suzuki et al.,2008; Das et al.,2013). To obtain direct evidence of the physical interaction between *LINC01279* and SIN3A, we carried out RIP on the RNA-SIN3A complex using SIN3A antibodies and measured the amount of *LINC01279* associated with SIN3A immunoprecipitates but not IgG (Fig. 6A). IgG was a negative control. Furthermore, immunoblotting indicated that knocking down *LINC01279* could have downregulated the expression of SIN3A, but not by qRT-PCR(Fig. 6B, **Supplementary Fig. S4A**), which can prove that SIN3A and *LINC01279* are mutually combined. In addition, knockout of SIN3A did not affect the expression of *LINC01279* (**Supplementary Fig. S4B**). In order to further study whether *LINC01279* exerts cell function by combining with SIN3A, we transferred

the knocked-out SIN3A into lung adenocarcinoma cell lines. The efficiency of siRNA-mediated knocking down of SIN3A were 40%-75% (Fig. 6C).

The protein level of SIN3A also reduced significantly after treatment with siRNA (Fig. 6E). Observing its growth ability, invasion, metastasis and apoptosis, the results show that knocking out SIN3A can inhibit the growth, proliferation, invasion and metastasis of lung adenocarcinoma cells, and promote apoptosis (Fig. 6D, Fig. F-G, **Supplementary Fig. S4C**). Knockout of SIN3A inhibits the expression of FAK and ERK proteins, but has no effect on the RNA level(Fig. 6H, **Supplementary Fig. S4D- H**). All this shows *LINC01279* is very possible to regulate downstream proteins by binding to SIN3A to affect cell function and promote the development of tumors. This result indicated that *LINC01279* combined with SIN3A through the downstream pathway of FAK/ERK to play the role of cancer cells in LUAD.

We have found that SIN3A affects the expression of FAK protein, so how does FAK affect SIN3A? In order to clarify the relationship between SIN3A and FAK, we knocked out FAK and found that the RNA and protein levels of SIN3A were reduced (**Supplementary Fig. S4I-J**). This suggests whether FAK is a transcription factor of SIN3A, which regulates its RNA level and leads to protein changes.

***LINC01279* induces autophagy via upregulating SIN3A and FAK protein** Autophagy is a catabolic process in which damaged or useless proteins or other cytoplasmic components are degraded by a lysosomal degradation system. Autophagy is an adaptive pathway that is an essential link in cell metabolism. In particular, it has been revealed in many studies that autophagy plays a key role in regulating various pathological processes, especially cancer (Nah et al.,2015; Roos et al.,2016). In the past few decades, more and more evidences have accumulated that point at a profound relationship between lncRNAs and autophagy. To be more specific, lncRNAs enters the regulatory network of autophagy by mediating the transcriptional and post- transcriptional regulation of autophagy-related genes (Chen et al.,2017; Ma et al.,2018). In cancer, autophagy plays both the role of a promoter and one of an inhibitor, acting as either pro-survival or pro-death mechanisms under different circumstances (Guo and White,2016; Liu et al.,2016). Recent evidence (Choudhry et al.,2016) draws a complicated picture of lncRNAs in cancer, activating or preventing autophagy which in turn promote or inhibit cancer. From the formation of autophagosomes to the maturation of autophagolysosomes, p62 can act as a receptor for vesicles to be degraded by autophagy. Increased expression indicates autophagy inhibition, and decreased expression indicates increased autophagy activity. During the initiation of autophagy, LC3-I is cleaved and lipidated to produce LC3-II, which in turn polymerizes to form a double-layer membrane structure to wrap broken organelles to form autophagic vesicles. Therefore, LC3-II can be used as a protein marker for the formation of autophagosome. The quantity of LC3-II, and the LC3- II/LC3-I ratio are positively correlated with the amount of autophagic vesicles in the cell. Beclin-1 (the mammalian ortholog of yeast Atg6) has an evolutionarily conserved role in macroautophagy (Liang et al.,1999). Emerging lines of evidence suggest that beclin-1 is a novel substrate of caspases (Zhu et al.,2010).

In last section, we have demonstrated that *LINC01279* stabilizes SIN3A protein (Fig. 6). Previous studies have revealed that SIN3A influences autophagy via regulating various proteins and pathways

related to autophagy (Bowman et al.,2014). Sun C reported that osteopontin-enhanced autophagy attenuates early brain injury via FAK/ERK pathway (Sun et al.,2019). FAK induces autophagy and apoptosis in human nuroblastoma cells (Pham et al.,2018). Therefore, we tested whether *LINC01279*-mediated down-regulation of SIN3A and FAK would promote autophagy in LUAD cells. As shown in Figures 7A, aberrant expression of *LINC01279* significantly increased Beclin-1 in LUAD cells, and promoted production of LC3-II while suppressed the expression of p62 (**Supplementary Fig. S5A-C**). In the meantime, it was found that under-expression of *LINC01279* drastically increased green fluorescent GFP-LC3 punctas, and increased the quantity of autophagosomes in LUAD cells (Fig. 7B-C, **Supplementary Fig. S5D-E**). All of these effects are obviously the same as using the corresponding siRNA that silences SIN3A and FAK (Fig. 7D-E, **Supplementary Fig. S5F-J**). All these were significantly alleviated once SIN3A and FAK is silenced. All these findings collectively point at that *LINC01279* inhibits autophagy of LUAD cells via upregulating the expression of SIN3A/FAK. These suggest that *LINC01279*/SIN3A/FAK has an important role in autophagy induction.

Discussion

Works have been accumulating that suggest dysregulation of lncRNAs may have key regulatory functions at different stages of various diseases, and play a substantial role in the pathogenesis and development of cancer (Peng et al.,2017; Munschauer et al.,2018). According to existing works, lncRNAs are associated with various cellular biological functions, such as cell development, differentiation, autophagy and apoptosis, inflammation (Ginger et al.,2006; Cesana et al.,2011; Batista and Chang,2013; Kretz et al.,2013; Wang et al.,2014). They also affect chromatin imprinting, tumorigenesis and drug resistance of cancer cells (Nagano and Fraser,2011).

In this study, a new and up-regulated *LINC01279* was used as the research object to verify its expression in LUAD and its clinical significance, and to explore its effect on the cell biological function of LUAD and its possible mechanism, so as to provide a theoretical basis for clinical diagnosis or prognosis prediction molecular markers and potential therapeutic targets. Subsequent loss of function experiments showed that *LINC01279* promoted the progress of NSCLC by enhancing the ability of proliferation, invasion and migration of NSCLC cells and weakening the ability of apoptosis. To unambiguously identify the mechanism of LUAD oncogenesis driven by *LINC01279* and to determine the alteration in gene expression downstream of *LINC01279*, we performed a protein screening experiment to evaluate the effects of *LINC01279* knockdown. Through protein screening, it was found that FAK and ERK protein expressions were significantly changed compared with the control group after silencing *LINC01279*. Focal adhesion kinase (FAK) is a multi-functional regulator of cell signaling within the tumor microenvironment, and controls cell movement, invasion, survival through FAK's kinase-dependent and -independent functions (Sulzmaier et al.,2014). The extracellular signal-regulated kinase and mitogen-activated protein kinase (ERK/MAPK) signaling pathway has also been implicated in multiple cellular processes such as proliferation, migration and apoptosis, and ERK phosphorylation is regulated by FAK (Provenzano et al.,2009; Zou et al.,2013; Yang and Huang,2015). Qi Cao repoted that miR-7 can inhibit the activation of ERK/MAPK signaling pathway by down-regulating FAK expression, thereby suppressing the

proliferation, migration and invasion of NSCLC cells (Cao et al.,2016).The function loss experiment of FAK show that FAK can promote the invasion and migration of tumor cells as well as inhibiting their apoptosis in our study. In particular, it leads to changes in downstream ERK. These results indicate that *LINC01279* affects the proliferation and metastasis of lung adenocarcinoma cells through downstream FAK and ERK proteins

According to the reported research on SIN3A (Gambi et al.,2019), the regulatory transcription factor SIN3A is significantly correlated to the pathogenesis and progression of cancer. Sin3A, a member of the Sin3 family of proteins linked to tumorigenesis that are thought to regulate gene expression through their role as histone deacetylases (HDACs) (Das et al.,2013). Berberine chloride (BBC) suppresses human NSCLC by deregulating Sin3A/TOP2B pathway, leading to DNA damage and apoptosis in human NSCLC in vitro and in vivo (Chen et al.,2020).We found the protein SIN3A may bind to *LINC01279* by searched the website GeneCards (https://www.genecards.org/). In order to explore whether SIN3A is related to *LINC01279*, we carried out RIP analysis and found that SIN3A combining with *LINC01279* in regulating NSCLC cells. In addition, we also proved the ubiquitin degradation pathway of SIN3A. Moreover, we further proved that *LINC01279*/SIN3A can promote the activation of FAK and ERK signaling pathway to inhibit NSCLC cell apoptosis and autophagy. Knockout of FAK caused a decrease in the RNA and protein levels of SIN3A, suggesting that FAK maybe a transcription factor for SIN3A. In summary, FAK directly binds to SIN3A in the nucleus to promote SIN3A transcription. *LINC01279* binds directly to SIN3A and exerts carcinogenic effects through the downstream pathway of FAK/ERK protein. FAK in turn will affect the expression of SIN3A, ultimately leading to a loop of positive feedback, thus exerting the function of affecting the proliferation, apoptosis and autophagy of lung adenocarcinoma cells.

As we all know, both apoptosis and autophagic death of cells are manifestations of programmed cell death (PCD) (Liu et al.,2017). Several lncRNAs have been found to take part in regulating autophagy (Peng et al.,2017; Munschauer et al.,2018). A large amount of evidence shows that autophagic flow impairment is closely related to the pathogenesis of many diseases, especially tumors. Methioninase (METase) suppressed autophagy to reduce CDDP resistance of drug-resistant gastric cancer cells through regulating HULC/FoxM1 pathway (Xin et al.,2019). LncRNA BLACAT1 was upregulated in DDP- resistant NSCLC cells, and promoted autophagy and chemoresistance of NSCLC cells through the miR- 17/ATG7 signaling pathway (Xin et al.,2019). KCNQ1OT1 promotes cell proliferation and autophagy and inhibits cell apoptosis via regulating miR-204-5p/ATG3 axis, providing a promising target for NSCLC therapy (Xin et al.,2019). In our study, it was found that LINC01279 had effects on apoptosis and autophagy of NSCLC cells. Our results found that low expression of *LINC01279* promotes autophagic cell death and attenuates proliferation of lung adenocarcinoma cells. Our results reveal that *LINC01279* could be a potential therapeutic target to promote the growth of lung adenocarcinoma cells by prevent autophagy. We know that LC3B, Beclin-1, and P62 are the most commonly used markers in autophagy research (Xin et al.,2019). If P62 protein decreases and the transformation of LC3B-I to LC3B-II increases, it indicates autophagy current activation (Xin et al.,2019). In addition, we can determine whether the autophagy stream is activated by observing the key intermediate protein Beclin-1 in the autophagy pathway (Xin et al.,2019). At the same time, we measured the change in the level of expression of autophagy pathway

protein after silencing *LINC01279*. It turns out that Beclin-1 and LC3B-II protein related to autophagy were significantly overexpressed, while P62 was significantly underexpressed. At the same time, immunofluorescence suggested that the expression of LC3B-II increased.

In this study, we carried out a detailed investigation of the key role of *LINC01279* in regulating the apoptosis and autophagy in NSCLC cells, as well as the regulatory pathway of autophagy in tumor cells. Silencing *LINC01279* increases the apoptosis and autophagy of lung adenocarcinoma, which can provide an option for identifying new therapeutic targets for NSCLC in the future. However, there are still several problems to be solved, including the interaction between autophagy and apoptosis in tumor cells, and the specific mechanism of autophagy giving therapeutic drug resistance. In the future, we still need to strengthen our understanding of autophagy and apoptosis pathway in cancer (Xin et al.,2019).

Conclusions

Our research found that *LINC01279* is significantly upregulated in NSCLC (both clinical tissues and lab-grown cell lines), and results in changes of FAK and ERK proteins in downstream pathways by combining with SIN3A (Fig. 8). This consequently promotes the proliferation of lung adenocarcinoma cells, while inhibiting their apoptosis and autophagy. Our discovery reveals a new potential diagnostic or prognostic molecular marker *LINC01279*, and will serve as a theoretical basis for the development of new diagnostic and therapeutic targets.

Abbreviations

LncRNAs: Long non-coding RNAs

NSCLC: Non-small cell lung cancer

LUAD: lung adenocarcinoma

OS: Overall survival

siRNA: small interfering RNA

RIP: RNA binding protein immunoprecipitation.

Declarations

Ethical Approval and Consent to participate

This project was approved by the Ethical Committee on Affiliated Hospital of Guangdong Medical University.

Consent for publication

Not applicable.

Availability of data and materials

All the data obtained and/or analyzed during the current study were available from the corresponding authors on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SWM and YZX conceived the projects and designed the experiments. WJC, HXB, LXF,ZHL,CXR and CYY performed the experiment, WJC and HXB wrote the manuscript. HHQ and HZ analyzed the data. HJ and CGA supervised this work. SWM, ZJ and YZX revised the paper.All authors read and approved the final manuscript.

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Figures

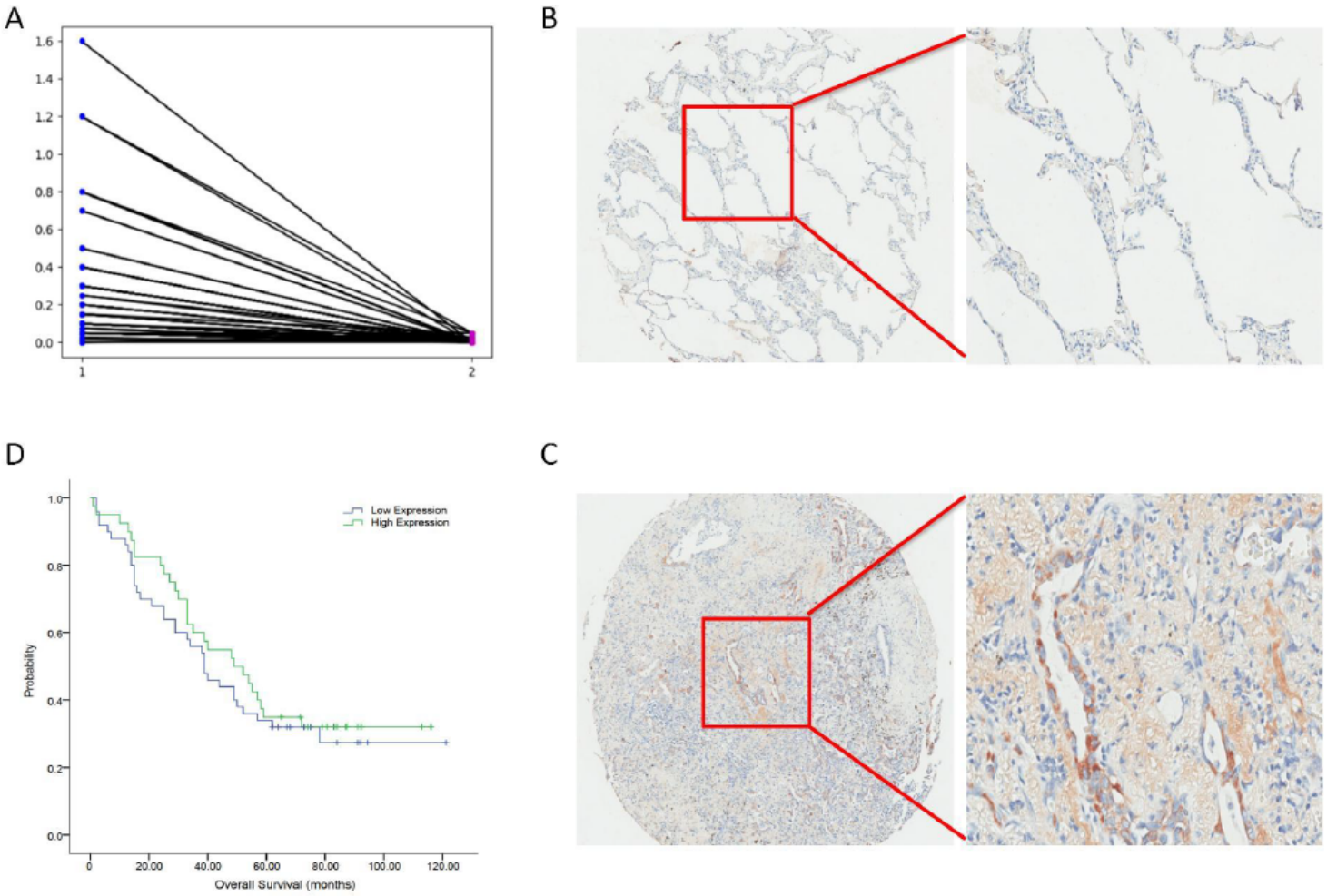

Figure 1

LINC01279 is high expression in patients with NSCLC (A) The differential expression of long Non-coding RNA LINC01279 between lung adenocarcinoma and noncancerous lung tissues (n=90 for each group). (B and C) Situ hybridization showed the high expression of LINC01279 in LUAD specimens (C) compared with noncancerous lung tissues (B). (D) Kaplan–Meier survival analysis indicates the difference in level of expression of LINC01279 and overall survival rates is not statistically significant.

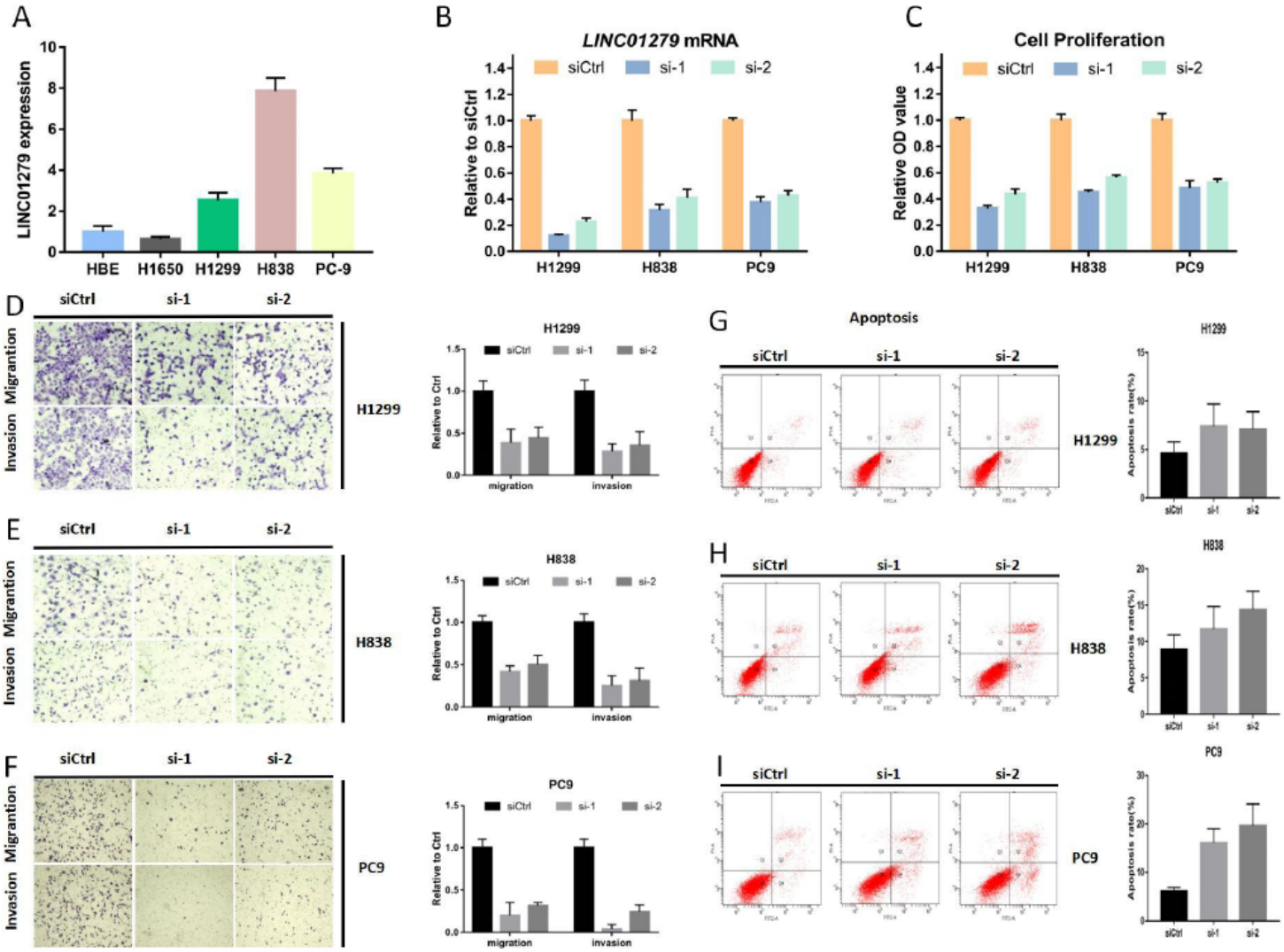

Figure 2

Knockdown of LINC01279 inhibits tumor progression, invasion and migration and promotes in-vitro apoptosis (A) qRT-PCR analyses the expression of LINC01279 mRNA in HEB, H1650, H1299, H838 and PC9 cells. (B) Treated with siRNA in H1299, H838 and PC9 cells respectively for 48h, the silencing efficiency of LINC01279 was measured by qRT-PCR. (C) Transfected with siRNA in LUAD cell for 96h, the cell proliferation was reduced tested by WST-1 assays. (D-F) Down regulation of LINC01279 via siRNA treatment reduced the ability of invasion and migration in H1299, H838 and PC9 cells. The right figures represented the relative quantified value, respectively. (G-I) Silencing of LINC01279 promoted apoptosis in H1299, H838 and PC9 cells. The right figures also represented the relative quantified value. * $P < 0.05$, ** $P < 0.01$.

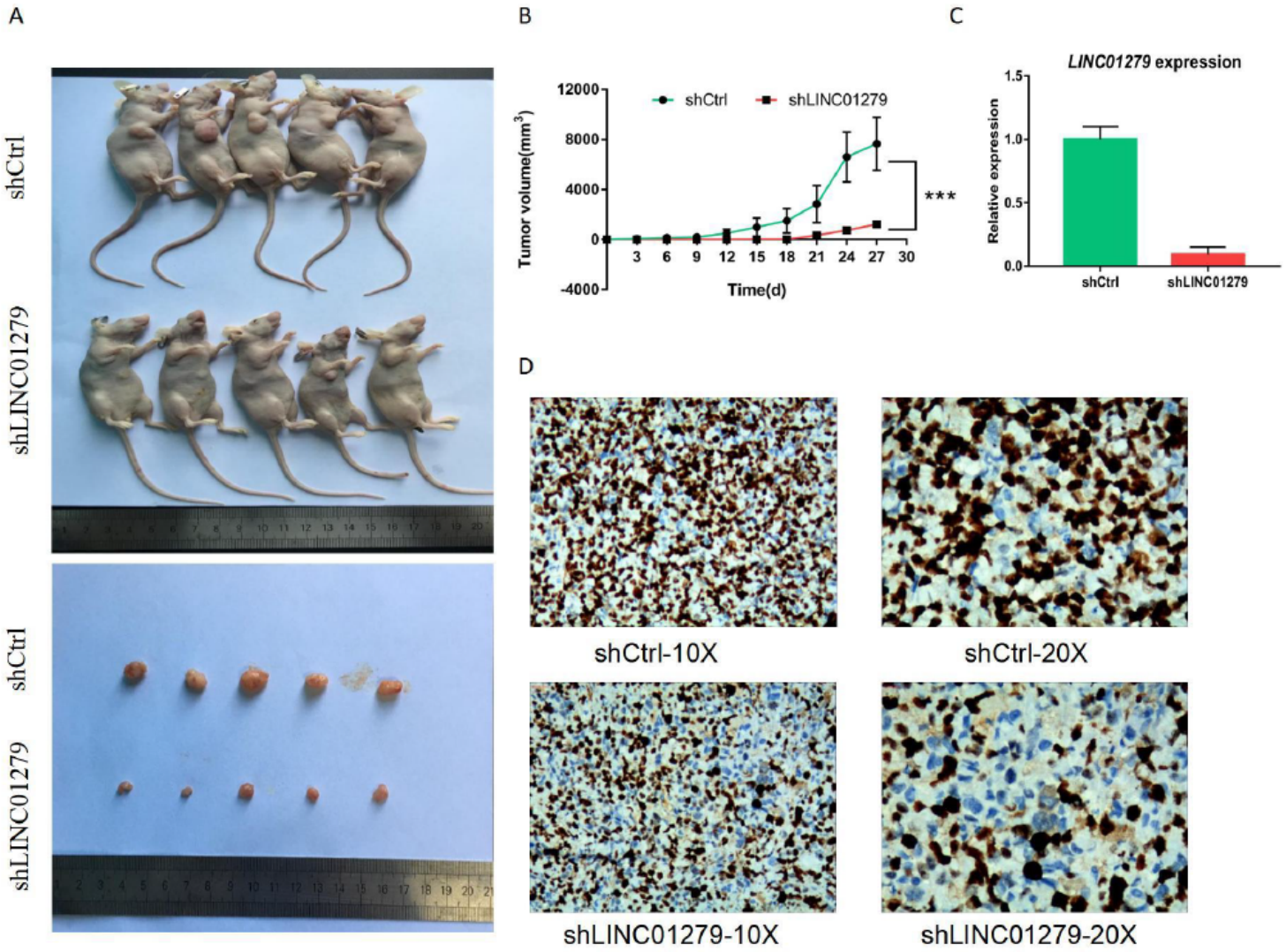

Figure 3

In-vivo regulation of LUAD cell proliferation by LINC01279 (A) H1299 cells were stably transfected with shLINC01279 or control vector, and then inoculated into nude mice to build the model of subcutaneous xenograft tumors. The sizes of tumors formed in the shLINC01279 group were substantially less than those formed in the shCtrl group (n=5 for each group). (B) Compared with shCtrl-treated nude mice, the tumor growth rate markedly decreased in shLINC01279 group. (C) qRT-PCR analysis showed the expression of LINC01279 mRNA was significantly reduced in shLINC01279-treated nude mice tumor tissues. (D) The positivity level of Ki-67 was measured using IHC staining in the neoplasm of each group nude mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.01$.

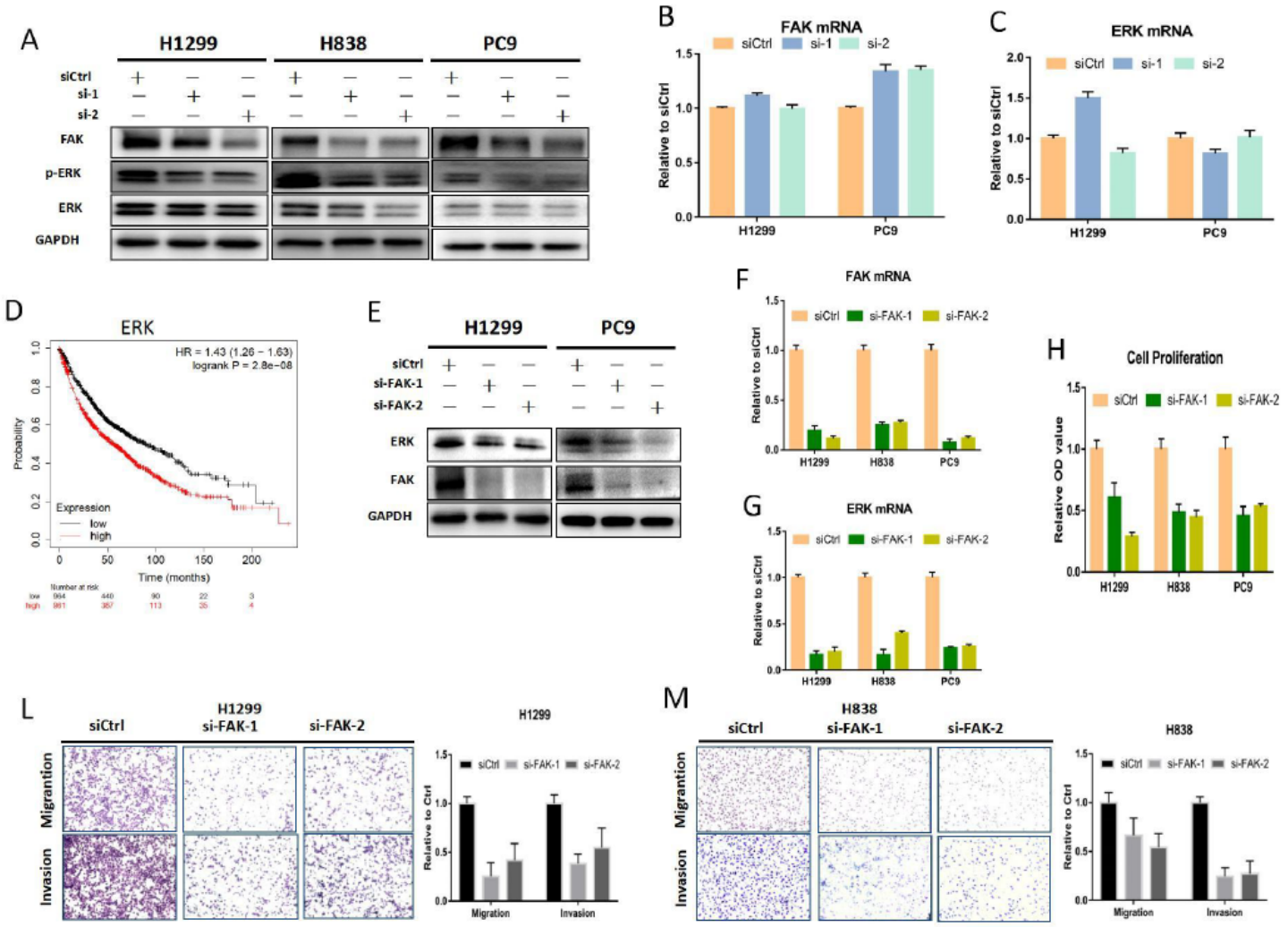

Figure 4

LINC01279 exerts carcinogenic effects through FAK/ERK protein (A) FAK, ERK and p-ERK proteins were decreased after LINC01279 silence. (B and C) The mRNA expression of FAK and ERK measured using qRT-PCR following LINC01279 knockdown. (D) Higher ERK expression was significantly relating with poor patient survival (HR=1.43, 1.26-1.63, logrank P= 2.8e-08). (E) FAK and ERK proteins were decreased after FAK inhibited. (F and G) The mRNA expressions of FAK and ERK were decreased after the silencing efficiency of FAK. (H) Cell proliferation was decreased measured by WST-1 assays following FAK knockdown in NSCLC cells. (L-M) Silencing of FAK via siRNA reduced the ability of cellular invasion and migration in H1299 and PC-9 cell lines. GAPDH is used as loading control.* P < 0.05, ** P < 0.01, *** P < 0.01.

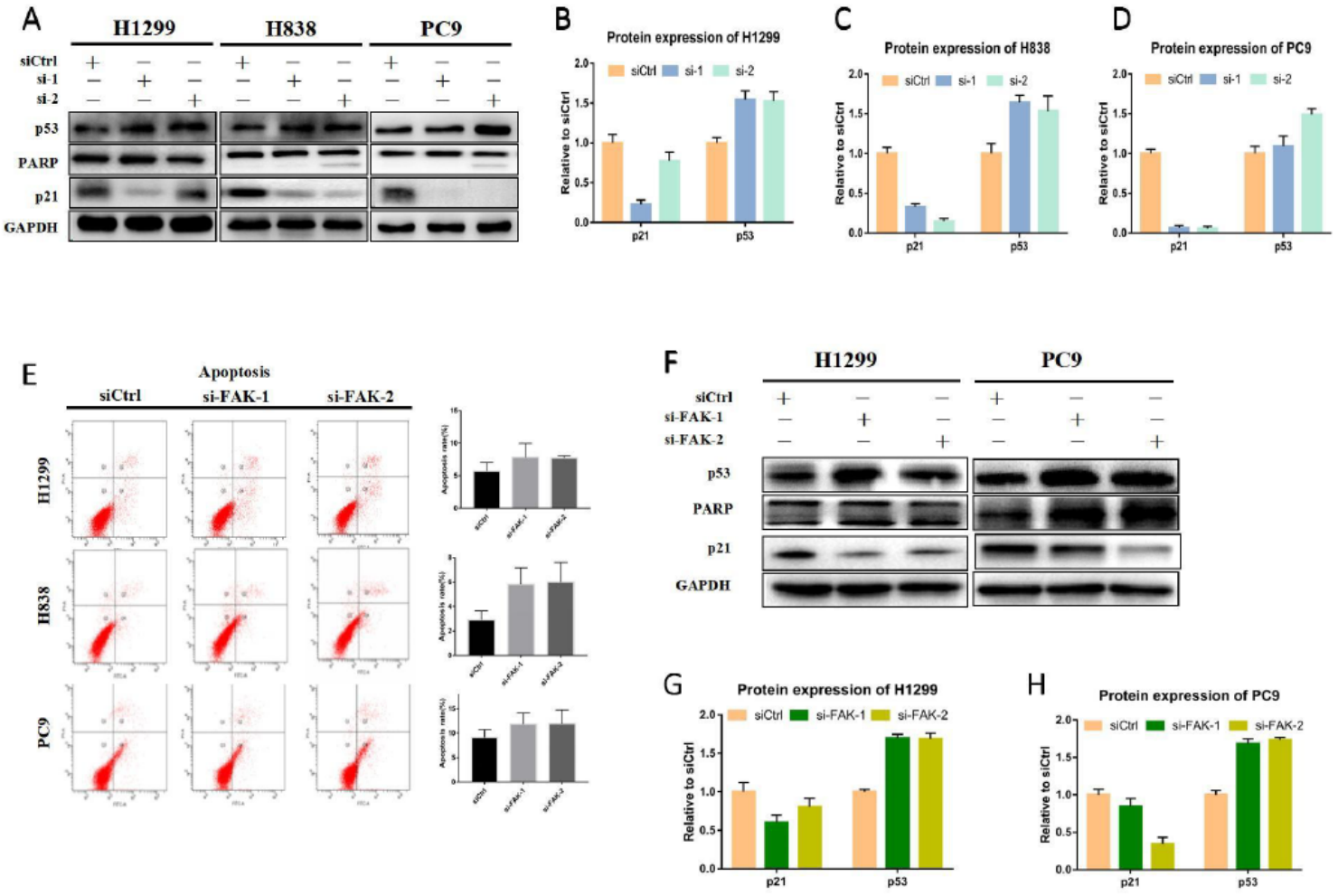

Figure 5

LINC01279 affects the apoptosis of lung adenocarcinoma through FAK (A) p53 protein were decreased, on the contrary p21 protein decreased following LINC01279 knockdown in H1299, H838 and PC9 cells, and the cleaved PARP protein detected in H838 and PC9 cells ,but not H1299 cell. (B-D) Relative quantified value of the expression of p21 and p53 after LINC01279 inhibited in H1299, H838 and PC9 cells. (E) Silencing of FAK promoted apoptosis in NSCLC cells. (F) The protein expression of p21, p53 and cleaved PARP after FAK inhibited in H1299 and PC9 cells measured by western blot. (G-H) Relative quantified value of the expression of p21 and p53 after FAK inhibited in H1299 and PC9 cells.

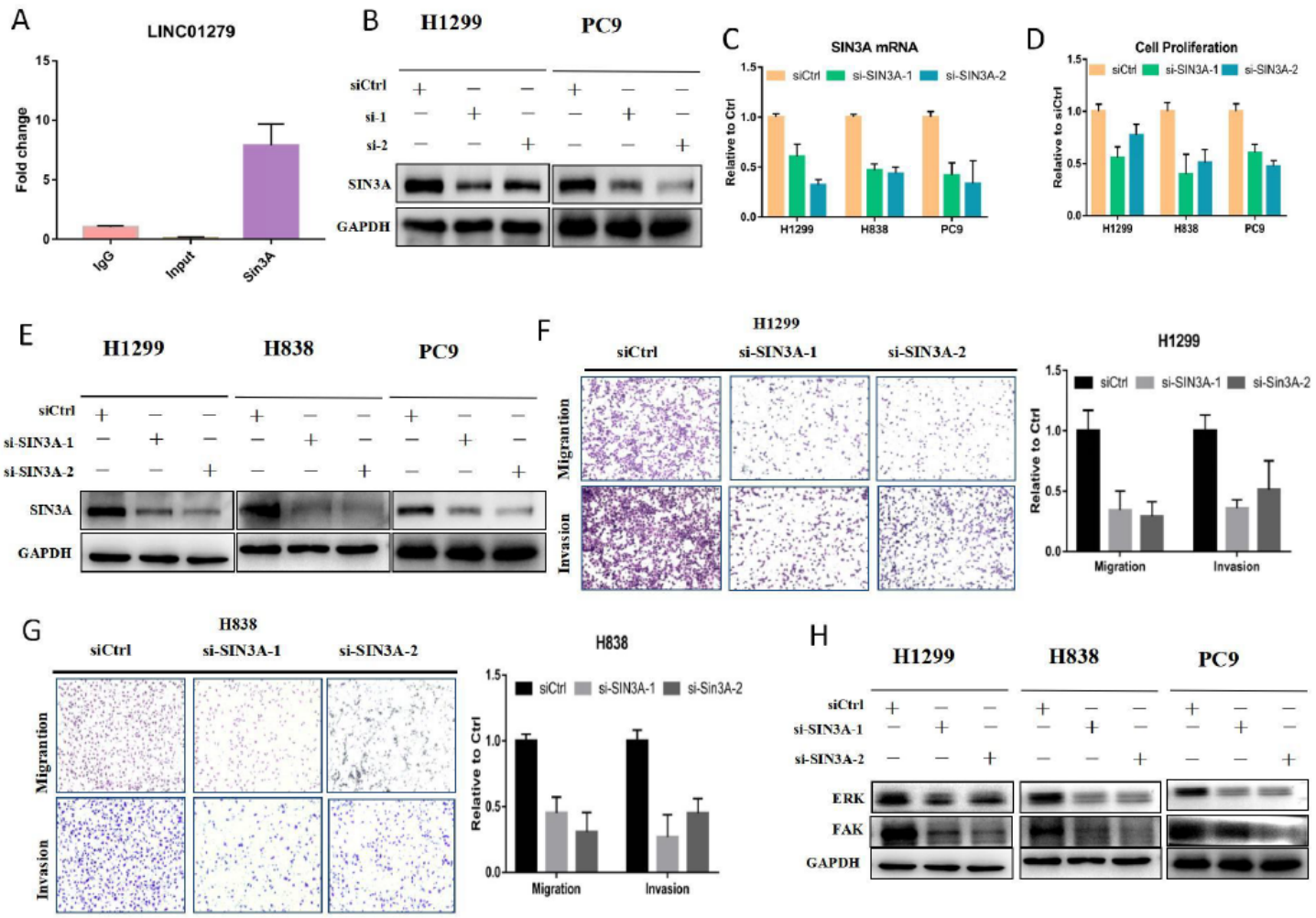

Figure 6

LINC01279 interacts with SIN3A protein and increases its stability (A) RIP assay showed the LINC01279 expression is 7.8 fold change compared with IgG (IgG is a negative control). (B) The protein levels of SIN3A were reduced after LINC01279 silence by siRNA. (C) The silencing efficiency of SIN3A was measured using qRT-PCR. (C) Cell proliferation was decreased measured by WST-1 assays following SIN3A knockdown in NSCLC cells. (D) Immunoblotting showed the silencing efficiency of SIN3A on protein level. (F-G) Silence of SIN3A reduced the ability of invasion and migration in NSCLCcells. (H) The protein expression of FAK and ERK after SIN3A knockdown in H1299, H838 and PC9 cells.

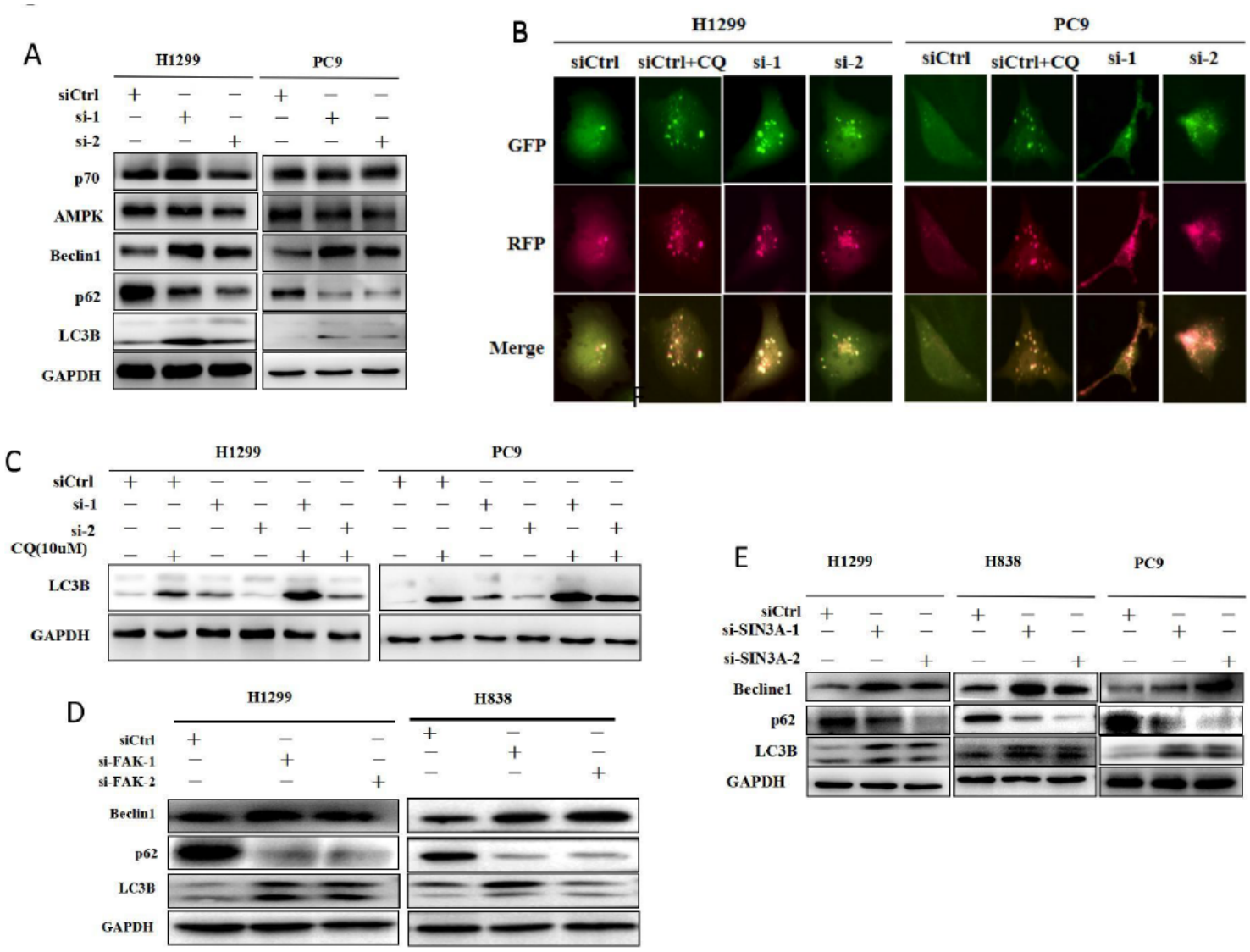

Figure 7

LINC01279 induces autophagy via upregulating SIN3A and FAK protein (A) In H1299 and PC9 cells transfected with si1/2, LC3B-II and Beclin1 proteins were significantly increased, whereas p62 protein decreased, and AMPK and p70 proteins had no obvious change. (B) The representative images of autophagic flow. CQ is a inhibitor of aytophagy, stopped the amalgamation between autophagosome and lysosome. Cells treated with si1/2 and mRFP-GFP-LC3 plasmid were visualized alive with fluorescence microscope. (C) Analysis of autophagy flux: immunoblotting analysis of LC3B-II accumulation in extracts from NSCLC cells with LINC01279 silence which untreated or treated with chloroquine. (D-E) The expression of autophagy-related proteins were tested after FAK and SIN3A knockdown, respectively.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTablesS1S3.pdf

- supplementary15.pdf