

# Long Non-Coding RNA GATA6-AS1 Inhibits Proliferation and Promotes Apoptosis of Lung Adenocarcinoma Through Sponging miR-331-3p and Regulating SOCS1/JAK2/STAT3 Pathway

**Xiaodong Huo**

Second Hospital of Tianjin Medical University

**Huixing Wang**

Second Hospital of Tianjin Medical University

**Ning Jiang**

Tianjing Institute of Urology

**Kuo Yang**

Tianjin Institute of Urology

**Bin Huo**

Second Hospital of Tianjin Medical University

**Lili Wang**

Second Hospital of Tianjin Medical University

**Jinhuan Wang**

Second Hospital of Tianjin Medical University

**Haitao Wang** (✉ [qitao7469667@163.com](mailto:qitao7469667@163.com))

Second Hospital of Tianjin Medical University

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## Research

**Keywords:** GATA6-AS1, miR-331-3p, SOCS1, JAK2/STAT3 signaling pathway, lung adenocarcinoma

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# Abstract

**Background:** Accumulating evidence has indicated the remarkable roles of long non-coding RNAs (lncRNAs) as oncogenes or tumor suppressors in many malignancies. The involvement of lncRNA GATA6-AS1 in cancers remains largely undiscovered. Herein, our research was aimed at elucidating the function and mechanism of GATA6-AS1 in lung adenocarcinoma (LUAD).

**Methods:** Gene expression was measured through qRT-PCR and WB. Cell proliferation ratio was determined using CCK-8 and EdU assays. Cell apoptosis ratio was determined using TUNEL and flow cytometry assays. Molecular interactions were examined through RIP, RNA pull-down and luciferase reporter assays.

**Results:** GATA6-AS1 expression was markedly down-regulated in LUAD cell lines. GATA6-AS1 could inhibit LUAD cell proliferation and promote cell apoptosis. Mechanistically, GATA6-AS1 was identified as the molecular sponge for miR-331-3p, whose knockdown in LUAD cells could reinforce the tumor-suppressing effects of GATA6-AS1 overexpression. Moreover, GATA6-AS1 functions as a competing endogenous RNA (ceRNA) through sequestering miR-331-3p to deregulate SOCS1, thus inhibiting JAK2/STAT3 signaling pathway and suppressing LUAD cell viability.

**Conclusions:** These results demonstrate the tumor-suppressing function and mechanism of lncRNA GATA6-AS1 in LUAD cells. The axis of GATA6-AS1/miR-331-3p/SOCS1/JAK2/STAT3 can be adopted as a novel approach for LUAD treatment.

## Background

Nowadays, the cancers gradually present an upward trend due to multiple factors, such as aging of the population, physical inactivity and smoking, etc. [1]. In terms of lung cancer, it is the main cause of death in men and women with high incidence rates, poor prognosis and low survival rates [2]. In addition, people have divided lung cancer into several subtypes like non-small cell lung cancer (NSCLC), which accounts for about 85% of lung cancer [3, 4]. Furthermore, NSCLC also include lung adenocarcinoma (LUAD), lung squamous carcinoma (LUSC) and large cell carcinoma (LCC) [5]. Thus LUAD is chosen as aim of study because of its aggression and malignancy in this study. In spite that abundant target therapies and treatments constantly spring up for LUAD patients at present, but its mortality remains high, so it is still urgent to explore other biomarkers method.

It has been known that the long non-coding RNAs are positively involved into the occurrence and development of varied tumor or cancer, whose imbalance expression affect the cancer cell proliferation, migration and etc. [6]. Some lncRNAs associated with LUAD have been reported and become novel master regulator for treatment of LUSD patients in the recent few years. For example, UCA1 over-expression is significantly associated with poor overall survival for LUAD patients [7]. Similarly, SOX21-AS1 devotes to push the proliferation of LUAD cells and cut down the cell cycle and apoptosis [8]. LINC01614 stimulates the development of LUAD [9]. SPRY4-IT1, SNHG6 and linc00665 has great

influence on the development of lung adenocarcinoma [10–12]. However, the function of GATA6-AS1 in the lung adenocarcinoma remains unknown and need to explore.

In this study, we attend to probe the biological function of GATA6-AS1 in LUAD. A good deal of functional experiments is performed to confirm the tumor-suppressor role of GATA6-AS1 in lung adenocarcinoma. In addition, GATA6-AS1/miR-331-3p/SOCS1 axis is discussed in detail. The abnormal expression of GATA6-AS1 makes great influence on JAK2/STAT3 signaling pathway as well.

## Methods

### Cell lines and reagent

Human normal lung epithelial cell line (BEAS-2B) and human lung adenocarcinoma cell lines (HCC827, H1650, H522, H1299, A549) were available from American Type Culture Collection (ATCC; Manassas, VA). All cell samples were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS; Gibco) and 1% mixture of Pen/Strep. Cell culture was performed in humidified incubator containing 5% CO<sub>2</sub> at 37 °C. 0.5 μM of Colivelin, STAT3 activator, was available from Santa Cruz Biotechnology (Dallas, TX).

### Isolation of total RNA and quantitative real-time PCR (RT-qPCR)

The isolation of total RNA was conducted with the application of Trizol reagent (Thermo Fisher Scientific, Waltham, MA). cDNA was then synthesized using PrimeScript™ RT reagent kit as instructed by supplier (Takara, Otsu, Japan). SYBR Premix Ex Taq II (Takara) was employed on StepOne™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA) for quantitative analysis, with GAPDH gene or U6 as internal reference.  $2^{-\Delta\Delta Ct}$  method was applied for calculating relative expression.

### Plasmid transfection

To overexpress GATA6-AS1, the full-length cDNA sequences of GATA6-AS1 was cloned into pcDNA3.1 vector (Invitrogen, Carlsbad, CA) after PCR amplification. To suppress GATA6-AS1 and SOCS1, the designed short hairpin RNAs (shRNAs) and control-shRNAs were purchased from GenePharma (Shanghai, China). Besides, miR-331-3p mimics and NC mimics, miR-331-3p inhibitor and NC inhibitor were acquired from RiboBio (Guangzhou, China). Transfection kit Lipofectamine2000 was used for 48 h of plasmid transfection as instructed (Invitrogen).

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

After transfection, cells of A549, H1299 and HCC827 were reaped at logarithmic growth phase and seeded to 96-well plates with  $2 \times 10^4$  cells/well. Cell viability was monitored every 24 h using CCK-8 Kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). 10  $\mu$ l of CCK-8 was added to each well for 1 h at 37°C with 5% CO<sub>2</sub>. Absorbance at 450 nm was examined by microplate reader.

## EdU staining

Cell proliferation was also examined by EdU staining Kit as required by supplier (Ribobio). After transfection, each well of 96-well plate were added with 100  $\mu$ L of EdU medium diluent, then cells were fixed by 4% paraformaldehyde and permeabilized by 0.5% Troxin X-100. DAPI dye was used for nuclear counterstain, and cell stained cells were evaluated by fluorescent microscope (Leica, Wetzlar, Germany).

## TUNEL staining

Cells on coverslips were fixed for 10 min, then permeabilized in PBS. Cell apoptosis was detected in accordance with the protocol of In Situ Cell Apoptosis Detection Kit (Roche, Basel, Switzerland). After DAPI staining, cell samples were analyzed by manual counting under fluorescent microscope.

## Flow cytometry assay

Transfected cancer cells ( $2 \times 10^5$ ) were reaped, then rinsed in precooled PBS and subjected to Annexin V-FITC/PI Apoptosis kit as instructed by provider (BD Biosciences, San Jose, CA). Following 15 min of incubation in darkroom, apoptotic cells were treated with FACS Calibur flow cytometer (BD Biosciences).

## RNA pull down

The miR-331-3p fragment covering the GATA6-AS1 or SOCS1 binding sites including wild-type and mutant, were biotinylated into Bio-miR-331-3p-WT/Mut probes. The cell protein lysates collected from RIPA lysis buffer were mixed with miR-331-3p probes and control probes in magnetic beads. The RNA enrichment in pull-down mixture was assayed via qRT-PCR.

## RNA immunoprecipitation (RIP)

Thermo Fisher RIP kit was commercially from Thermo Fisher Scientific for performing RIP assay in A549 and H1299 cells in light of the user manual. The collected cell lysates from RIP lysis buffer were employed for immunoprecipitation with magnetic beads and human Argonaute2 (Ago2) antibody (Millipore, Billerica, MA). Normal mouse immunoglobulin G (IgG; Millipore) served as negative control.

## Luciferase reporter assay

The GATA6-AS1 or SOCS1-3'-UTR fragment covering the miR-331-3p binding sequences (wild-type and mutant) were used to insert into pmirGLO vector, and the constructs were named as GATA6-AS1-WT/Mut and SOCS1-3'-UTR-WT/Mut. Following co-transfection with indicated transfection plasmids, HEK-293T cells (ATCC) were subjected to Luciferase Reporter Assay System (Promega, Madison, WI) 48 h later.

## Western blot

Cell protein samples collected from RIPA lysis buffer were subjected to electrophoresis on 12% SDS-PAGE, and then separated proteins were shifted to PVDF membranes. The primary antibody against SOCS1 (ab62584; Abcam, Cambridge, MA) and GAPDH (ab8245; Abcam) were used to probe membranes after specific dilution. At last, membranes were analyzed using ECL detection system as instructed (Bio-Rad lab, Hercules, CA).

## Statistical analysis

All assays in this study were bio-repeated for more than two times, the measurement data were exhibited as the means  $\pm$  Standard Deviation (S.D.) and analyzed by use of PRISM 6 (GraphPad, San Diego, CA). P-values below 0.05 were seen as the significant levels for statistical analyses in form of one-way analysis of variance (ANOVA) or Student's t-test.

## Results

### GATA6-AS1 expression is down-regulated in the LUAD tissues and cells

To examine the biological function of GATA6-AS1 in LUAD tissues and cells, we use NONCODE and GEPIA databases to respectively analyze their expression in adjacent normal tissues and LUAD tissues. As shown in Fig. 1A and 1B, GATA6-AS1 expression was remarkably up-regulated in the lung organ. Furthermore, its expression was obviously down-regulated in the lung adenocarcinoma tissues compared to the matched adjacent normal tissues. Additionally, the KM PLOT displayed that the high expression of GATA6-AS1 tended to increase the likelihood of survival compared with low GATA6-AS1 expression, simultaneously indicating that the low expression gave rise to poor prognosis (Fig. 1C). Next, we took advantage of RT-qPCR to examine the GATA6-AS1 expression in the normal human bronchial epithelial cells BEAS-2B and LUAD cell lines HCC827, H1650, H522, H1299, and A549. The results demonstrated that GATA6-AS1 expression was strikingly down-regulated in the LUAD five cell lines compared with the normal bronchial epithelial cell BEAS-2B (**Figure. 1D**). According to this data, we firstly transfected pcDNA3.1/GATA6-AS1 or sh-GATA6-AS1 #1, sh-GATA6-AS1 #2 and sh-GATA6-AS1 #3 into

A549, H1299 and HCC827 cells lines. And then we used RT-qPCR to examine the change of GATA6-AS1 expression. The results revealed that GATA6-AS1 expression was remarkably up-regulated due to overexpressed GATA6-AS1 in comparison with pcDNA3.1 groups. However, GATA6-AS1 expression was obviously knocked down due to silencing of GATA6-AS1 compared with sh-NC groups. Taken together, the expression of GATA6-AS1 shows low level in LUAD tissues and cells.

## **LncRNA GATA6-AS1 suppresses the growth of lung adenocarcinoma cells**

How the ectopic expression of GATA6-AS1 effects the biological behavior of LUAD cells was continuously probed and analyzed. As presented in Fig. 2A and 2B, the absorbance and EdU positive cells rate were apparently reduced by up-regulation of GATA6-AS1 compared to pcDNA3.1 groups in A549 and H1299 cells. However, knockdown of GATA6-AS1 was prone to result in the high absorbance and EdU positive cells rate in HCC827 cell. It also reflected that increasing of GATA6-AS1 had great suppressor function on LUAD growth. Inversely, the LUAD cells apoptosis ratio was prominently elevated after transfection pcDNA3.1/GATA6-AS1 into A549 and H1299 cells using TUNEL assay (Fig. 2C). Similarly, the same consequence was uncovered when treated with flow cytometry analysis (Fig. 2D). It also disclosed that GATA6-AS1 up-regulation enhanced the apoptosis of LUAD cells. All in all, GATA6-AS1 effectively restrains the proliferation of LUAD cells, induces cell apoptosis.

## **MiR-331-3p is sponged by upstream gene GATA6-AS1**

To further investigate the reciprocal function between GATA6-AS1 and its downstream gene-miRNA, we made use of LncBase database and LncRNASNP2 database to filter 2 possible target genes (miR-331-3p and miR-708-5p), which was seen from Fig. 3A. Considering that miR-708-5p had tumor-suppressor role for LUAD, so we hypothesized miR-331-3p as downstream gene of GATA6-AS1. Next, ENCORI was performed to predict the underlying binding sites between GATA6-AS1 and miR-331-3p. The finding demonstrated that the wild type of GATA6-AS1 was capable of combining with miR-331-3p (Fig. 3B). In addition, the results of RIP assay uncovered that GATA6-AS1 and miR-331-3p were enriched in Anti-Ago2 groups but not in IgG group in LUAD cells, thus we conjectured that GATA6-AS1 and miR-331-3p were in RNA-induced silencing complex (RISC) and GATA6-AS1 possibly served as competing endogenous RNA (ceRNA) to exert its regulatory mechanism (Fig. 3C). Additionally, the wild type of biotinylated miR-331-3p significantly enhanced GATA6-AS1 level, however, there was not obvious change with mutant type of biotinylated miR-331-3p (Fig. 3D). Finally, the luciferase reporter assay illustrated that the luciferase activity of GATA6-AS1-WT was evidently declined by miR-331-3p up-regulation compared with NC mimics groups, while GATA6-AS1-Mut luciferase activity was not apparently changed (Fig. 3E). Collectively, these data suggested that miR-331-3p could directly bind to GATA6-AS1 and be sponged by GATA6-AS1.

# MiR-331-3p inhibition and overexpressed GATA6-AS1 declined cell proliferation and promoted cell apoptosis

To further the miR-331-3p biological function in LUAD cells, we co-transfected pcDNA3.1/GATA6-AS1 and miR-331-3p inhibitors into A549 and H1299 cells. As suggested in Fig. 4A and 4B, overexpression of GATA6-AS1 and miR-331-3p knock-down contributed to cut down the ability of LUAD cells proliferation, which were explored using CCK8 assay and EdU assay. In the contrary, the findings of TUNEL assay and flow cytometry analysis illustrated the GATA6-AS1 up-regulation and miR-331-3p silence both raised cell apoptosis together (Fig. 4C-4D). On the whole, miR-331-3p knockdown and GATA6-AS1 up-regulation simultaneously benefit to restrain LUAD development.

## SOCS1 is regulated by miR-331-3p and GATA6-AS

To further probe the target gene of miR-331-3p, we availed of PicTar, TargetScan, PITA and miRmap database to winnow 33 mRNAs that potentially combined with miR-331-3p (Fig. 5A). Moreover, we used ENCORI software to judge out that SOCS1 was equipped with complementary base pairing with miR-331-3p (Fig. 5B). In the light of above findings, we further explored association of GATA6-AS1 and SOCS1 in the LUAD cells. Firstly, the RT-qPCR analysis defined that expression of SOCS1 was apparently increased by up-regulated GATA6-AS1 (Fig. 5C). Additionally, the results of western blot assay showed that the protein levels of SOCS1 in both cell lines (A549 and H1299) were also increased compared with pcDNA3.1 groups (Fig. 5D). To detect if GATA6-AS1, miR-331-3p and SOCS1 are in the same RISC complex, we performed an RIP assay. As expected, the GATA6-AS1, and miR-331-3p and SOCS1 were especially enriched with Ago2 compared to IgG group, indicating that three RNAs indeed co-existed in RISC (Fig. 5E). Furthermore, the RNA pull down assay also presented that Bio-miR-331-3p-WT strengthened the SOCS1 expression, whereas, not any change with Bio-miR-331-3p-MUT in contrast to Bio-NC (Fig. 5F). In the end, up-regulation of miR-331-3p reduced the luciferase activity of SOCS1-3'-UTR-WT, not SOCS1-3'-UTR-MUT compared with NC mimics, which was inferred from Fig. 5G. And overexpressed GATA6-AS1 recovered the luciferase activity of SOCS1-3'-UTR-WT. In conclusion, SOCS1 is regulated by miR-331-3p and GATA6-AS as well.

## Knockdown of SOCS1 blocks LUAD cells proliferation and induces cell apoptosis

We co-transfected pcDNA3.1/GATA6-AS1, sh-SOCS1#1, sh-SOCS1#2 and sh-SOCS1#3 into A549 and H1299 cells to further assess the relation of GATA6-AS1 and SOCS1. As shown in the Fig. 6A, the SOCS1 expression was sharply increased by up-regulation of GATA6-AS1 compared with pcDNA3.1 groups, and it was naturally knocked down via SOCS1 silencing compared with pcDNA3.1/GATA6-AS1 + sh-NC groups. In like manner, the results of western blot assays displayed that the protein level of SOCS1 was remarkably by up-regulated SOCS1, however was cut down by silencing of SOCS1 (Fig. 6B). Next we

performed western blot assays again to verify if SOCS1 down-regulation activates JAK2 and STAT3 function. The results showed that the p-JAK2 level was obviously decreased with up-regulation of GATA6-AS1 and it was increased by sh-SOCS1#1, and it totally recovered by Colivelin. The p-STAT3 level was cut down by pcDNA/GATA6-AS1, and then sh-SOCS1#1 and Colivelin fully saved it (Fig. 6C). And then the CCK8 assay and EdU assay were used to detect the change of down-regulated SOCS1 or co-transfection with Colivelin for cell proliferation and apoptosis. The results showed that overexpressed GATA6-AS1 declined the ability of cell proliferation, however SOCS1 knockdown partly increased cell proliferation, the Colivelin fully abolished the function of overexpressed GATA6-AS1 (Fig. 6D-6E). In addition, the results of TUNEL assay and flow cytometry analysis suggested that the ability of cell apoptosis was sharply elevated due to GATA6-AS1 up-regulation, whereas was partly rescued with down-regulated SOCS1, was completely recovered by Colivelin (Fig. 6F-6G). Taken together, GATA6-AS1/SOCS1 axis regulates LUAD cells proliferation and apoptosis; GATA6-AS1/SOCS1 down-regulation could inactivate the JAK2 /STAT3 signal path

## Discussion

Long non-coding RNA (lncRNA) is a type of noncoding RNA with over 200 nucleotides in length and has no protein coding ability and [13, 14]. As for the GATA6-AS1 mentioned in this study, it has been confirmed that it is close associated with lung squamous cell carcinoma [15, 16]. However, its function and mechanism in LUAD are still not studies until now. In this investigation, we used a string of assay to detect the change of LUAD cell proliferation and apoptosis. All data show that GATA6-AS1 up-regulation is able to suppress the cell proliferation and induce cell apoptosis. So we primitively guess that GATA6-AS1 is tumor-suppressor gene in LUAD.

lncRNAs have been widely reported to target to miRNAs and serve as sponge to regulate miRNAs [17, 18]. In this study, GATA6-AS1 up-regulation and knockdown of miR-331-3p jointly contribute to cut down the LUAD cells proliferation, motivate the cell apoptosis. As for the miR-331-3p, it has been reported to play its particular function in cancers. For example, miR-331-3p is a tumor-promoting gene in pancreatic cancer cells [19]. Similarly, miR-331-3p is able to target RCC2 to inhibit the cell proliferation, migration and invasion of ovarian cancer [20]. Up-regulation of miR-331-3p reduces prostate cancer cell growth, migration and colony formation [21]. Based on above three examples, we could get a conclusion that miR-331-3p has double characters—oncogen and tumor-inhibitor gene. As for this study, it plays an oncogene role in LUAD tumorigenesis and progression. Besides, we also analyze suppressor of cytokine signaling (SOCS1), the target gene of miR-331-3p and confirm the combination relation by RIP assay, RNA pull down and luciferase reporter assay. In the matter of SOCS1, SOCS1 has been reported for its tumor-inhibitor role [22, 23]. What's more, SOCS1 and JAK2/STAT3 pathway are discussed in hepatocellular carcinoma [24]. David et al. uncover that the overexpression of SOCS1 give rises to cell morphology changes and obviously reduced tumor cell invasion in vitro [25]. In this study, the results of rescue assays demonstrates that JAK2/STAT3 signaling pathway inactivation caused by GATA6-AS1 overexpression, is reversed by silencing of SOCS1 and Colivelin. Knockdown of SOCS1 counteracts the inhibitory effect of GATA6-AS1 overexpression on LUAD proliferation.



# Conclusions

In summary, our results show that GATA6-AS1 effectively suppresses the proliferation of LUAD cells through sponging miR-331-3p to regulating SOCS1/JAK2/STAT3 pathway. The suppressor effects of GATA6-AS1 on LUAD progression may imply that GATA6-AS1 has potential use in LUAD treatment. The finding provides a novel and promising biomarker for patient of LUAD.

# Abbreviation

Lung adenocarcinoma (LUAD)

GATA binding protein 6 antisense RNA 1 (GATA6-AS1)

Suppressors of cytokine signaling 1 (SOCS1)

lncRNAs (long noncoding RNAs)

miRNAs (microRNAs)

mRNA (messenger RNA)

ceRNA (competing endogenous RNA)

WT (wild-type)

Mut (mutant)

5-ethynyl-20-deoxyuridine (EdU)

The terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL)

Isolation of total RNA and quantitative real-time PCR (RT-qPCR)

Cell counting kit-8 (CCK-8)

FBS (fetal bovine serum)

RNA immunoprecipitation (RIP)

DMEM (Dulbecco's Modified Eagle's Medium)

GAPDH (glyceraldehyde-3-phosphate dehydrogenase)

SD (standard deviation)

ANOVA (Analysis of Variance)

## Declarations

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Authors confirmed that this work can be published. The content of this manuscript is original and it has not yet been accepted or published elsewhere.

## Availability of data and materials

Not applicable.

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## Competing interests

The authors declare that they have no competing interests

## Authors' contribution

XDH conceived this study. XDH, HXW, NJ performed experiments and analyzed data. KY, BH and LLW contributed to materials and methods. JHW investigated background. HTW wrote this paper. All authors read and approved the final manuscript.

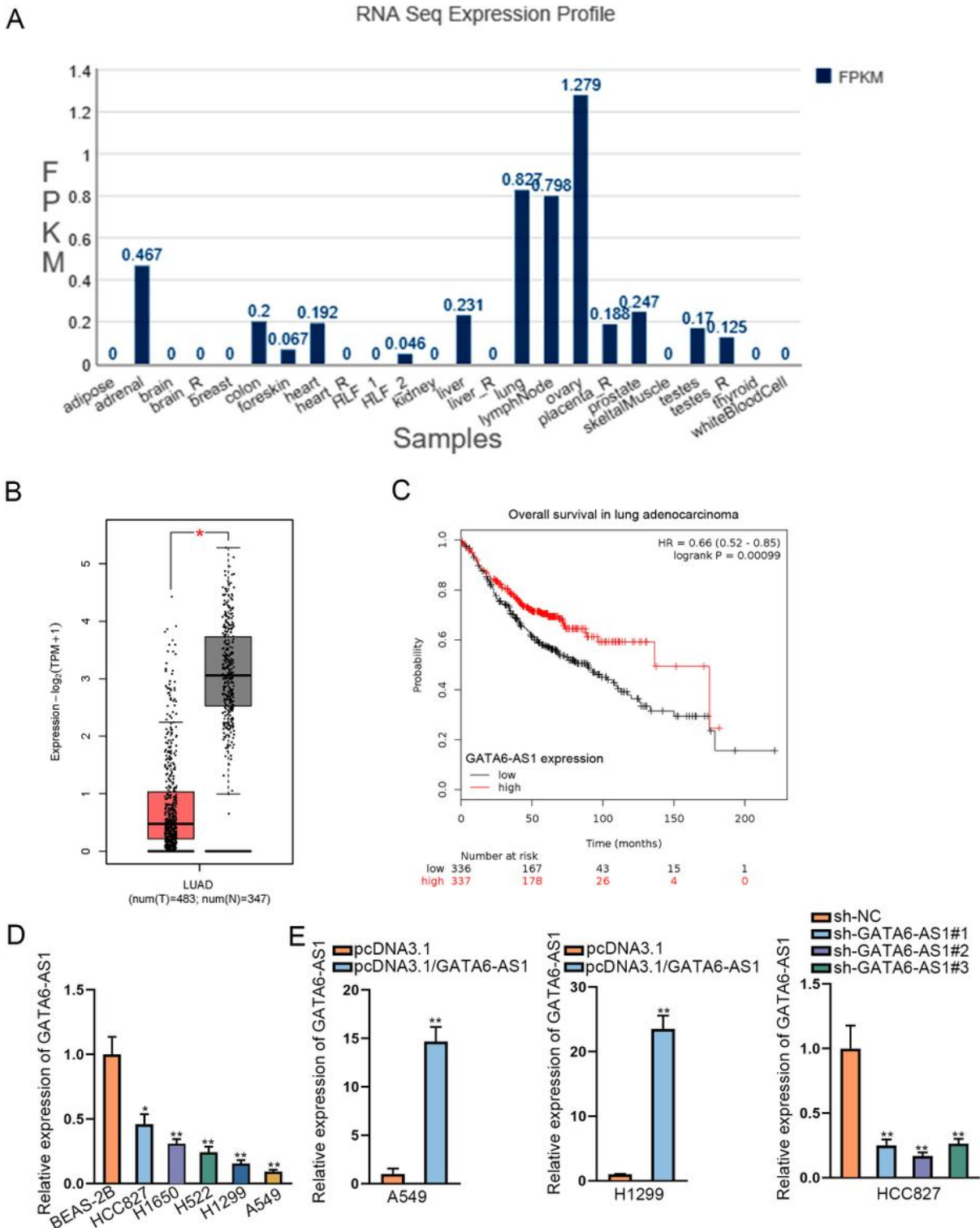
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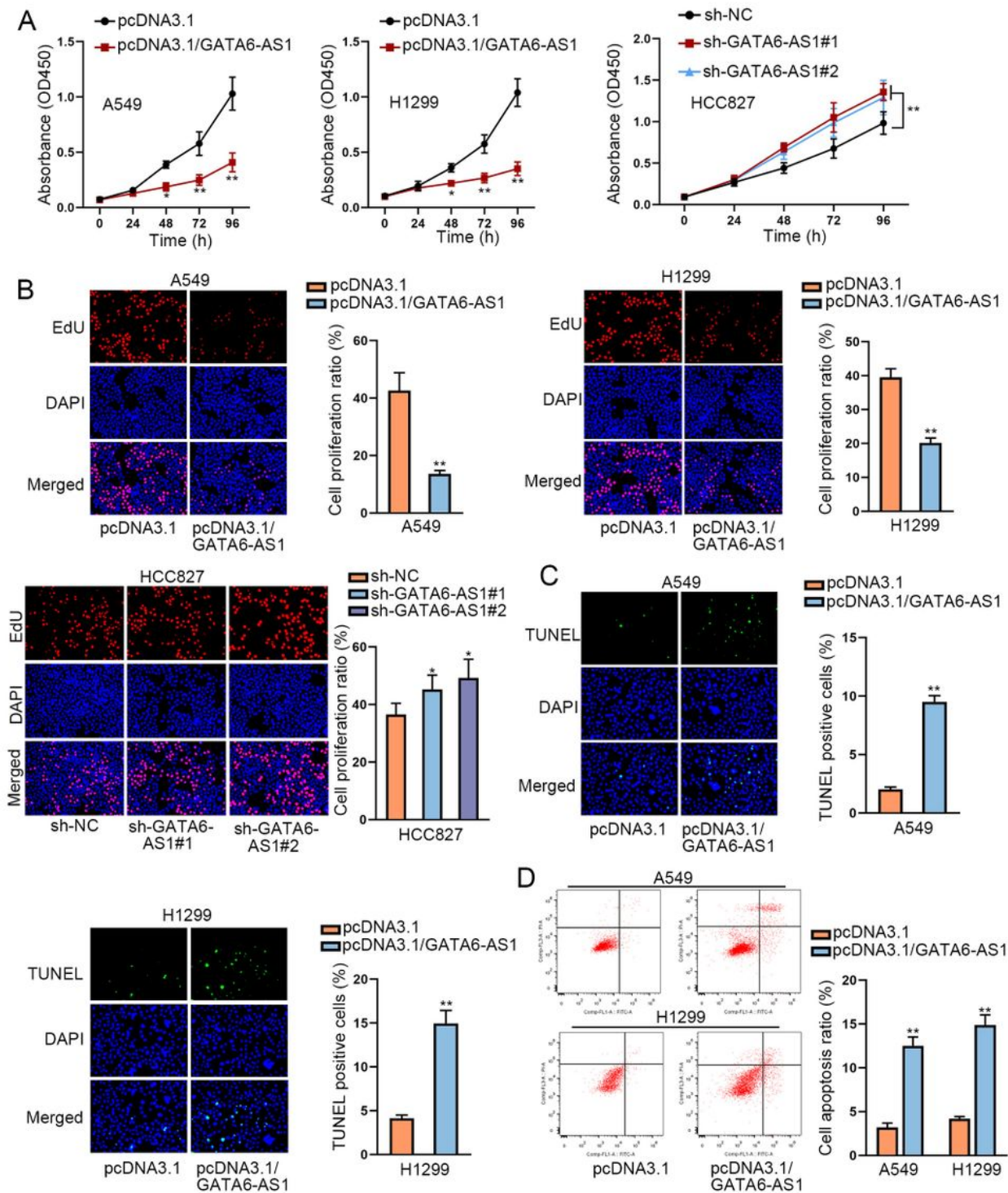
## Figures



**Figure 1**

GATA6-AS1 expression is down-regulated in the LUAD tissues and cells A. GATA6-AS1 expression was measured in the normal tissues using NONCODE database (<http://www.noncode.org/>). B. GATA6-AS1 expression was examined in lung adenocarcinoma tissues and the matched adjacent normal tissues via GEPIA (<http://gepia.cancer-pku.cn/>). C. The survival rates of low expression and high expression of GATA6-AS1 was accessed through KMPLLOT (<http://www.kmplot.com/>). D. The RT-qPCR was used to

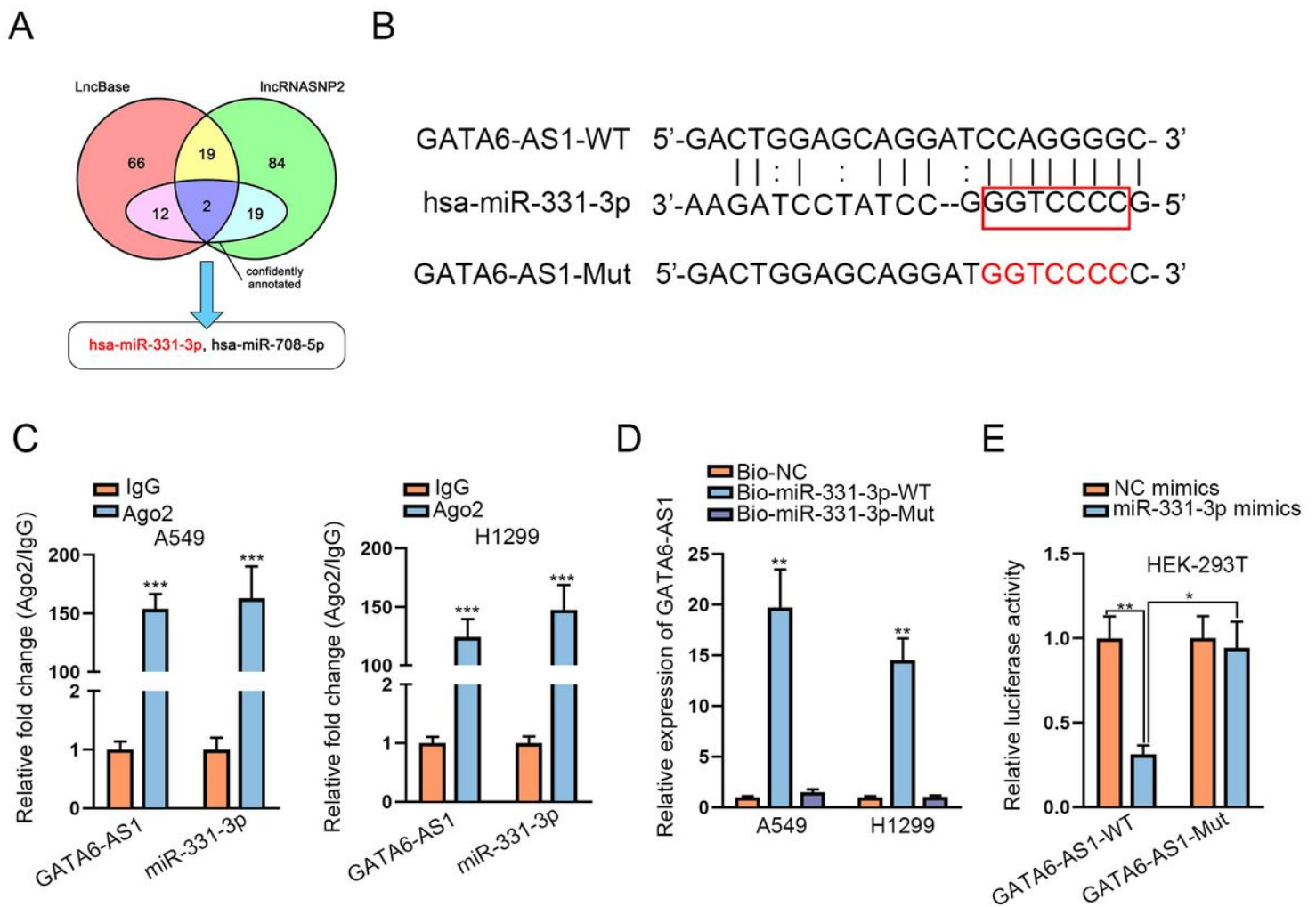
detect the expression of GATA6-AS1 in LUAD cells and normal human bronchial epithelial cells. E. The efficiency of overexpression or knockdown of GATA6-AS1 was analyzed through RT-qPCR. \*P < 0.05, \*\*P < 0.01.



**Figure 2**

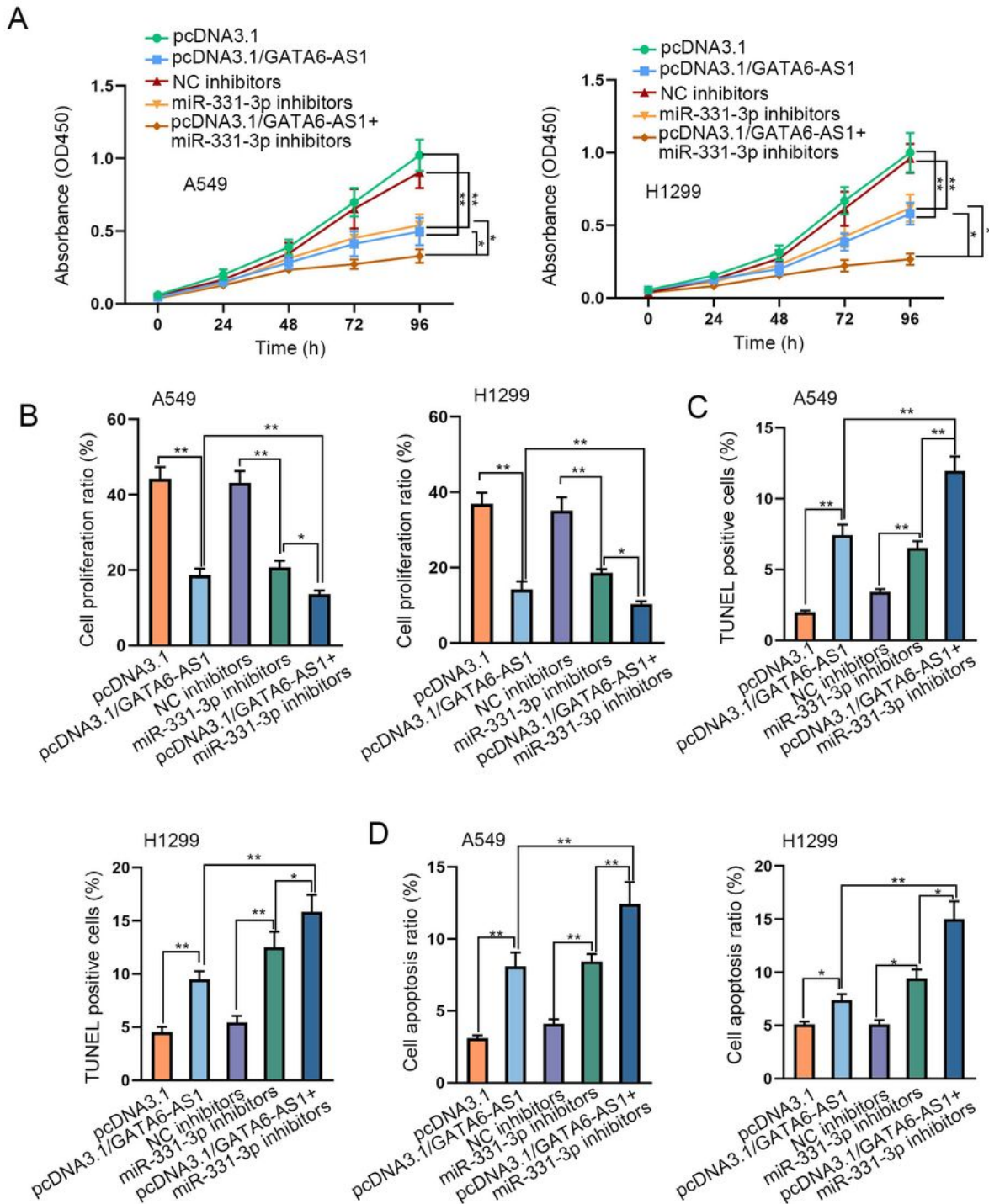
LncRNA GATA6-AS1 suppresses the growth of human lung adenocarcinoma cells A and B. The cell counting kit-8 (CCK8) and EdU assay were utilized to access the LUAD cells proliferation. C and D. TUNEL

assay and flow cytometry analysis were conducted to examine the ability of LUAD cells apoptosis.  $**P < 0.01$ .



**Figure 3**

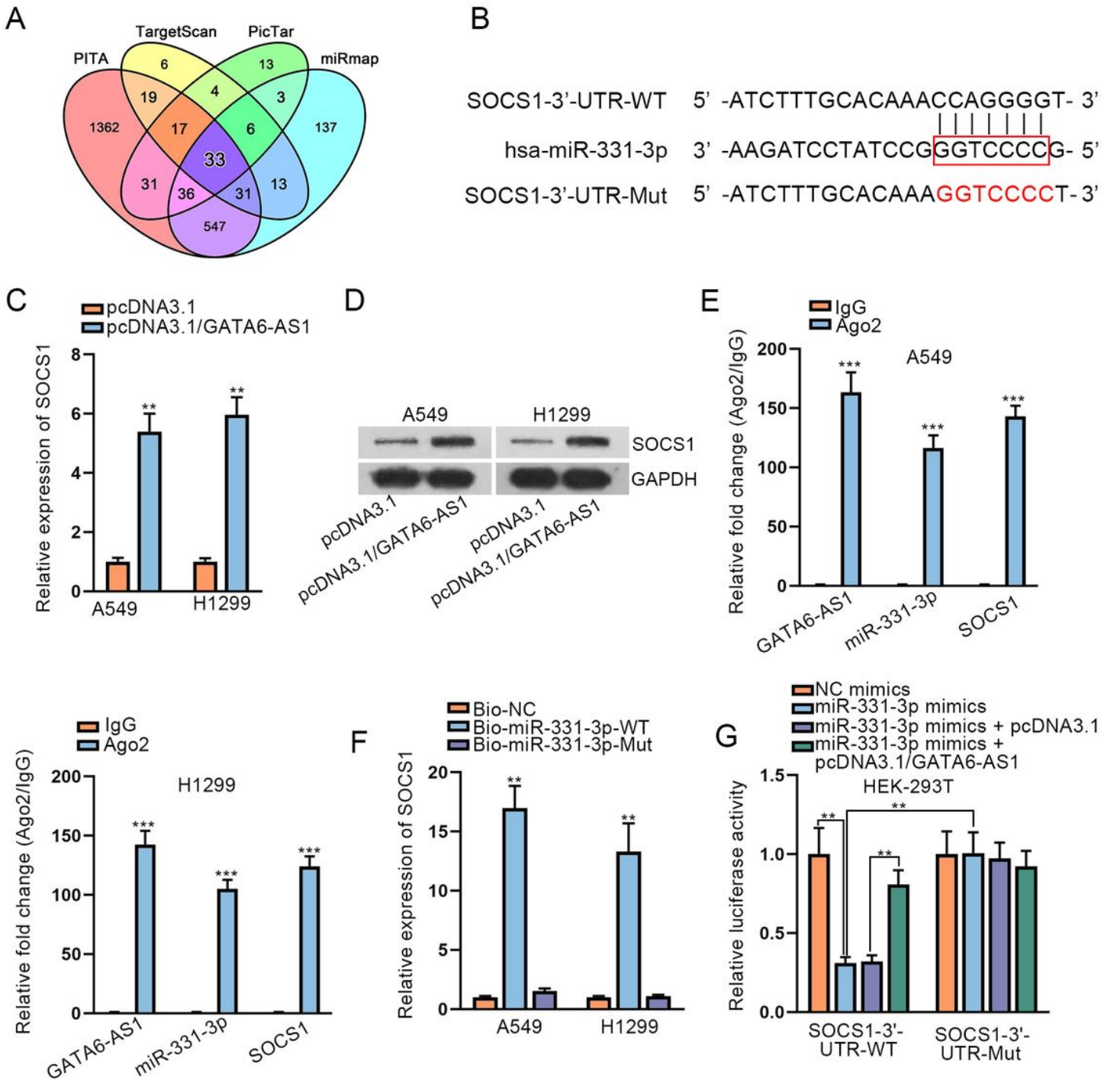
MiR-331-3p is sponged by upstream gene GATA6-AS1. A. Schematic representation of the predicted target gene of GATA6-AS1. B. The ENCORI database (<http://starbase.sysu.edu.cn/index.php>) was used to predict underlying binding sites. C. RIP assay was used to assess whether two RNAs co-exist in RISC. D and E. RNA pull down and luciferase reporter assay were conducted to confirm the combined association between GATA6-AS1 and miR-331-3p. All results were suggested as the mean  $\pm$  SD.  $**P < 0.01$ .



**Figure 4**

MiR-331-3p inhibition and overexpressed GATA6-AS1 declined cell proliferation and promoted cell apoptosis A and B. The cell proliferation ability was examined by CCK8 and EdU assay. C and D. TUNEL assay and flow cytometry analysis were used to measure the capacity of LUAS cell apoptosis. All results were suggested as the mean  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.01$ .

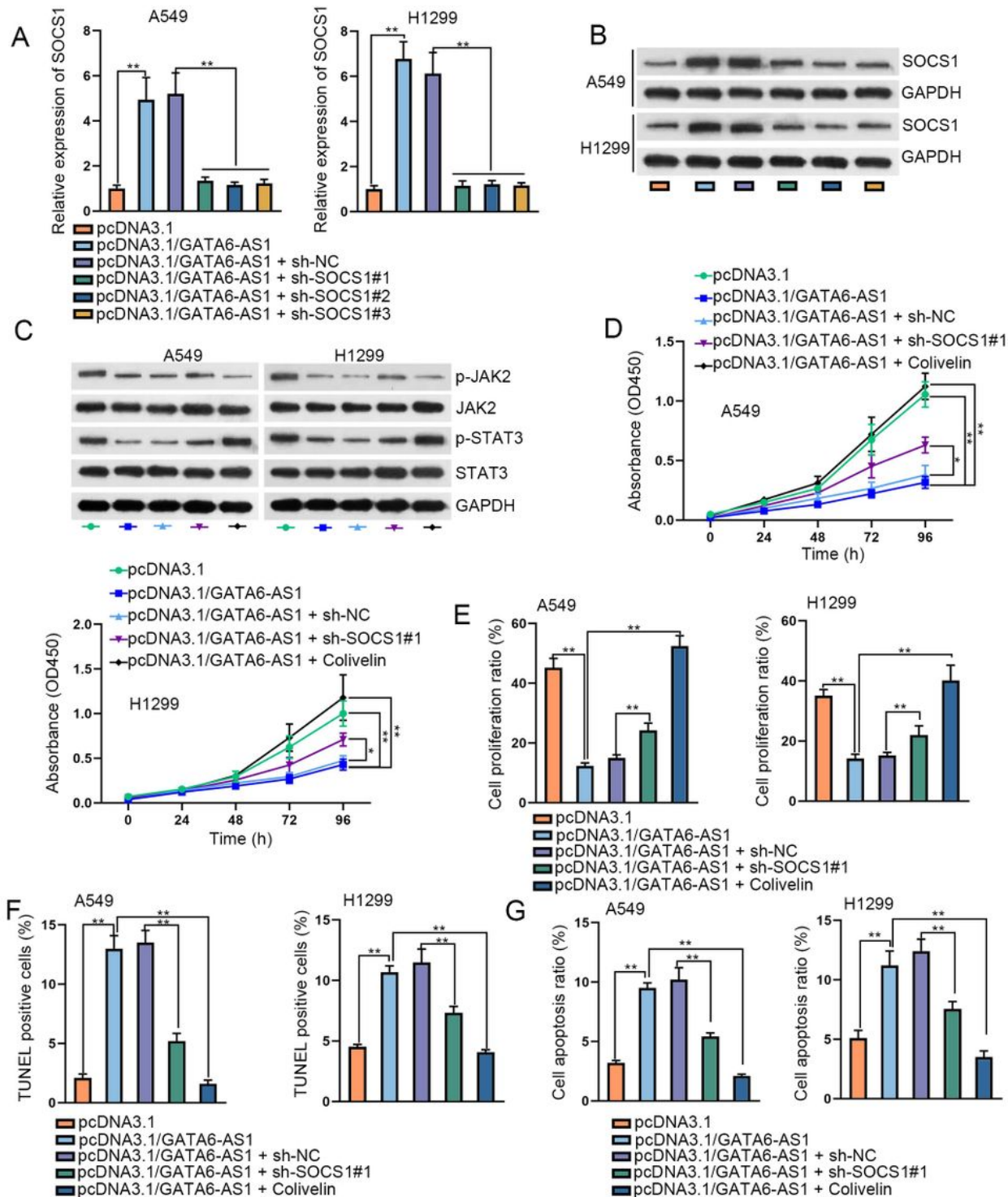




**Figure 5**

SOCS1 is regulated by miR-331-3p and GATA6-AS A. PicTar, TargetScan, PITA and miRmap database were used to presume target gene of miR-331-3p. B. The underlying binding sites were predicted between miR-331-3p and SOCS1 via ENCORI database. C. RT-qPCR was access the expression of SOCS1. D. The protein levels of SOCS1 were assessed through western blot assay. E. RIP assay was used to access whether three RNAs whether co-exist in RISC. F and G. RNA pull down and luciferase reporter assay were

conducted to confirm the combined association between miR-331-3p and SOCS1. All results were suggested as the mean  $\pm$  SD. \*\*P < 0.01.



**Figure 6**

Knockdown of SOCS1 blocks LUAD cells proliferation and induces cell apoptosis A and B. RT-qPCR and western blot assay were carried out to evaluate the expression of SOCS1 by transfection with pcDNA/GATA6-AS1 in both cells. C. The western blot was used to confirm whether down-regulation

activates JAK2 and STAT3. D and E, The CCK8 assay and EdU assay were used to test the abilities of LUAD cells after down-regulation of SOCS1 or transfecting with SOCS1. F and G. TUNEL assay and luciferase reporter assay were conducted to assess the capacity of LUAD cells apoptosis. All results were displayed as the mean  $\pm$ SD, n = 3, Student's t test or one-way analysis of variance was carried out for evaluation of differences between groups, \*\*P < 0.01.

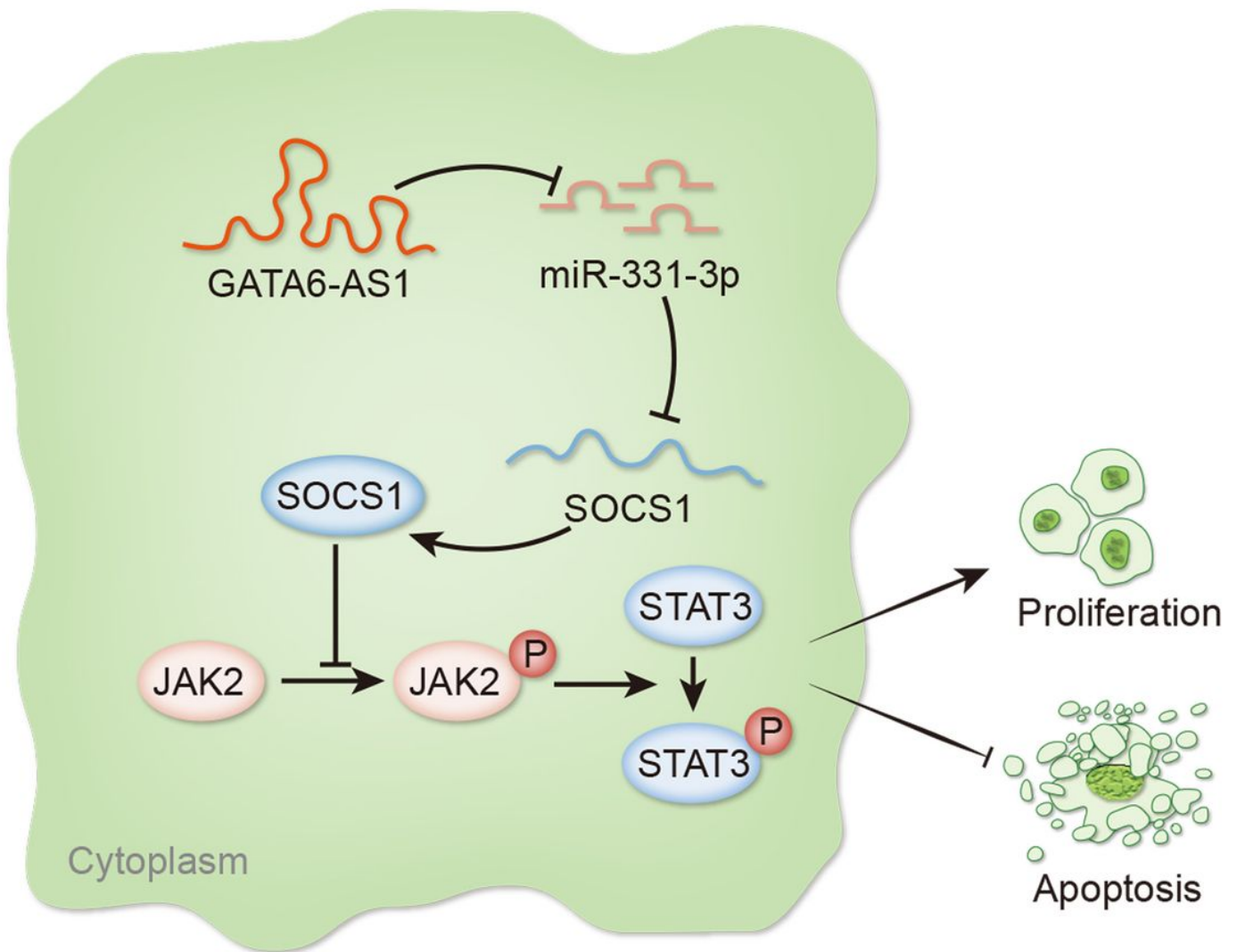


Figure 7

Figure 7