# LINC00958 promotes the proliferation of TSCC via miR-211-5p/CENPK axis and activating the JAK/STAT3 signaling pathway

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# Abstract

**Background:** Tongue squamous cell carcinoma (TSCC) is one of the most common oral tumors. Recently, long intergenic noncoding RNA 00958 (LINC00958) has been identified as an oncogene in human cancers. Nevertheless, the role of LINC00958 and its downstream mechanisms in TSCC is still unknown.

**Methods:** The expression levels of LINC00958 in human TSCC tissues and adjacent normal tissues were detected. The effect of LINC00958 on TSCC cells proliferation and growth were assessed by CCK-8, colony formation, 5-Ethynyl-2'-deoxyuridline (EdU) assay, and flow cytometry assays in vitro and tumor xenograft model in vivo. Bioinformatics analysis was used to predict the target of LINC00958 in TSCC, which was verified by RNA immunoprecipitation and luciferase reporter assays.

**Results:** We found LINC00958 was increased in TSCC tissues, and patients with high LINC00958 expression had a shorter overall survival. LINC00958 knockdown significantly decreased the growth rate of TSCC cells both in vitro and in vivo . In mechanism, LINC00958 acted as a competing endogenous RNA (ceRNA) by competitively sponging miR-211-5p. In addition, we identified centromere protein K (CENPK) as a direct target gene of miR-211-5p, which was higher in TSCC tissues than that in adjacent normal tissues. Up-regulated miR-211-5p or down-regulated CENPK could abolish LINC00958-induced proliferation promotion in TSCC cells.

**Conclusion:** Furthermore, CENPK promoted the expression of oncogenic cell cycle regulators and activated the JAK/STAT3 signaling. Our findings suggest that LINC00958 is a potential prognostic biomarker in TSCC.


# Introduction

Tongue squamous cell carcinoma is one of the most frequently diagnosed malignancies in the oral cavity [1, 2]. Because of its high metastatic and proliferative ability, TSCC is a considerable threat to human health worldwide [3, 4]. It caused about 12,060 new cases and 2030 deaths in the United States in 2011 [5], and about 48,100 new cases and 22,100 deaths were recorded in China in 2015 [1]. Over the past decades, new developments have been achieved in the therapeutic management of TSCC, such as surgery, chemotherapy and radiotherapy; however, the five-year survival rate of TSCC patients is still less than 50 % [3, 6]. Therefore, increased understanding of the complex mechanisms of TSCC cells is imperative to the development of effective and mechanism-based therapeutic modalities for this malignancy.

Increasing evidence has proved that Long non-coding RNAs (lncRNAs) modulate diverse physiologic and pathologic processes, including proliferation, migration, invasion, and autophagy[7].It has been reported as a core regulator in the processes of chromatin modification, transcriptional regulation, alternative splicing and interaction with RNAs and protein complex at transcriptional or posttranscriptional levels[8].lncRNA-SRLR is responsible for the sorafenib tolerance in renal cell

carcinoma via binding to NF-κB and promotes IL-6 transcription[9].lncRNA NORAD promotes hepatocellular carcinoma progression, it functions as a ceRNA to target miR-202-5p,leading to the enhanced activation of TGF-β pathway[10]. H19 facilitated TSCC migration and invasion by sponging miR-let-7 [11], and LncRNA KCNQ1OT1 regulated proliferation and cisplatin resistance in tongue cancer via miR-211-5p-mediated Ezrin/Fak/Src signaling [12]. It is reported that High expression of LINC00958 is associated with malignancy, proliferation, and poor prognosis in various cancers, such as oral squamous cell carcinoma, head and neck squamous cell carcinoma, and pancreatic cancer [13,14] suggesting that LINC00958 is closely connected with cancer development. However, its role and the associated downstream mechanisms in TSCC have remained unknown.

MicroRNAs (miRNAs) are evolutionarily conserved small RNAs (20–22 nucleotides long) without protein coding potential. MiRNAs can negatively regulate gene expression post-transcriptionally by binding to complementary sequences on their target mRNAs [15, 16]. A previous study has reported that miR-3064 could promote pancreatic cancer cell growth, invasion, and sphere formation by down-regulating the levels of PIP4K2B—a tumor suppressor [17]. Some studies have indicated that miR-211-5p-mediated inhibition of SNAIL1 expression contributes to the suppression of gastric carcinoma progression [18]. MiR-211-5p has been linked to short survival in hepatocellular carcinoma patients, and the restoration of miR-211-5p expression inhibited hepatocellular carcinoma cell proliferation, migration, and invasion *in vitro* by targeting ZEB2[19]. However, little is known about the role of miR-211-5p in TSCC.

In this study, lncRNAs in TSCC tissues from different patients at different stages were screened based on TCGA database analysis, and we identified that LINC00958 was the most up-regulated lncRNA in the TSCC tissues. In addition, we found that high LINC00958 expression was closely related to poor prognosis of TSCC patients. *In vitro* and *in vivo* functional experiments showed that LINC00958 promoted TSCC cell proliferation. Furthermore, LINC00958 sponged miR-211-5p to increase CENPK expression and activate the JAK/STAT3 pathway, and thus mediated proliferation. Our study elucidated the clinical significance and regulatory mechanism of LINC00958 in TSCC and provides a prognostic indicator as well as a promising therapeutic target for TSCC patients.

# Materials And Methods

### RNA sequence data analysis

RNASeq and miRNASeq data from TSCC samples were downloaded from the Cancer Genome Atlas (TCGA) database (https://cancergenome.nih.gov/). All the data are publicly available. lncRNAs were identified according to the Ensembl database (http://www.ensembl.org/index.html, version 89) lncRNAs that were not included in this database were excluded. The edgeR package (Robinson, McCarthy & Smyth, 2010) was used to normalize gene expression of mRNAs (DEmRNAs), miRNAs (DEmiRNAs), and lncRNAs (DElncRNAs) in TSCC and normal tissues. Absolute log2FC ≥ 2 and FDR < 0.01 were used as cut-off criteria. Survival R package were further used to exclude the differentially expressed lncRNA without OS

at P-value < 0.05. Next, the screened lncRNAs were used to predict lncRNA-miRNA interactions according to TargetScan (Fromm et al., 2015). lncRNAs included in these interactions were used for further study.

## Clinical specimens

Patients with TSCC, who were diagnosed, treated, and followed up at the Department of Oral Surgery, Stomatological Hospital, Southern Medical University, were included in the study. This study was approved by the hospital institutional review board and written informed consent was obtained from all the patients. All the protocols were reviewed by the Joint Ethics Committee of the Southern Medical University and performed following national guidelines. Tissue samples were collected at surgery, immediately frozen in liquid nitrogen, and stored until total RNA or proteins were extracted.

## Cell Lines and Culture

HOK and five TSCC cell lines (CAL-27, SCC-9, SCC-4, SCC-15, SCC-25) were obtained from the Tumor Cell Bank of the Chinese Academy of Medical Science (Shanghai, China). CAL-27 cells were maintained in DMEM medium (Gibco, Grand Island, USA), which was supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, USA), and the other cells were cultured in RPMI-1640 (Gibco, Grand Island, USA) supplemented with 10% FBS. For all cell lines, 100 IU/ml penicillin and 100 μg/mL streptomycin were added to the culture medium, and all of the cells were incubated at 37℃ in a humidified atmosphere of 95% air 5% $CO_2$.

## Real-time quantitative PCR (RT-qPCR)

Total RNA was isolated from cells or tissues using TRIzol reagent (Invitrogen, California, USA) and then was converted to cDNA using a PrimeScript RT reagent kit (TaKaRa, Tokyo, Japan). RT-qPCR analysis were carried out in triplicate for each sample using SYBR Green Master Mix (TaKaRa, Tokyo, Japan). All primers are listed in Table S1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control. For detecting miRNA expression level, cDNA was synthesized using MicroRNAs qPCR Kit (SYBR Green Method) (Sangon Biotech, Shanghai, China), and U6 small nuclear RNA served as the endogenous control.

## CCK-8 assay

Cell viability was determined using the CCK-8 assay. Briefly, $2\times10^3$ cells/well were seeded into 96-well plates, and the absorptions of the cells were measured using a CCK-8 kit (Dojindo, Kyushu, Japan) according to the manufacturer's instructions at different indicated time points. Data were from three separate experiments with four replications each time.

## Clone formation assay

From each group, nearly $1\times10^3$ cells were plated in each well of a 6-well culture plate. Each cell group consisted of three wells. The cells were incubated at 37 °C for 14 days with growth media being replaced

every third day. Then, the cells were washed twice with PBS and stained with 0.5% crystal violet.

### EdU Incorporation Assays

Cells were cultured in 24-well plates, and 10 μM EdU was added to each well. Then, the cells were cultured for 2 h at 37°C and were fixed with 4% formaldehyde for 20 min at RT. After washing with PBS, the incorporated EdU was detected with a kFluor488-EdU kit (KeyGEN, Jiangsu, China) for 30 min at RT, and subsequently, the cells were stained with Hoechst 33342 for 20 min and were captured using a fluorescence microscope (Olympus, Tokyo, Japan) and were merged using Adobe Photoshop 6.0 software. All experimental procedures were repeated at least three times.

### Cell cycle analysis by flow cytometry

Cell cycle analysis were performed using the Cell Cycle Analysis Kit (Beyotime, Jiangsu, China) as per the manufacturer's instructions. Cells were harvested and fixed in 70% ethanol overnight at 4 ℃. Then, the cells were stained with 25 μg/mL propidium iodide containing 1 μg/mL RNase at 37 ℃ for 30 min. The cells were analyzed for their distribution in different phases of the cell cycle on FACSCalibur flow cytometer using CellQuestPro software (Becton Dickinson, New Jersey USA).

### RNA immunoprecipitation (RIP)

A Thermo Scientific RIP kit (Thermo, Waltham, MA, USA) was used to carry out RIP according to the manufacturer's instructions. CAL-27 and SCC-9 cells were transfected with miR-211-5p mimics or mimics NC. Complete RIP lysis buffer was used to lyse cells. Magnetic beads conjugated with anti-Argonaute 2 (AGO2, Millipore, Massachusetts, USA) or control anti-immunoglobulin G (IgG, Abcam, Cambridge, England) antibody were used to incubate the cell extract. The cell extract was incubated for 6 h at 4℃. As the protein beads were removed, RT-qPCR was conducted for the purification of RNA.

### Western blot analysis

Cell lysates was separated by SDS-polyacrylamide gel (4%–10%) electrophoresis, and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Massachusetts, USA). The membranes were then blocked with 5% skimmed milk and incubated overnight at 4°C with the following primary detection antibodies: anti-CENPK (1:1000, Abcam, Cambridge, England), anti-C-MYC (1:1000, Abcam, England), anti-Cyclin D (1:1000, CST, Boston, USA), anti-Cyclin E (1:1000, Abcam, Cambridge, England), anti-Rb (1:1000, CST, Boston, USA), anti-p-Rb (1:1000, CST, Boston, USA), anti-GAPDH (1:10000, Abcam, Cambridge, England), anti-JAK1 (1:1000, Abcam, Cambridge, England), anti-STAT3 (1:1000, Abcam, Cambridge, England), or anti-p-STAT3 (1:1000, Abcam, Cambridge, England). The species-matched secondary antibodies were then incubated for 1 h at room temperature and the proteins were detected using BeyoECLPlus (Beyotime, Jiangsu, China).

### Luciferase reporter assay

The wild-type or mutant LINC00958 fragment or CENPK 3'-UTR containing the predicted binding sites of miR-211-5p. were subcloned into a psiCHECK2 Dual-luciferase vector (Promega, Madison, USA). The luciferase reporter plasmids were co-transfected into TSCC cells with miR-211-5p mimics or the negative control. The relative luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega, Madison, USA) according to the manufacturer's instructions.

### IHC staining

We used anti-Ki67 antibody (CST, Boston, USA), anti-PCNA antibody (Abcam, Cambridge, England), anti-CENPK antibody (Abcam，Cambridge, England) and anti-p-STAT3 (CST, Boston, USA) to detect the expression in the mouse xenografts. The sections were visualized using a Nikon ECLIPSE Ti microscope system and were processed with Nikon software.

### Plasmids, Virus Production, siRNA, and Transfection

The full length of LINC00958 and shRNAs targeting LINC0058 was synthesized and cloned into the lentiviral plasmid pSin-EF2-puromycin (Addgene, Cambridge, MA, USA). pSin-EF2-LHX2-puromycin or negative control pSin-EF2-puromycin vector was then co-transfected into 293T cells with the VSVG and PSPAX2 packaging plasmid (Addgene, Cambridge, MA, USA) using Lipofectamine 3000 reagent (Invitrogen). The supernatants were harvested and used to infect CAL-27 and SCC-9 cells, and stable clones were selected using 0.5 μg/mL puromycin. Small interfering RNA targeting CENPK (si-CENPK) was synthesized by RiboBio (Guangzhou, China). For CENPK knockdown, CAL-27 and SCC-9 were transfected with si-CENPK (50 nM) with Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's instruction and harvested for assays (48 h after transfection). miR-211-5p mimics, miR-211-5p inhibitors, and miR-controls were purchased from RiboBio (Guangzhou, China). MiRNA mimics, miRNA inhibitors, and miR-controls were transfected into cells at a concentration of 50 nM using Lipofectamine 3000 (Invitrogen, California, USA).

### *In vivo* nude mouse models

A total of twenty-four 5-week-old male nude mice were purchased from Guangdong Medical Laboratory Animal Center, and kept under specific pathogen-free conditions. Mice were divided into four groups at random and inoculated with Cal-27 and SCC-9 cells subcutaneously on the right flank with $2 \times 10^6$ cells (n = 6 per experimental group). The growth of the tumors was observed and the volumes of tumors were measured every 4 days. The animals were sacrificed after 4 weeks of growth, and the tumors were excised for pathological examination.

### Subcellular Fractionation and Fluorescent *in Situ* Hybridization (FISH) assay

Nuclear and cytoplasmic RNA was separated with the NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Invitrogen) accordingly, and then analyzed by quantitative RT-PCR. FISH was performed for subcellular localization of LINC00958. The cover glasses were placed into a 24-well plate and cells were

inoculated at the density of $6\times10^4$ cells/well. When cell confluence reached 80 %, the cover glasses were removed, and the cells were washed by PBS and fixed with 1 mL 4 % paraformaldehyde at room temperature. The experiment was conducted according to the instructions of RiboTMlncRNA FISH Probe Mix (Red) (RiboBio Co., Ltd., Guangdong, China). The cells were observed and photographed under a fluorescence microscope.

### Statistical Analysis

Data were expressed as mean ± SD of three independent experiments with GraphPad Prism softwareversion 6.0. Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) software (version 16.0). Depending on the type of data, the appropriate statistical methods were used, including the t-test, one-way ANOVA, chi-square test, and linear correlation analysis. The Kaplan–Meier method with the log-rank test was used to compare the survival rate of TSCC patients based on LINC00958 expression. Survival data were evaluated using univariate and multivariate Cox proportional hazards models. $p < 0.05$ was considered statistically significant.

## Results

### LINC00958 is specifically up-regulated in tongue cancer tissues and cell lines

Using TCGA database analysis, we detected aberrantly expressed lncRNAs between adjacent normal tissues and TSCC tissues from TSCC patients. Furthermore, we found that LINC00958 was highly up-regulated in tumor tissues, compared with normal tissues from the TCGA database, (Fig.S1A-B and Fig.1A-B). Furthermore, the correlation between LINC00958 expression and clinicopathological traits was analyzed. LINC00958 expression was much higher in T3 and T4 than in T1 and T2 (Table S2). In addition, Kaplan-Meier survival analysis indicated that increased LINC00958 expression was significantly associated with a lower rate of overall survival in TSCC patients (Fig. 1C), and high expression of LINC00958 predicted poor survival in some other solid tumors (Fig. S1C). We also examined the relative expression of LINC00958 in TSCC tissues (n = 24) and adjacent normal tissues (n = 24). RT-qPCR results showed that the expression of LINC00958 was significantly increased in TSCC samples than in adjacent normal tissues (Fig. 1D). This change in LINC00958 expression was further evaluated in TSCC cell lines including SCC-15, SCC-25, SCC-4, SCC-9, and CAL-27. As shown in Fig. 1E, the expression level of LINC00958 was up-regulated in TSCC cell lines, compared with HOK cells. These findings indicate that LINC00958 was significantly up-regulated in TSCC and predicte poor survival.

### LINC00958 promotes the proliferation of TSCC cells and affecting cell cycle distribution

To evaluate the influence of LINC00958 on TSCC cell growth, CAL-27 and SCC-9 cells were transfected with two different shRNAs against LINC00958 (sh-LINC00958-1 and sh-LINC00958-2), a LINC00958-overexpressing plasmid (pcDNA-LINC00958) and their corresponding controls. The transfection efficiency was confirmed by RT-qPCR analysis (Fig. S2A). CCK-8 assays and colon formation assays revealed a significant inhibition in the proliferation of TSCC cells transfected with sh-LINC00958, compared with

those transfected with scrambled vectors, whereas the opposite effects were observed in TSCC cells with LINC00958 overexpression (Fig. 2A-B). In addition, the knockdown of LINC00958 decreased cell division, whereas overexpression of LINC00958 significantly increased cell division as shown by EdU assays (Fig. S2B). Morover, flow cytometry analysis indicated that overexpression of LINC00958 promoted cell cycle progression, while the knockdown of LINC00958 induced cell cycle arrest at the G0/G1 phase in CAL-27 and SCC-9 cells (Fig. 2C).Taken together, these findings suggest that LINC00958 enhances the cell proliferation ability of TSCC cells and promotes cell cycle progression.

### Down-regulation of LINC00958 suppresses TSCC tumor growth *in vivo.*

To explore the effect of LINC0095 on the tumorigenesis of TSCC cells *in vivo*, CAL-27 cells with stably knockdown LINC00958 was subcutaneously transplanted into nude mice. The growth rate of tumors in the LINC00958 knockdown group was substantially suppressed compared with that in the control group (Fig. 3A-B). The tumor weights in the LINC00958 knockdown group were significantly lower than those of in the control group (Fig. 3C). Furthermore, LINC00958 knockdown reduced Ki-67 and proliferating cell nuclear antigen (PCNA) positivity as confirmed by immunohistochemistry results (Fig. 3D). These results indicate that LINC00958 knockdown inhibits TSCC cell growth *in vivo*.

### LINC00958 acts as a ceRNA via binding to miR-211-5p in TSCC

LINC00958 is highly expressed in various types of cancers, and could act as a ceRNA[20, 21].. To explore if LINC00958 acts as a miRNA sponge that competes with mRNA, we first predicted its subcellular localization with lncATLAS (http://lncatlas.crg.eu/). LINC00958 was predicted to be located mainly in the cytoplasm in a variety of cell types (Fig. 4A). RT-qPCR and FISH assays verified that LINC00958 was localized mainly in the cytoplasm of TSCC cells (Fig. 4B-C). Using LncRNABase (www.lncrnadb.org) and NONCODE (www.noncode.org) online database, we found potential binding sites between LINC00958 and miR-211-5p (Fig. 4D). Based on this prediction, luciferase reporter constructs carrying LINC00958 reporter were generated. The luciferase reporter assays showed that miR-211-5p mimics significantly decreased the luciferase activity of the wild type (Wt) reporter, but not that of mutant (Mut) reporter (Fig. 4E), indicating that the binding sequences were synergistically responsible for the interaction of LINC00958 and miR-211-5p. To further test if LINC00958 regulated miR-211-5p by acting as a ceRNA, RIP assays were performed with TSCC cells extracts using anti-Ago2. As shown in Fig. 4F, LINC00958 and miR-211-5p were substantially enriched in the Ago2 immunoprecipitation compared with the negative control IgG. In addition, miR-211-5p significantly increased the enrichment of LINC00958 by Ago2 immunoprecipitation. We also found the expression of miR-211-5p was significantly decreased in TSCC samples compared with the adjacent normal tissues (Fig. S3A-B). And the expression of miR-211-5p correlated negatively with that of LINC00958 in the TSCC tissues (Fig. 4G, Fig. S3C). Further investigations showed that LINC00958 negatively affected miR-211-5p expression levels (Fig. 4H), whereas LINC0095 expression was obviously suppressed by overexpression of miR-211-5p and elevated by ihibition of miR-211-5p ((Fig. 4I). All these results indicate that the modulating effect of LINC00958 on miR-211-5p expression may be possibly mediated by its ability to act as a ceRNA.

## MiR-211-5p binds to CENPK and represses its expression

The preceding results suggest that LINC00958 regulates miR-211-5p possibly by acting as a ceRNA. Thus, we searched the possible targets of miR-211-5p by using a bioinformatics prediction tool—miRcode (http://www.mircode.org/) and analyzed the differentially expressed mRNAs in TCGA database between adjacent normal tissues and TSCC tissues (Fig. 5A). Among the common genes, eight of the most up-regulated mRNAs were subjected to validation by RT-qPCR analysis in CAL-27 cells with miR-211-5p inhibitor or miR-211-5p mimics administration. The results revealed that CENPK was the most notably up-regulated transcript in the miR-211-5p inhibitor group and down-regulated in the mimics group (Fig. 5B). The binding site between CENPK and miR-211-5p is shown in Fig. 5C. The results of luciferase reporter assays showed that the binding sequences were synergistically responsible for the interaction between CENPK and miR-211-5p (Fig. 5D). We also verified a negtive correlation bwtween CENPK and mir-211-5p based on the TSCC TCGA database and frozen clinical specimen (Fig. 5E-F). Furthermore, the expression of CENPK was significantly increased in TSCC samples compared with the adjacent normal tissues (Fig. S3D-E). Further investigations showed that miR-211-5p negatively regulated CENPK expression (Fig. 5G). Therefore, we confirmed that miR-211-5p suppressed CENPK expression in TSCC cells.

## LINC00958 promotes CENPK-mediated proliferation through miR-211-5p sponging *in vitro*

Next, we examed the effect of LINC00958 on CENPK. LINC00958 increased CENPK expression, which could be attenuated by miR-211-5p mimics (Fig. 6A). In addition, miR-211-5p inhibition successfully rescued the effect of LINC00958 silencing on CENPK expression (Fig. 6B). Pearson correlation analysis showed that the expression of CENPK positively correlated with that of LINC00958 in TSCC tissues (Fig. 6C-D). What's more, the expression of miR-211-5p was dramatically increased in xenografts derived from LINC00958 knockdown group, while CENPK expression was decreased (Fig. S3F). To verify if LINC00958 modulate TSCC progression in a CENPK-dependent manner, we co-transfected CAL-27 and SCC-9 cells,with pcDNA-LINC00958 and si-CENPK or miR-211-5p mimics. CCK-8 assays, colony formation assays and Flow cytometry analysis demonstrated that the CENPK silencing or miR-211-5p overexpression weaken the promotion effects of LINC00958 over-expression on TSCC cell proliferation (Fig. 6E-G). These findings demonstrate that LINC00958 is an oncogenic lncRNA that promotes TSCC cell proliferation via the LINC00958/ miR-211-5p/ CENPK axis.

## LINC00958/mir-211-5p/CENPK axis promotes TSCC proliferation by regulating cell cycle process and activating the JAK/ STAT3 signaling pathway

To better understand the mechanism by which CENPK regulates cell proliferation in TSCC, we referred to gene set enrichment analysis (GSEA) and found that CENPK expression was related to c-MYC target and cell cycle function. (Fig. 7A, Fig. S4A). CENPK over-expression increased the levels of c-myc, cyclinD, cyclinE, and p-RB, whereas the knockdown of CENPK resulted in the opposite effects (Fig. 7B-C). Furthermore, GSEA analysis suggested that CENPK might influence the JAK/STAT3 signaling pathway (Fig. 7D and Table. S3). IHC analysis showed a decreased CENPK and p-STAT3 levels in LINC00958 silenced xenografts (Fig. 7E).Western blot assays further verified that the overexpression of CENPK

positively affected the expression of p-JAK1 and p-STAT3 at the protein levels (Fig. 7F). Moreover, LINC00958 could also elevated the above protein levels, while CENPK silencing or miR-211-5p overexpression could restain these promotion effects (Fig.7G).Together, these findings demonstrate that LINC00958-induced CENPK promotes TSCC tumorigenesis through the modulation of the cell cycle process and the activation of the JAK/STAT3 signaling pathway (Fig.8).

# Discussion

Accumulating evidence indicates that lncRNAs dysregulation plays important roles in tumorigenesis and in the progression of various types of cancers, which provides new perspectives for the treatment of malignant cancers. Some reports have pointed out that some lncRNAs serve as a scaffold of protein complex to promote or inhibit gene expression at the transcription level [22], but lncRNAs can also act as a mediator in the ceRNA pathway to regulate tumorigenesis [23]. LINC00958 has been identified as an oncogene in human cancers. It could bind with miR-330-5p, and silencing LINC00958 could inhibit EMT, tumor growth, invasion, and metastasis of prostate cancer cells [21]. LINC00958 could promote lymph node metastasis by inducing tumor-associated lymphangiogenesis and promoting bladder cancer cell invasiveness [24].. In addition, cell proliferation could be suppressed and apoptosis could be elevated by combining radiotherapy with LINC00958 silencing in cervical cancer cells [20]. Moreover, overexpression of long non-coding facilitated cell proliferation, migration and invasion in lung adenocarcinoma.[25]. In the present study, LINC00958 was also up-regulated in TSCC tissues when compared with pair matched normal tissues, and the upregulation of LINC00958 was associated with clinical characteristics and poor overall survival. We also determined the effects of LINC00958 on TSCC cells. The inhibition of LINC00958 suppressed the proliferation of TSCC cells *in vitro* and *in vivo*, and LINC00958 promoted TSCC cell proliferation by sponging miR-211-5p, leading to enhanced CENPK expression and the activation of the JAK/STAT3 signaling pathway. Collectively, our results indicate that LINC00958, acting as an oncogene, may be a potential diagnostic and prognostic biomarker, as well as a therapeutic target, in TSCC.

Growing evidence supports the existence of a novel and extensive interaction network involving ceRNAs [26, 27], in which lncRNAs regulate miRNAs by competitively binding their target sites on protein-coding mRNA molecules. To a large extent, the level of regulation depends on the cellular location of the lncRNA [28]. LncRNAs located in the cytoplasm always function as endogenous miRNA sponges for miRNA response elements, thereby impairing the function of target mRNA at the posttranscriptional level [29]. In the present study, using FISH assay and RT-qPCR detection of RNAs in the cell cytoplasm or nucleus, we noted that LINC00958 is a cytoplasmic long noncoding RNA, which suggested that LINC00958 might exert its function as a ceRNA at the post-transcription level. Thus, to verify the ceRNA mechanism of LINC00958, we searched for candidate miRNAs.

A large number of lncRNAs have shown the capacity to act as sponges for miRNA and exert functions in tumorigenesis and tumor progression. For example, LINC01234 acts as a ceRNA to regulate CBFB expression by sponging miR-204-5p to promote gastric cancer cell proliferation [30]. UICLM promoted

colorectal cancer metastasis by acting as a ceRNA which sponges miRNA-215 to regulate ZEB2 expression [31]. In this study, using bioinformatics tools as well as dual luciferase reporter and RNA pull-down assays, we showed that miR-211-5p directly bound to LINC00958. In addition, the expression of miR-211-5p was inversely related to that of LINC00958 in TSCC samples. Knockdown of LINC00958 promoted the expression of miR-211-5p.  As previously reported in a mount of researches,when working as a ceRNA, lncRNA could binds with miRNA in MREs and downregulate its expression. The mechanism for how lncRNA decrease miRNA was not fullly understand, One way through which a relatively lncRNA can nevertheless regulate the activity of typically miRNAs is through binding sites with special sequences or pairing topology, that would trigger miRNA degradation upon binding.In detail,.binding of miRNAs loaded in Ago1 to targets with extensive sequence complementarity triggers tailing of the miRNA with non -templated nucleotides (mostly adenines and uridines), miRNA trimming, and eventual miRNA degradation a phenomenon referred to as target RNA-directed miRNA degradation, or TDMD[32].

MiRNAs directly bind to the 3’-UTR of downstream targeting genes involved in tumor progression [33]. miR-211-5p-mediated inhibition of SNAIL1 expression contributes to the suppression of RCC progression [18]. Moreover, miR-211-5p overexpression suppressed the proliferation, migration, and invasion of triple-negative  breast cancer [34]. Using an online database, we predicted CENPK as a potential target of miR-211-5p, which was confirmed by luciferase reporter and RT-qPCR assays. CENPK is known to be a subunit of the CENPH-I complex, which is essential for proper kinetochore assembly [35]. Several studies have found that CENPK is overexpressed in several tumor types and promotes tumor progression. In the present study, our results showed that CENPK was up-regulated in clinical samples. In addition, the expression of miR-211-5p was inversely related to that of CENPK in TSCC samples. The overexpression of miR-211-5p inhibited CENPK protein expression. Further investigation found that the expression of miR-211-5p was negatively associated with that of CENPK in TSCC samples. The overexpression of CENPK promoted the expression of proliferation-related protein, such as c-myc, cyclin D, cyclin E, p-Rb. It demonstrated that LINC00958 up-regulated CENPK expression by competitively sponging miR-211-5p, thus promoting TSCC cell proliferation.

Signal transducer and activator of transcription 3 plays a crucial role in a wide variety of biological processes such as cell proliferation, invasion, apoptosis and immunity [36]. Numerous studies have reported that STAT3 activation is associated with a poor prognosis of diverse cancers including ,colorectal cancer [37], and hepatocellular carcinoma [38]. In addition, LncRNA ATB activated AKT and the JAK/STAT3 signaling pathway through down-regulated miR-494 in lung cancer [39]. However, the molecular mechanisms responsible for the activation of JAK/STAT3 signaling pathway in TSCC are still poorly understood. The data from GSEA demonstrated that JAK/STAT3 signaling pathway related-genes are enriched in TSCC patients expressing high level of CENPK. Our study dissected the mechanisms by which CENPK mediates the JAK/STAT3 signaling pathway activation. The overexpression of CENPK promoted the expression of p-JAK1 and p-STAT3. In addition, we also demonstrated that LINC00958 knockdown decreased the expression of CENPK and p-STAT3 *in vivo*. Thus, these findings uncover the novel mechanism associated with the activation of STAT3 in TSCC.

# Conclusions

In conclusion, we identified LINC00958 as an oncogenic lncRNA in TSCC. Functional and mechanistic analysis revealed that LINC00958 promotes TSCC cell proliferation by acting as a ceRNA which sponges miR-211-5p, causing enhanced CENPK expression and the activation of the JAK/STAT3 signaling pathway. Our study demonstrates that LINC00958 plays an important role in TSCC tumorigenesis and progression. Furthermore, it elucidates the clinical significance and regulatory mechanism of LINC00958 in TSCC and provides a prognostic indicator as well as a promising therapeutic target for TSCC patients.

# Abbreviations

lncRNAs: Long non-coding RNAs; LINC00958: long intergenic noncoding RNA 00958; RT-qPCR: quantitative real-time polymerase chain reaction; EdU:5-Ethynyl-2'-deoxyuridline; CENPK: centromere protein K; TSCC: Tongue squamous cell carcinoma; IHC: Immunohistochemistry; ceRNA: Competing endogenous RNA; shRNA: Short hairpin RNA; SMC: smooth muscle cell; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; PVDF: polyvinylidene fluoride.

# Declarations


### Funding

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### Ethics approval and consent to participate

Not applicable.

### Consent for publication

All contributing authors agree to the publication of this article.

### Availability of data and materials

All data are fully available without restrictions.

### Competing interests

We confirmed that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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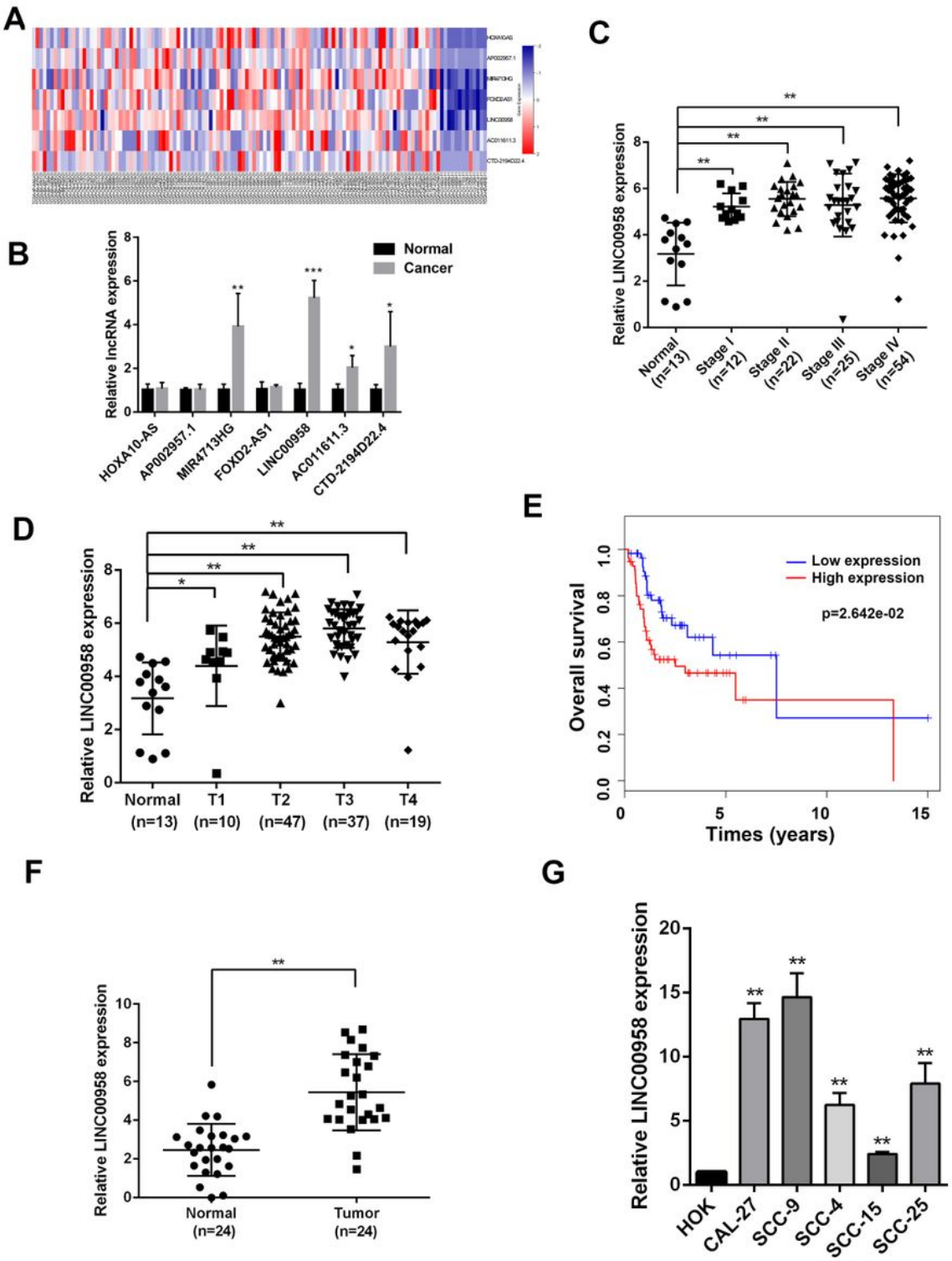
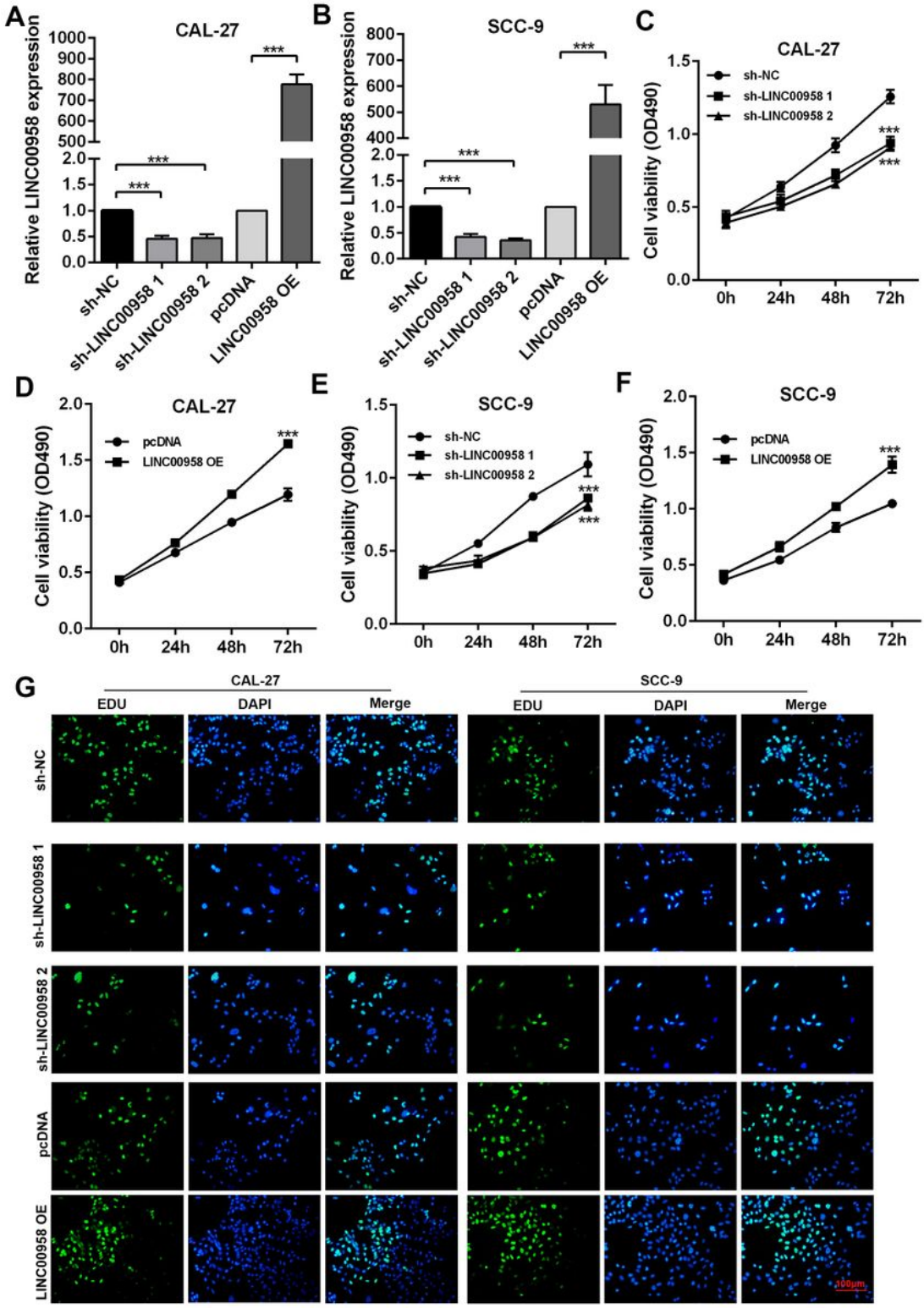
# Figures




## Figure 1

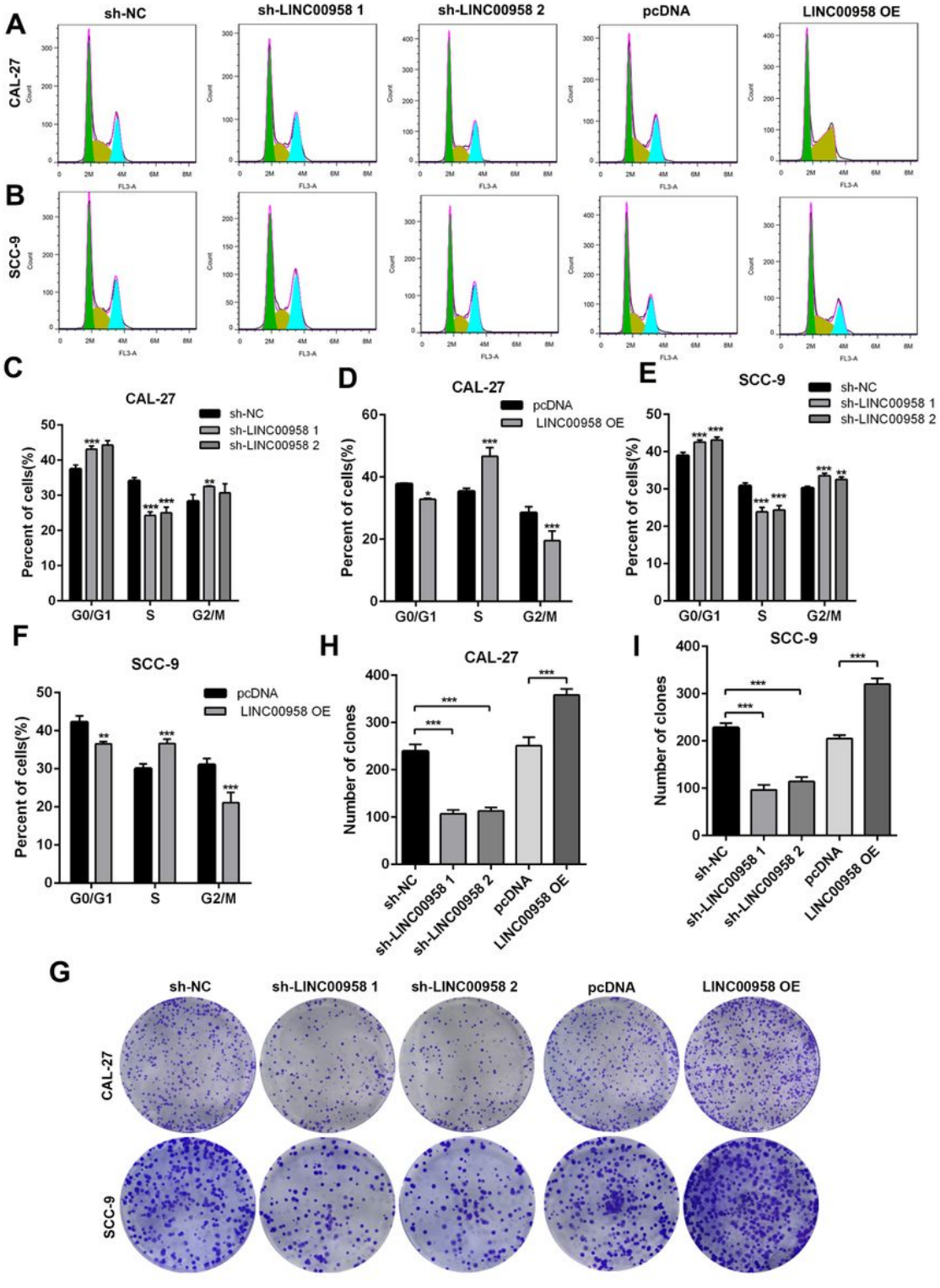
LINC00958 is specifically upregulated in tongue cancer tissues and tumor cell lines. (A) Hierarchical clustering analysis the most differentially expressed lncRNAs in TSCC tumors tissues from TCGA database.□B□The expression of different expressed lncRNAs were subject to validation by q-PCR. (C) Relative expression of LINC00958 in normal epithelial tissues and different stages of TSCC.The data were downloaded and analyzed from the TCGA database. (D) Relative expression of LINC00958 in

normal epithelial tissues and different clinical T-staging of TSCC. The data were downloaded and analyzed from the TCGA database. (E) Kaplan-Meier analysis of the correlation between LINC00958 expression and overall survival. The data were downloaded and analyzed from the TCGA database. (F) The expression of LINC00958 were detected in TSCC (n=24) and normal epithelial tissues (n=24) by RT-qPCR. The LINC00958 expression was normalized to β-actin (ΔCt) and compared with the maximum ΔCt. Data were presented as -ΔΔCt. (G) The expression of LINC00958 was detected in HOK and 5 TSCC cell lines by RT-qPCR. *P<0.05, **P<0.01, ***P<0.001.



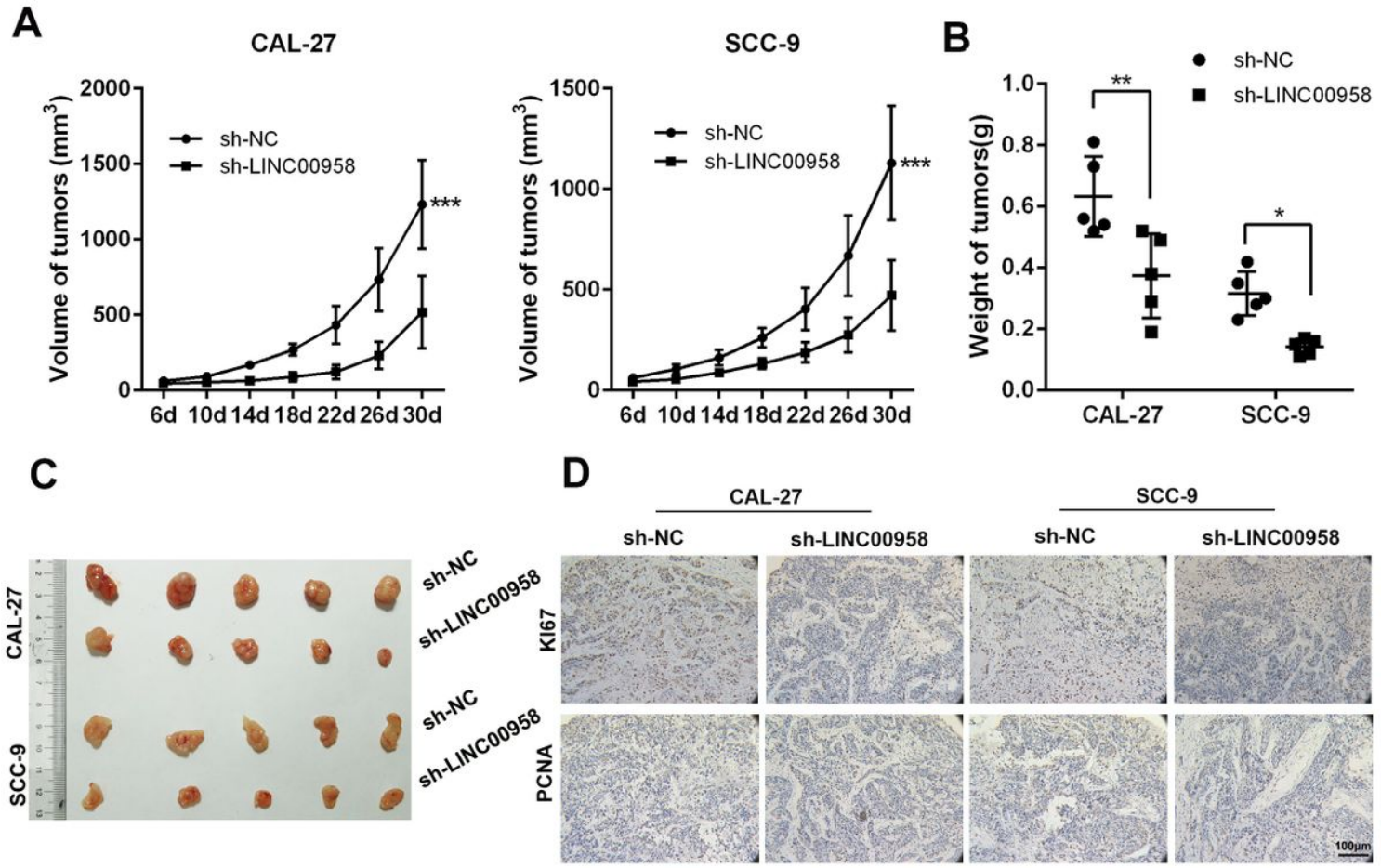
## Figure 2

LINC00958 promotes the growth of TSCC cells. (A-B) The expression of LINC00958 was detected in CAL-27 and SCC-9 cells stably transfected with sh-LINC00958 or pcDNA-LINC00958 by RT-qPCR. (C-F) Influence of LINC00958 knockdown or over-expression on cell viability of CAL-27 and SCC-9 cells by the CCK8 assay. (G) CAL27 and SCC9 cells were stably transfected with sh-LINC00958 or pcDNA-LINC00958 and the cell viability were examined by EDU assay. *P<0.05, **P<0.01, ***P<0.001.



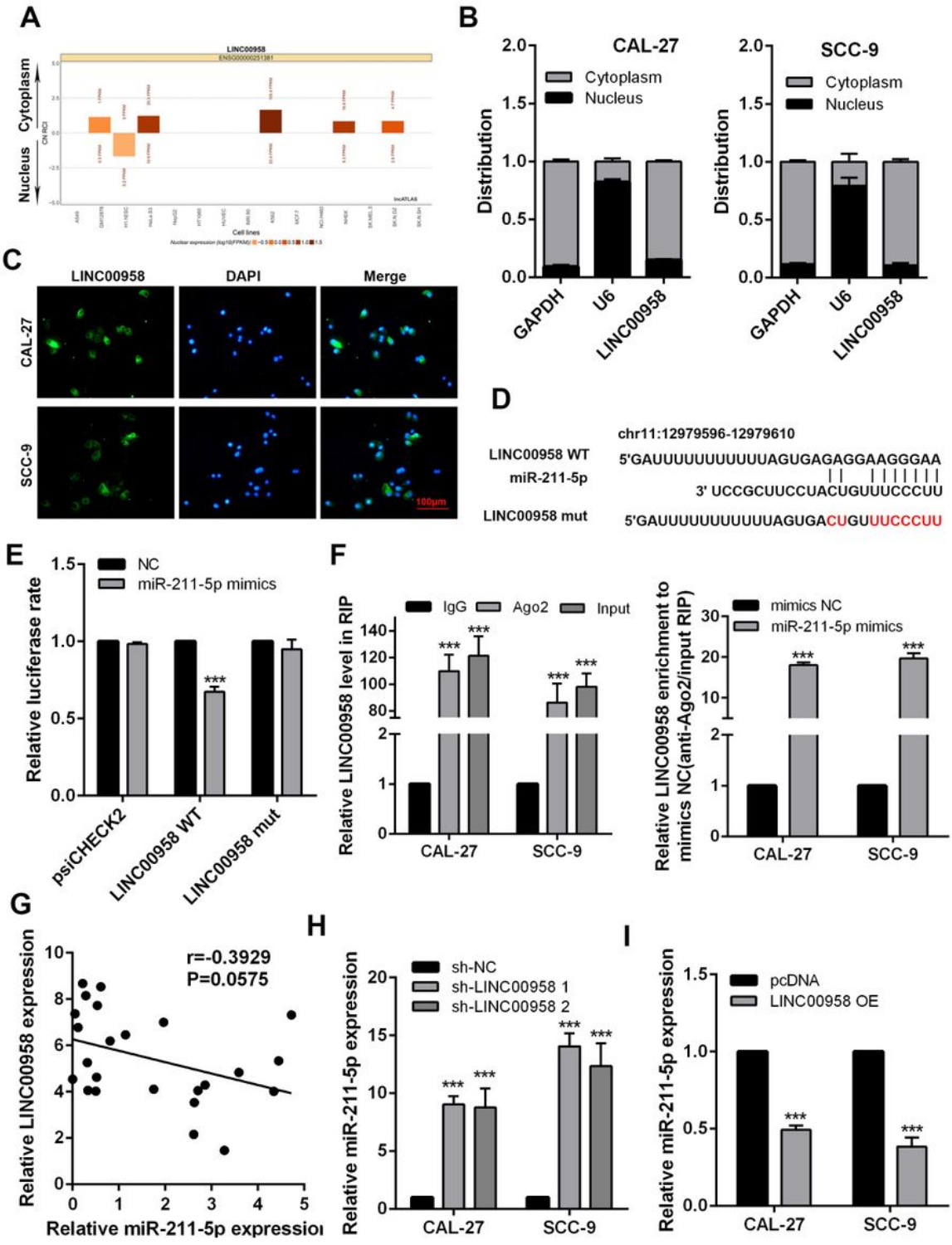
## Figure 3

LINC00958 promotes TSCC cells proliferation by regulating cell cycle. (A-F) Flow cytometric analysis of cell cycle distribution in CAL-27 and SCC-9 cells transfected with sh-LINC00958 or pcDNA-LINC00958. (G-I) Influence of LINC00958 knockdown or over-expressed on cell proliferation of CAL-27 and SCC-9 cells by the colony formation assay. *P<0.05, **P<0.01, ***P<0.001.




## Figure 4

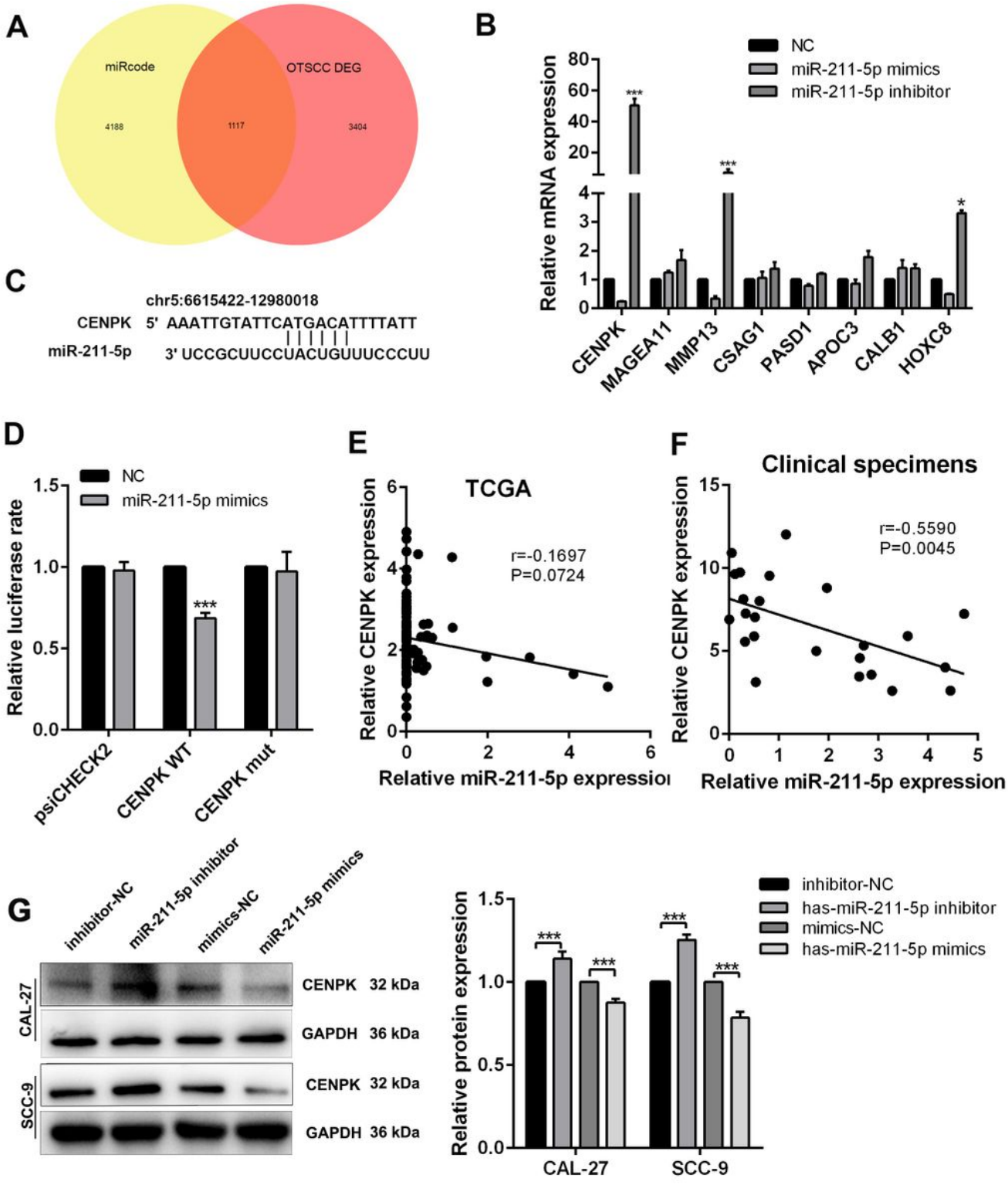
Downregulation of LINC00958 suppresses TSCC tumor growth in vivo. Nude mice were transplanted subcutaneously with CAL-27 and SCC-9 cells stably transfected with sh-LINC00958 or the control shRNA. A representative picture of the morphology of tumor xenografts after excision at 30 days of treatment. (B) Tumor volumes were measured every four days. (C) Tumor weights were measured after the mice were sacrificed. (D) The expression of Ki67 and PCNA in the xenografts was examined by IHC. *P<0.05, **P<0.01, ***P<0.001.




## Figure 5

LINC00958 acts as a ceRNA via binding to miR-211-5p. (A) LINC00958 was predicted to be located mainly in the cytoplasm using the bioinformatics tools in lncATLAS. (B) RT-qPCR analysis of subcellular LINC00958 expression in the nucleus and cytoplasm of CAL-27 and SCC-9 cells. GAPDH, β-actin and U6 were used as endogenous controls. (C) Subcellular localization of LINC00958 in CAL-27 and SCC-9 cells detected by RNA-FISH. LINC00958 is stained green and nuclei are stained blue (DAPI). (D) The predicted

miR-211-5p binding sites in the LINC00958 transcript. (E) The potentail binding sites were constructed into different vector. The wild-type (WT) vector and mutant-type vector were cotransfected into CAL-27 cells, together with miR-211-5p mimics or miR-NC. The reporter vectors were normalized to Renilla luciferase vector. (F) RIP experiments revealed the enrichment of LINC00958 and miR-211-5p in the Ago2 immunoprecipitation compared with the control IgG precipitation. (G) Correlation between LINC00958 and miR-211-5p expression based on the TCGA database. (H-I) The expression of miR-211-5p was detected in CAL-27 and SCC-9 cells transfected with sh-LINC00958 or pcDNA-LINC00958 by RT-qPCR. *P<0.05, **P<0.01, ***P<0.001.




## Figure 6

MiR-211-5p binds to CENPK and represses its expression. The overlapping mRNAs from the online analysis tools miRcode (http://www.mircode.org/) and analyzed TCGA database, were identified with a Venn diagram. (B)The level of these mRNAs was detected in CAL-27 cells transfected with miR-211-5p mimics or miR-211-5p inhibitor. (C) Schematic illustration of the predicted binding sites between CENPK and miR-211-5p. (D) Luciferase assays in CAL-27 cells transfected with wild-type or mutant CENPK and

miR-211-5p mimics. (E) Correlation between CENPK and miR-211-5p expression based on the TCGA analysis. (F) Correlation between CENPK and miR-211-5p expression of TSCC clinical samples. (G) The expression of CENPK was detected in CAL-27 and SCC-9 cells transfected with miR-211-5p mimics or miR-211-5p inhibitor by western blotting. *P<0.05, **P<0.01, ***P<0.001.

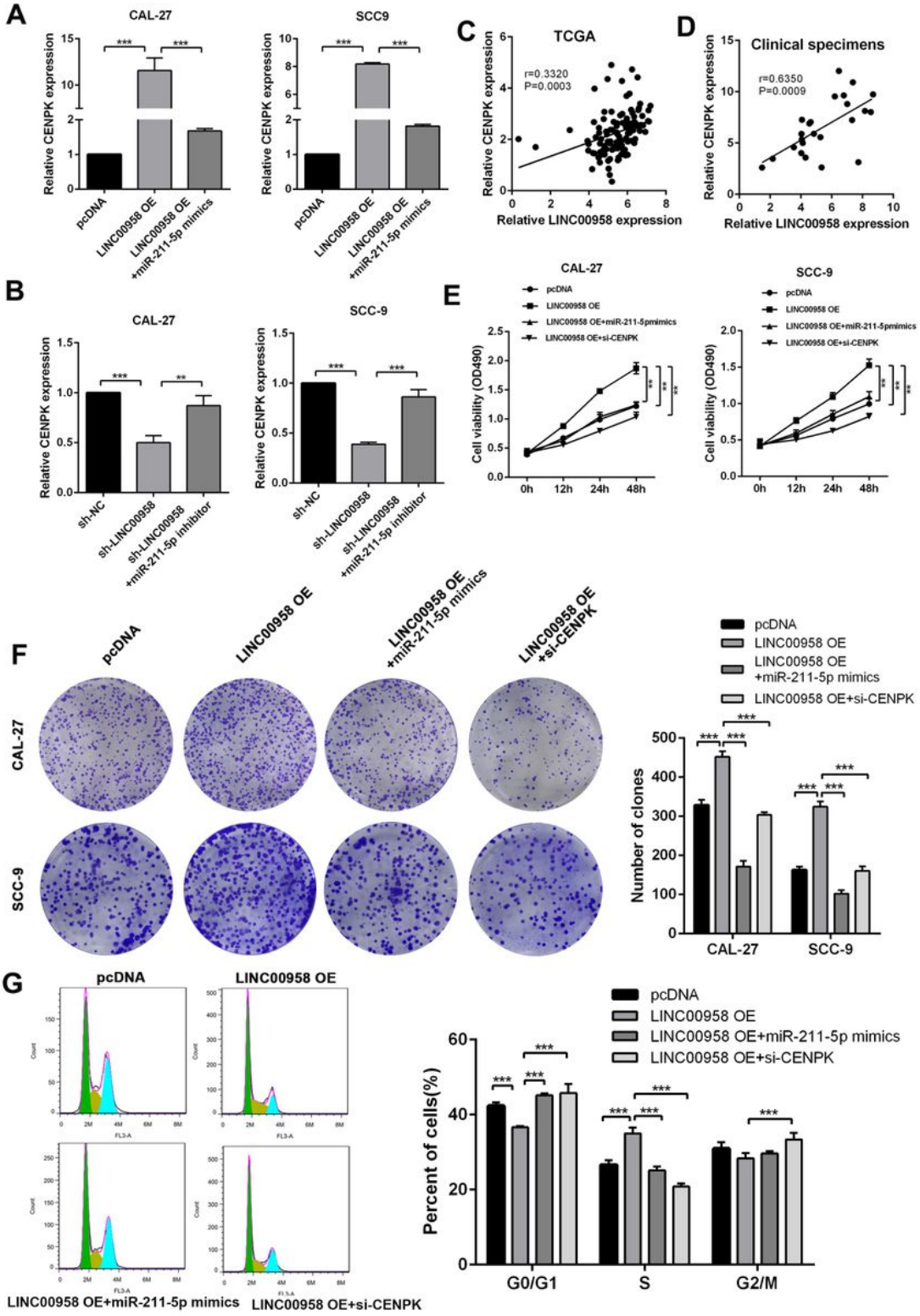
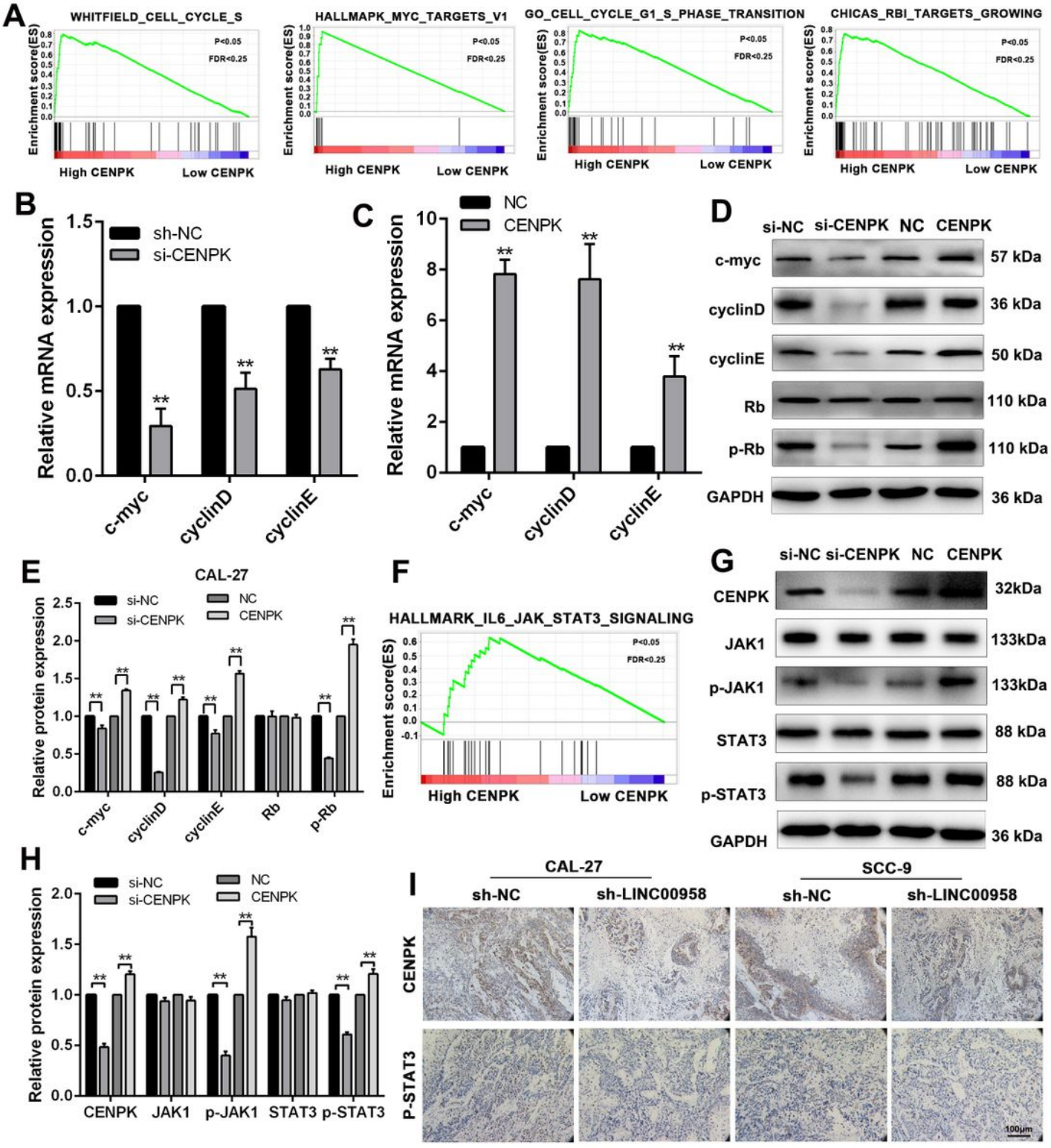



Figure 7

LINC00958 promotes CENPK mediated proliferation through miR-211-5p sponging in vitro. The expression of CENPK was detected in CAL27 and SCC-9 cells transfected with pcDNA-LINC00958 or pcDNA simultaneously with miR-211-5p mimics by RT-qPCR. (B) The expression of CENPK was detected in CAL27 and SCC-9 cells transfected with sh-LINC00958 or sh-NC simultaneously with miR-211-5p inhibitor by RT-qPCR. (C) Correlation between CENPK and LINC00958 expression based on the TCGA database. (D) Correlation between CENPK and LINC00958 expression of TSCC clinical samples. (E) CAL27 and SCC9 cells were transfected with pcDNA-LINC00958 or pcDNA simultaneously with miR-211-5p mimics or si-CENPK and the cell viability were examined by CCK8 assay. (F) CAL27 and SCC9 cells were transfected with pcDNA-LINC00958 or pcDNA simultaneously with miR-211-5p mimics or si-CENPK and the cell viability were examined by Clones formation assay. (G) CAL27 and SCC9 cells were transfected with pcDNA-LINC00958 or pcDNA simultaneously with miR-211-5p mimics or si-CENPK and the cell cycle were examined by Flow cytometric analysis. *P<0.05, **P<0.01, ***P<0.001.




## Figure 8

LINC00958/ mir-211-5p/ CENPK axis promotes TSCC proliferation via regulating cell-cycle process and activating the JAK/ STAT3 signaling pathway. (A) GSEA revealed that cell cycle process was related to CENPK expression in TSCC. (B) The expression of mRNAs were detected in CAL-27 cells transfected with si-CENPK by RT-qPCR. (C) The expression of mRNAs were detected in CAL-27 cells transfected with CENPK overexpression by RT-qPCR. (D-E) The expression of proteins were detected in CAL-27 cells

transfected with CENPK overexpression plasmid or si-CENPK by western blotting. (F) GSEA revealed that CENPK is related to the JAK/STAT3 signaling pathway. (G-H) The expression of proteins were detected in CAL-27 cells transfected with CENPK or si-CENPK by western blotting. (I) The expression of CENPK and p-STAT3 in the xenografts was examined by IHC. *P<0.05, **P<0.01, ***P<0.001.

## Supplementary Files

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