

## Original Article

# UBE2S targets RPL26 for ubiquitination and degradation to promote non-small cell lung cancer progression via regulating c-Myc

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**Abstract:** Multiple studies have shown that E2 conjugating enzyme family are dysregulated in various cancers and associated with tumor progression and poor prognosis. In present study, we screened and confirmed that UBE2S is one of the E2 conjugating enzymes highly expressed in non-small cell lung cancer (NSCLC), and it plays an oncogenic role by enhancing cell proliferation, migration and stemness in vitro. Using immunoprecipitation technology combined with mass spectrometry assay, we identified ribosomal protein RPL26 as the substrate protein of UBE2S in NSCLC. At the molecular level, overexpression of UBE2S accelerated the ubiquitination and degradation of RPL26, thus upregulating c-Myc to enhance the progression of NSCLC. In addition, the results of a xenograft experiment showed that inhibiting UBE2S could suppress RPL26-c-Myc mediated NSCLC tumor growth in vivo. Our data provided mechanistic evidence supporting the existence of a novel UBE2S-RPL26-c-Myc axis and its critical contribution to progression of NSCLC.

**Keywords:** Non-small cell lung cancer, ubiquitination, UBE2S, RPL26, c-Myc

## Introduction

Ubiquitination, an important post-translational modification of proteins, plays a key role in the regulation of protein homeostasis and cell function. The ubiquitination process usually involves the cooperation of three enzymes including E1 ubiquitin-activating enzymes (E1s), E2 ubiquitin-conjugating enzymes (E2s) and E3 ubiquitin ligases (E3s) [1]. Among them, E2s are considered as “ubiquitin carriers”, which regulate the formation of chains and establish the topology of chains, thereby determining the ubiquitylation of the modified specific substrate proteins and affecting the localization, activity and/or stability of proteins. Under certain circumstances, some E2s can also interact with target substrate proteins and directly ubiquitinate substrates without the cooperation of E3s [2-4]. All E2s contain a conserved ubiquitin-conjugating (UBC) domain of 150-200 amino acids that binds Ub/Ubl proteins activated by ATP. It

can be divided into four groups: those containing only UBC domain are class I E2s. In addition to the UBC domain, class II E2s contain an additional N-terminal domain, class III E2s contain an additional C-terminal domain, and class IV E2 contains N-terminal and C-terminal domains. These additional domains not only create E2s of diverse molecular size, but also control intracellular localization, confer regulatory properties, and enable specific interactions with particular E3s [2, 4-7].

In recent years, there has been a growing understanding of E2s in a variety of cellular processes including the cell cycle, genome stability, DNA damage repair, apoptosis and immune responses, etc. Multiple studies have shown that E2s are dysregulated in various cancers, and overexpression of E2s is linked to tumor-promoting processes and associated with tumor progression and poor prognosis [8]. For instance, in hepatocellular carcinoma (HCC) cells, UBE2L3 degrades GSK3 $\beta$  via ubiquitin-

proteasome pathway, thus making p65 unable to be phosphorylated and inhibiting the expression of target genes including PUMA, Bax, Bim, Bad and Bid, consequently promotes the proliferation and anti-apoptosis of cancer cells [9]. In glioma, UBE2D3 interacted with SHP-2 and promoted its ubiquitination, which activated STAT3 pathway and promoted glioma proliferation as well as glycolysis [10]. In lung cancer, UBE2O facilitates tumorigenesis and radio-resistance by promoting Mxi1 ubiquitination and degradation [11]. In prostate cancer and breast cancer, UBE2O specifically targets AMPK $\alpha$ 2 for ubiquitination and degradation, and thereby promotes activation of the mTOR-HIF1 $\alpha$  pathway and tumor metabolic reprogramming [12, 13]. Also, some small molecule inhibitors developed for E2s have been shown good application prospects in tumor therapy. Ceccarelli DF et al. identified a small molecule termed CC0651 that selectively destroys the activity of E2 enzyme CDC34 and effectively inhibits cell proliferation [14]. Leucettamol A, a peptide compound isolated from a marine sponge by Tsukamoto S et al., can destroy the interaction between UBC13 and UEV1A, thus upregulating p53 protein activity and playing an anti-cancer role [15]. The above work suggests that E2s may be a powerful candidate target for tumor diagnosis and therapy.

Lung cancer is the most commonly diagnosed cancer and the leading cause of cancer death worldwide [16]. Non-small cell lung cancer (NSCLC) is accounting for about 85% of the total number of lung cancer cases [17]. A recent American study reported that 60% of lung cancers are diagnosed in an advanced stage, and the 5-year survival of patients with stage IV NSCLC was 6%. The addition of adjuvant or neoadjuvant chemotherapy only improves 5 years survival by 5%~10% [18]. With the development of molecular targeted drugs and immunotherapy, the choice of therapeutic strategies for NSCLC and the survival rate of advanced patients have been greatly improved. Despite all this, some major concerns remain challenging, such as the resistance to targeted therapy drugs, optimal combinations of the current treatment regimens [18, 19]. Therefore, the identification for new potential therapeutic targets is still one of the clinical medical needs of NSCLC.

The importance of E2s in cancers has been particularly emphasized [8, 20]. Here, we con-

firmed that UBE2S is one of the E2 enzymes highly expressed in NSCLC, and it plays an oncogenic role by enhancing cell proliferation, migration and stemness. Using immunoprecipitation (IP) technology combined with mass spectrometry assay, we identified ribosomal protein RPL26 as the substrate protein of UBE2S, and elucidated the mechanism of UBE2S ubiquitinated RPL26 through ubiquitin-proteasome pathway, consequently upregulated c-Myc protein to promote the progress of NSCLC in vitro and in vivo.

### Materials and methods

#### *Cell culture, transfection, cycloheximide (CHX), chloroquine (CHQ) and MG132 treatment*

Lung cancer cell lines including A549, H1299, H520, H460 and normal lung epithelial cell line BEAS-2B were presented by Cancer Research Institute, Central South University. These cells were cultured with DMEM (Gibco) containing 10% fetal bovine serum (Gibco). Penicillin and streptomycin (100  $\mu$ g/mL) were added to the medium. The culture condition was 37°C, 5% CO<sub>2</sub>. The overexpressing plasmid pcDNA3.1-3 $\times$ HA-UBE2S and pEGFP-RPL26 were purchased from Miaoling Biological Company. The RNA interference sequences for RPL26 (si-RPL26: CACAUUCGAAGGAAGAUUAdTdT), UBE2S (si-UBE2S-1 sense: 5'-GACACGUCACGUCGACCAUTT-3', antisense: 5'-AUGGUCAGCAGUACGUGUCTT-3'; si-UBE2S-2 sense: 5'-UCAUCCGCUGGUGUACAATT-3', antisense: 5'-UUGUACACAGGCGGAUGATT-3') and negative control (NC) siRNA (sense: 5'-UUCUCCGAACGUGUCACGUTT-3', antisense: 5'-ACGUGACACGUUCGGGAAATT-3') were purchased from Guangzhou RiboBio Company. According to the instructions of TurboFect transfection Reagent (Thermo), the overexpression plasmid or siRNA were transfected into cells for 24 h, 48 h or 72 h for subsequent experiments, respectively. The A549 cell was infected with lentiviruses expressing UBE2S shRNA (sh-UBE2S-1, Shanghai Genechem Co., Ltd.) and selected using puromycin to establish the stable cell line knocking down of UBE2S. Protein degradation assay was performed by treating cells with CHQ (MedChem-Express, HY-17589A, 25  $\mu$ M), CHX (MedChem-Express, HY-12320, 75  $\mu$ M) or MG132 (MedChemExpress, HY-13259, 20  $\mu$ M) at indicated time points, then cells were lysed and followed by Western blotting analysis.

## UBE2S-RPL26-c-Myc promotes NSCLC progression

### *Total RNA extraction and RT-qPCR*

The total RNA of the cells was extracted by RNAiso kit (Takara, Japan), and mRNA was reverse transcribed by PrimeScript RT kit with gDNA Eraser (Takara Bio, Inc.). Then cDNA was subjected to qPCR using SYBR-Green PCR Master Mix (Tiangen Biotech, Co., Ltd., Beijing, China) and an MX3000 instrument (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA). Following initial denaturation for 10 min at 95°C, 40 cycles of PCR were performed. Each thermal cycle includes 95°C for 10 s, 58°C renaturation for 30 s, 72°C for 30 s. The forward (F) and reverse (R) primer sequences are as follows: UBE2S, F: 5'-AGAACCTACCCCG-CACATC-3', R: 5'-TCCTCGTTGGGAAAGACCTTGA-3'; UBE2C, F: 5'-GCCCGTAAAGGAGCTGAG-3', R: 5'-GGGAAGGCAGAAATCCCT-3'; UBE2T, F: 5'-CT-TGGAGACCATCCCTCAACATC-3', R: 5'-TCAGCCT-TTTGTTTCTGTCTTGC-3'; RPL26, F: 5'-AGCGGG-TGTAATCAGCAGCC-3', R: 5'-TCGAGTTCCCAAAT-CCCCGG-3';  $\beta$ -Actin, F: 5'-CCAACCGCGAGAAG-ATGA-3', R: 5'-CCAGAGGCGTACAGGGATA-3'.

### *Western blotting*

The protein was extracted with ice-cold RIPA (Beyotime, China) supplemented with protease inhibitors (Selleck.cn) and PMSF (Biotool Biotech), and the protein concentration was measured with the BCA protein detection kit (Beyotime). The 50  $\mu$ g protein was electrophoresed by SDS-PAGE, then transferred to a PVDF membrane and blocked with 5% skimmed milk in TBST. The membrane was incubated with the specific primary antibody at 4°C overnight, then following with HRP-coupled secondary antibodies (Beyotime) of the same species at room temperature for 1~2 hours. Finally, the protein bands are detected by a chemiluminescent substrate (Beyotime) and an imaging system (Tanon, China). The following are the information of primary antibodies: UBE2S (Proteintech, 14115-1-AP, 1:2000); RPL7A (Proteintech, 15340-1-AP, 1:1000); RPL30 (Sangon, D15-2710, 1:1000); RPL14 (Proteintech, 14991-1-AP, 1:1000); RPL23 (Proteintech, 16086-1-AP, 1:1000); RPL26 (ABclonal, A16680, 1:1000); ALDH1A1 (CUSABIO, P00352, 1:1000); Nanog (Wanleibio, WL03273, 1:1000); c-Myc (Proteintech, 10828-1-AP, 1:1000); P73 (Wanleibio, WL01604, 1:500); P53 (Wanleibio, WL01919; 1:500);  $\beta$ -Actin (Bioworld, AP0714, 1:5000).

### *Cell counting kit-8 (CCK8) assay*

Cells were seeded into 96-well plates at an initial density of  $3 \times 10^3$  cells per well. After different treatments, cells were treated with CCK8 (Shanghai Taosu Biochemical Technology Co., Ltd., C0005) in each well at a ratio of 1:10 (CCK8: complete medium), then incubated at 37°C for 2 h, and the absorbance was measured at the wavelength of 450 nm using a microplate reader (Thermo, USA).

### *Wound healing assay*

The cells in logarithmic growth phase were inoculated into 6-well plates, and three parallel wells were set in each group. Transfection was performed when the cells grew to 80%. After 24 h of transfection, the cells were scraped with a sterile 200  $\mu$ L pipette tip and the cell debris was washed with D-hanks solution, and then cultured in serum-free medium to synchronize the cell cycle. The images were taken under bright field by inverted fluorescence microscope (Olympus) at 0 h and 48 h after scraping, respectively.

### *Cell spheroid formation assay*

The spheroid formation assay was performed to assess the stemness of tumor cells. Cells (1000 cells/well) were seeded into 6-well plates, which were coated with 1% gel and 10% FBS DMEM in a ratio of 1:1, to generate cell spheroids. After 5~10 days, the number of cell spheroids was counted under an inverse microscope (Olympus).

### *Co-immunoprecipitation (Co-IP)*

Total protein was extracted from cells with an IP lysis buffer (Beyotime) and prepurified with 30  $\mu$ L protein A/G magnetic beads (Selleck, USA) at 4°C. Meanwhile, 50  $\mu$ L of magnetic beads were mixed with 2  $\mu$ g of anti-RPL26 antibody (ABclonal, A16680) or anti-UBE2S antibody (Proteintech, 14115-1-AP) or 2  $\mu$ g of control IgG antibody (Santa Cruz, SC-2025, USA), preincubated at 4°C for 4 hours, and then immunoprecipitated with total protein overnight at 4°C. The beads were washed three times with washing buffer (Beyotime) for 15 min each time, and resuspended in 50  $\mu$ L lysis buffer containing 1 $\times$  SDS loading buffer. The protein was denatured at 100°C for 10 min and then used for Western blot analysis.

## UBE2S-RPL26-c-Myc promotes NSCLC progression

### *Immunofluorescence analysis*

Cells were fixed with 4% paraformaldehyde (Sigma) at room temperature for 15 min. The fixative formaldehyde was sucked dry, and washed with PBS three times, 5 min each time. After blocked with buffer containing Triton™ X-100 and fetal bovine serum (FBS) for 1 h at room temperature, cells were incubated with primary antibody UBE2S (Sangon, D199081, 1:100) or RPL26 (ABclonal, A16680, 1:100) overnight at 4°C. Then cells were washed with PBS and incubated for 1 h at room temperature with a fluorescent-labeled secondary antibody (1:200, Proteintech, SA00013-2, SA00013-3). Finally, cells were washed with PBS and sealed with an anti-fluorescence quenching tablet containing DAPI (Beyotime Biotec, China), and photographed with a LSM 780 microscope (Zeiss, Oberkochen, Germany).

### *In vivo ubiquitination assay*

A549 cells were cultured and divided into four groups: si-NC transfection group, si-UBE2S-1 transfection group, pcDNA3.1 plasmid transfection group and pcDNA3.1-3×HA-UBE2S plasmid transfection group. After 48 hours of transfection, cells were treated with 20 μM MG132 for 14 hours. Then, the whole cell lysates were prepared with IP lysis mixtures (IP lysis buffer: cocktail: PMSF=100:1:1) at 4°C for 30 min. The lysates were centrifuged to obtain the endogenous protein components. A portion of the proteins was used for input analysis. The remaining proteins were placed on a hot plate immediately to boil for 10 min. The denatured proteins were used for immunoprecipitation analysis as follows: 50 μL of magnetic beads were mixed with 3 μg of anti-RPL26 antibody (ABclonal, A16680) or 2 μg of control IgG antibody (Santa Cruz, SC-2025, USA), preincubated at 4°C for 4 hours, and then immunoprecipitated with total protein overnight at 4°C. The proteins were released from the beads by boiling the beads in SDS/PAGE sample buffer, and the levels of RPL26 ubiquitination were detected by Western blotting with an anti-Ub antibody (Proteintech, 10201-2-AP, 1:1000).

### *Immunohistochemistry (IHC) staining*

IHC was performed according to standard procedures. The NSCLC tissue microarray slices (Shanghai OUTDO BIOTECH) were used to

detect the expression of UBE2S in clinical samples. The paraffin sections of xenograft were randomly selected to detect the expression of UBE2S, RPL26 and c-Myc proteins. The following are the information of primary antibodies: anti-UBE2S antibody (1:300, Proteintech, 14115-1-AP), anti-RPL26 (1:200, Proteintech, 17619-1-AP), anti-c-Myc (1:500, Proteintech, 67447-1-Ig). The results were obtained by digital slice scanner (3DHISTECH, Hungary).

### *In vivo xenograft experiment*

The animal studies were approved by the Experimental Animal Ethics Committee of College of Biology, Hunan University. A549 cells ( $5 \times 10^6$  cells per mouse) stably infected with sh-UBE2S-1 or sh-NC lentivirus were collected, washed and suspended with PBS, then subcutaneously injected into the armpit of four-week-old female nude mice (BALB/C). Five mice were in each group. Tumor formation was investigated every 2 days, the tumor volume was calculated using the formula  $V = a \times b^2/2$ , where “a” is the long axis and “b” is the short axis. After 32 days of subcutaneous injection, the tumors were removed, weighed, and embedded in paraffin-embedded tissue sections for IHC detection.

### *Statistics*

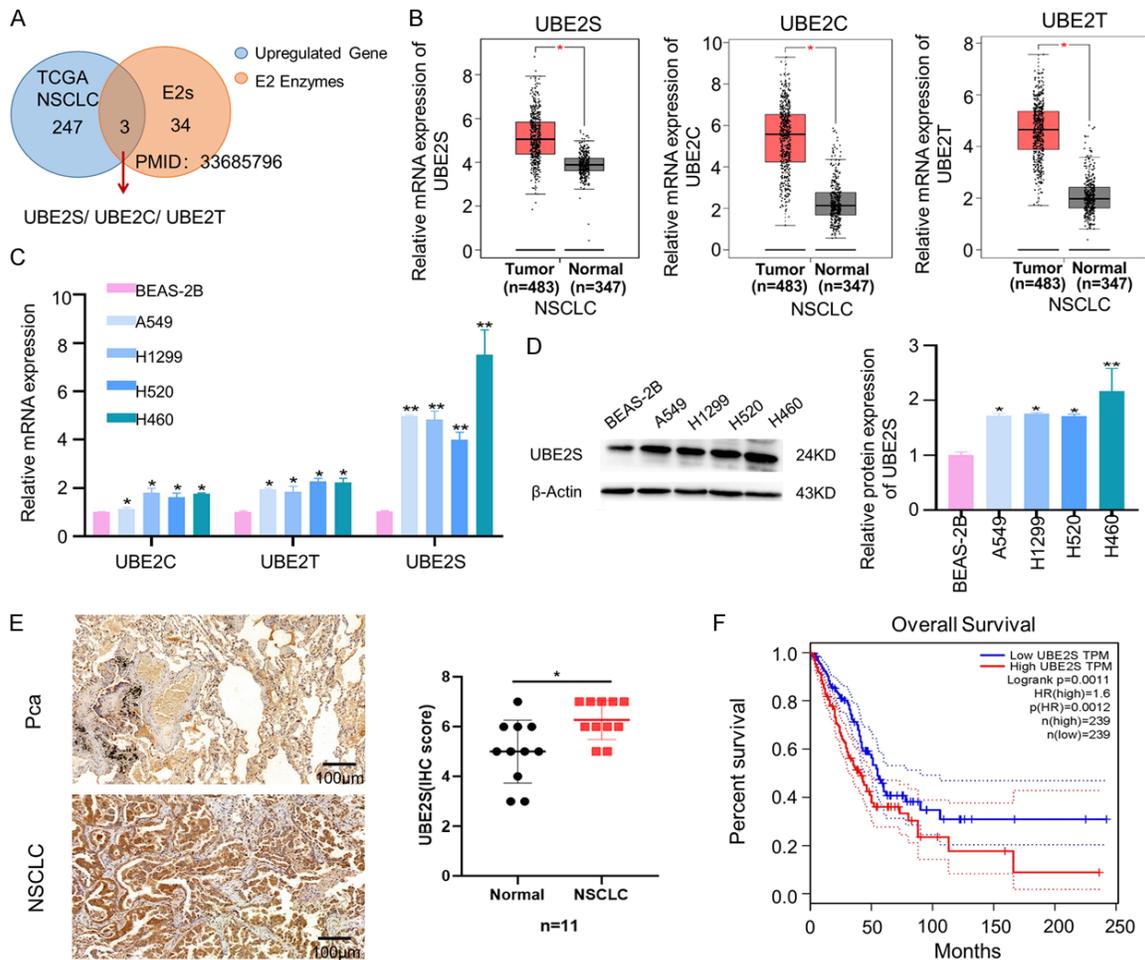
The data are expressed as mean ± standard deviation (SD). Statistical analyses were performed using Graphpad Prism 8.0 software. Student's t test was used to compare the differences between the two groups, and one-way ANOVA was used for comparisons of multiple independent groups. All assays were repeated independently three times, and representative images are shown. “ns” showed that there was no significant difference between the data. The statistical significances were defined as: \* $P < 0.05$ ; # $P < 0.05$ ; \*\* $P < 0.01$ ; ## $P < 0.01$ ; \*\*\* $P < 0.001$ .

## **Results**

### *UBE2S is upregulated and associated with poor prognosis in NSCLC*

In order to screen the abnormally high expression E2s in NSCLC, we analyzed the intersection of 250 genes significantly up-regulated in NSCLC in the Cancer Genome Atlas (TCGA)

## UBE2S-RPL26-c-Myc promotes NSCLC progression



**Figure 1.** UBE2S is upregulated and associated with poor prognosis in NSCLC. A. The intersection analysis of 250 genes up-regulated in NSCLC in the Cancer Genome Atlas (TCGA) database and 37 members of E2 enzyme family; B. The mRNA expression of UBE2S, UBE2C and UBE2T in NSCLC by GEPIA database analysis; C. The mRNA level of UBE2S, UBE2C and UBE2T in lung cancer cell lines including A549, H1299, H520, H460 and normal lung epithelial cell line BEAS-2B were analyzed by RT-qPCR; D. Endogenous expression of UBE2S in lung cancer cells and BEAS-2B cells were analyzed by Western blot; E. IHC analysis of UBE2S in clinical NSCLC and para-carcinoma (Pca) tissues, Scale: 100  $\mu$ m; F. The overall survival rate of NSCLC patients with high or low expression of UBE2S by GEPIA database analysis. \* $P < 0.05$ , \*\* $P < 0.01$ .

database and 37 members of E2 enzyme family [7]. The three overlapping E2 enzyme genes including UBE2S, UBE2C and UBE2T were selected (Figure 1A). The analysis of GEPIA database showed that the mRNA levels of UBE2S, UBE2C and UBE2T in NSCLC were higher than those in normal lung tissues of para-carcinomas (Figure 1B). The RT-qPCR results indicated that UBE2S mRNA was most significantly differentially expressed between normal lung epithelial cell line BEAS-2B and lung cancer cell lines (A549, H1299, H520, H460) (Figure 1C). Western blot analysis showed that the endogenous expression of UBE2S in lung cancer cell lines was significantly higher than that in BEAS-2B cell line as well (Figure 1D). IHC

staining confirmed that UBE2S was upregulated in clinical NSCLC samples compared to para-carcinoma tissues (Figure 1E). The data from GEPIA showed that the overall survival rate of NSCLC patients with high expression of UBE2S was low (Figure 1F), implying a strong association between the prognosis of NSCLC patients and high UBE2S expression. These results suggest that UBE2S plays an oncogenic role in NSCLC.

### *UBE2S promotes proliferation, migration and stemness of NSCLC cells*

In NSCLC A549 and H1299 cell lines, UBE2S was RNA interfered or overexpressed to explore

its biological role. Western blot results confirmed that both RNA interference and overexpression of UBE2S in NSCLC cells was successful (**Figure 2A**). CCK8 analysis showed that the proliferation of A549 and H1299 cells was inhibited after UBE2S knockdown compared with the si-NC control group, while the proliferation of cells increased after UBE2S overexpression (**Figure 2B**). The results of wound healing assay showed that RNA interference of UBE2S inhibited the migration ability of A549 and H1299 cells. On the contrary, overexpression of UBE2S promoted the cell migration (**Figure 2C**). Knockdown of UBE2S also led to decreased spheroid formation ability of cells, while overexpression of UBE2S reversed this effect (**Figure 2D**). The Western blot results showed that the expression of stemness and proliferation markers Nanog and c-Myc were inhibited after UBE2S knockdown. Conversely, the expression level of the two markers was up-regulated after UBE2S overexpression (**Figure 2E**). In addition, the rescue experiments including Western blot analysis (**Figure 3A**), CCK8 detection (**Figure 3B**), wound healing assay (**Figure 3C**) and spheroid formation analysis (**Figure 3D**) were performed, and the results verified that UBE2S overexpression can rescue the phenotype of si-UBE2S in NSCLC cells. These data demonstrate that UBE2S can promote the proliferation, migration and stemness of NSCLC cells.

*UBE2S down regulates RPL26 expression by ubiquitination in NSCLC*

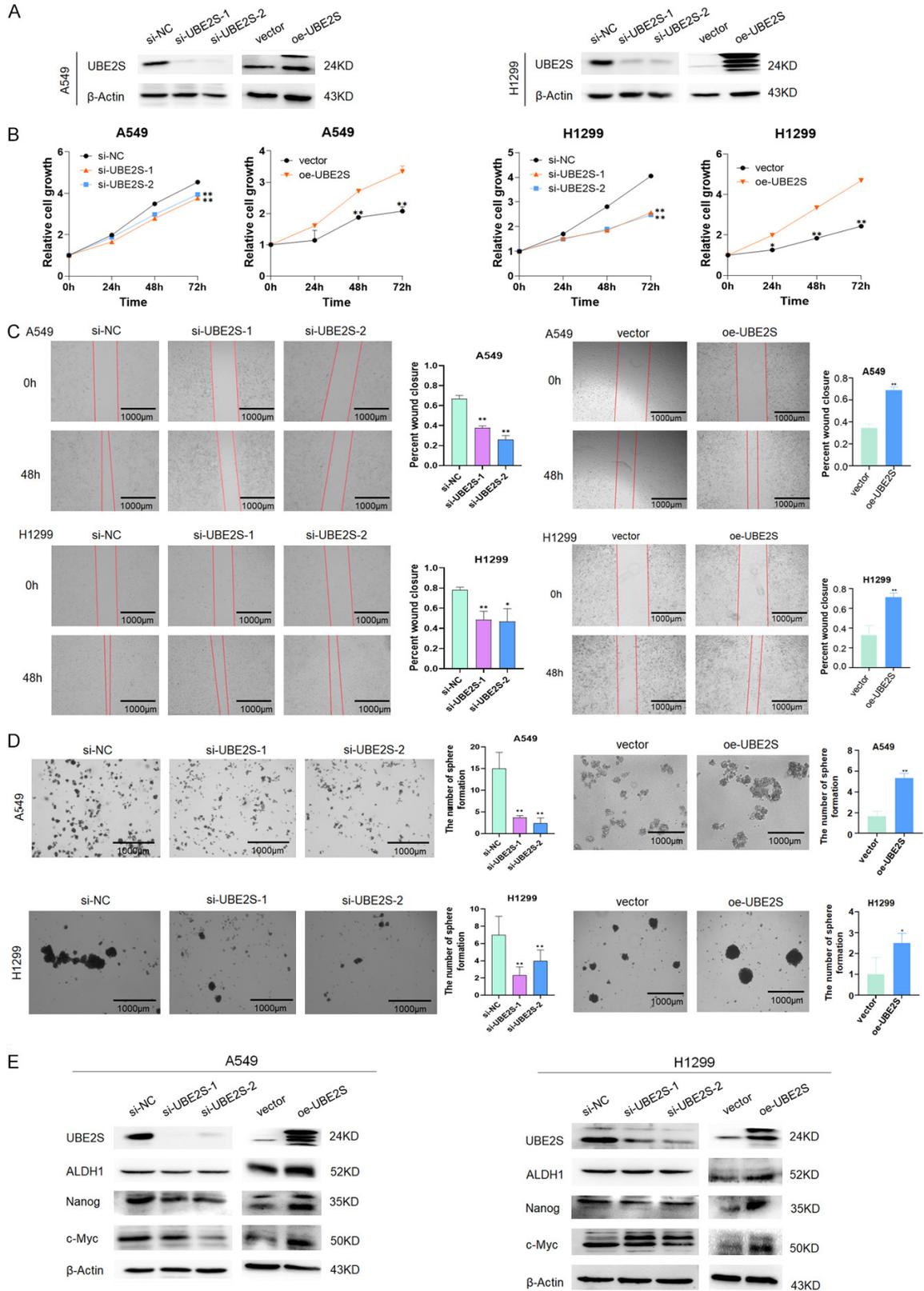
To explore the molecular mechanism of UBE2S in NSCLC, immunoprecipitation of UBE2S in A549 and H1299 cells was performed (**Figure 4A**) and 50 candidate proteins interacting with UBE2S were screened by mass spectrometry. Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) analysis showed that most of these interacting proteins belong to ribosomal proteins, and their molecular functions and biological processes are related to ribosomes biogenesis (**Figure S1**). Among all ribosomal proteins in the mass spectrometry data, the top five were RPL7A, RPL26, RPL23, RPL30 and RPL14 (**Figure 4B**). Western blot results indicated that only RPL26 was negatively regulated by UBE2S after overexpressing or RNA interference of UBE2S in both A549 and H1299 cells (**Figure 4C**). In addition, RPL26 is highly expressed in BEAS-2B cells, but low

expressed in lung cancer cell lines at mRNA level (**Figure 4D**) and protein level (**Figure 4E**), which was negatively correlated with the expression pattern of UBE2S. These data imply that RPL26 may be the substrate of UBE2S.

Subsequently, we confirmed the binding between UBE2S and RPL26 by the co-IP experiment in A549 cells (**Figure 5A**). Then immunofluorescence analysis was performed to verify the co-location of UBE2S and RPL26. The non-specific staining signals were excluded by data of siRNA (si-UBE2S-1 or si-RPL26) treatment group. It was shown that UBE2S and RPL26 were co-located to the nucleus and cytoplasm in A549 cells. After knockdown of UBE2S or RPL26 respectively, the co-location signal of both proteins was significantly weakened (**Figures 5B, S2**). At the transcriptional level, RT-qPCR analysis showed that knockdown or overexpression of UBE2S did not affect the mRNA expression of RPL26 (**Figure 5C**), implying that UBE2S may affect the expression of RPL26 through ubiquitination mechanism. After A549 cells were treated with CHX (75  $\mu$ M) for an indicated time course (2 h, 4 h, 8 h), it was found that the gradually reduction of RPL26 protein was directly regulated by UBE2S interference rather than CHX treatment (**Figure S3A**). We speculated that RPL26 protein may have a long half-life. When A549 cells were respectively treated with CHX for 12 h, 24 h, 36 h and 48 h, it was shown that the protein expression of RPL26 exist a slight decline after 12 h and 24 h of CHX treatment, and the obvious degradation was observed from time point of 36 h (**Figure S3B**). Thus, the following half-life of RPL26 protein experiment was performed at the three selected time points: 24 h, 36 h and 48 h. The Western blot results showed that knockdown of UBE2S led to prolonged half-life of RPL26 protein and the degradation rate of RPL26 protein became slower, while overexpression of UBE2S shortened the half-life of RPL26 protein and resulted in the faster degradation of RPL26 (**Figure 5D**), indicating that the stability of RPL26 protein was regulated by UBE2S.

After knockdown of UBE2S and treatment with proteasome inhibitor MG132 (20  $\mu$ M, 14 h) in A549 cells, the protein level of RPL26 showed a slight upregulation but with no statistical difference. However, after overexpression of UBE2S and treatment with proteasome inhibi-

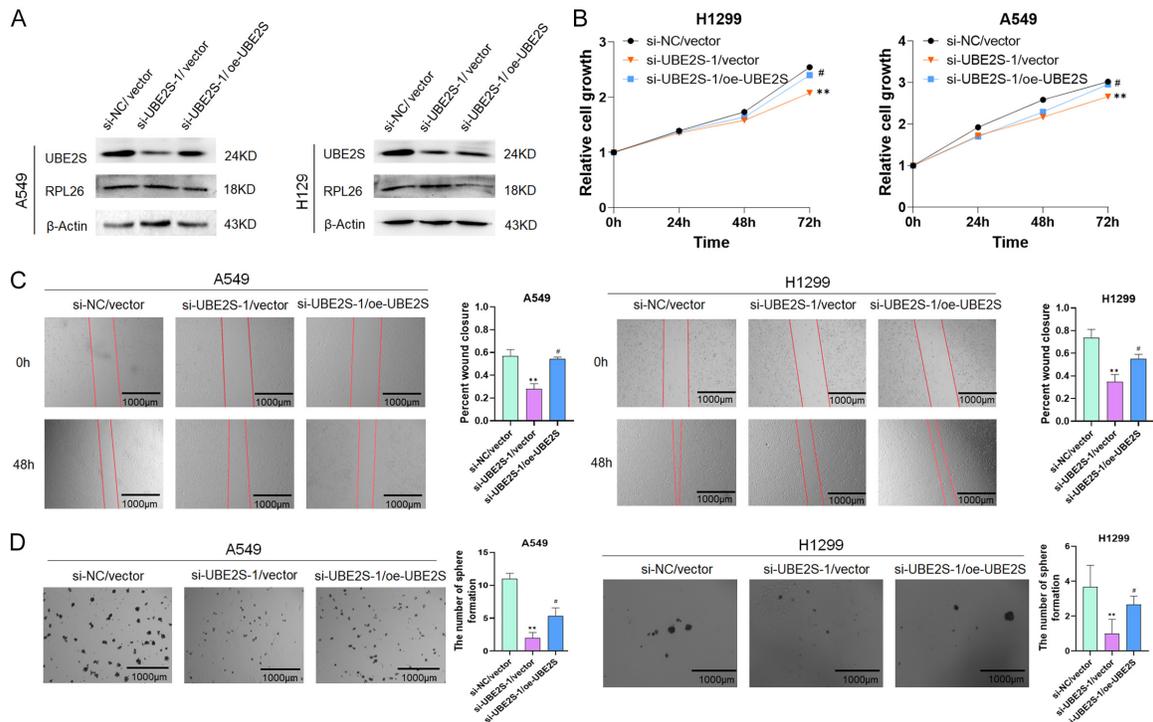
## UBE2S-RPL26-c-Myc promotes NSCLC progression



**Figure 2.** UBE2S promotes proliferation, migration and stemness of NSCLC cells. **A.** The RNA interference and overexpression efficiency of UBE2S in A549 and H1299 cells was analyzed by Western blot; **B.** The proliferation of A549 and H1299 cells was detected by CCK8 analysis after UBE2S knockdown or overexpression; **C.** The migration ability of A549 and H1299 cells was detected by wound healing assay after UBE2S knockdown or overexpression,

## UBE2S-RPL26-c-Myc promotes NSCLC progression

Scale: 1000  $\mu$ m; D. The spheroid formation ability of A549 and H1299 cells was analyzed after UBE2S knockdown or overexpression, Scale: 1000  $\mu$ m; E. The expression of ALDH1, Nanog and c-Myc in A549 and H1299 cells was analyzed by Western blot after UBE2S knockdown or overexpression. \* $P < 0.05$ , \*\* $P < 0.01$ .



**Figure 3.** UBE2S overexpression can rescue the phenotype of si-UBE2S in NSCLC cells. A. The RNA interference and overexpression efficiency of UBE2S in A549 and H1299 cells were analyzed by Western blot; B. The proliferation of A549 and H1299 cells were detected by CCK8 analysis after treatment with si-NC/vector, si-UBE2S-1/vector, si-UBE2S-1/oe-UBE2S, respectively; C. The migration ability of A549 and H1299 cells were detected by wound healing assay after treatment with si-NC/vector, si-UBE2S-1/vector, si-UBE2S-1/oe-UBE2S, respectively, Scale: 1000  $\mu$ m; D. The stemness of A549 and H1299 cells were analyzed by the spheroid formation ability after treatment with si-NC/vector, si-UBE2S-1/vector, si-UBE2S-1/oe-UBE2S, respectively, Scale: 1000  $\mu$ m. Data are shown as the mean  $\pm$  SD. \* $P < 0.05$  vs. si-NC; \*\* $P < 0.01$  vs. si-NC; # $P < 0.05$  vs. si-UBE2S-1/vector.

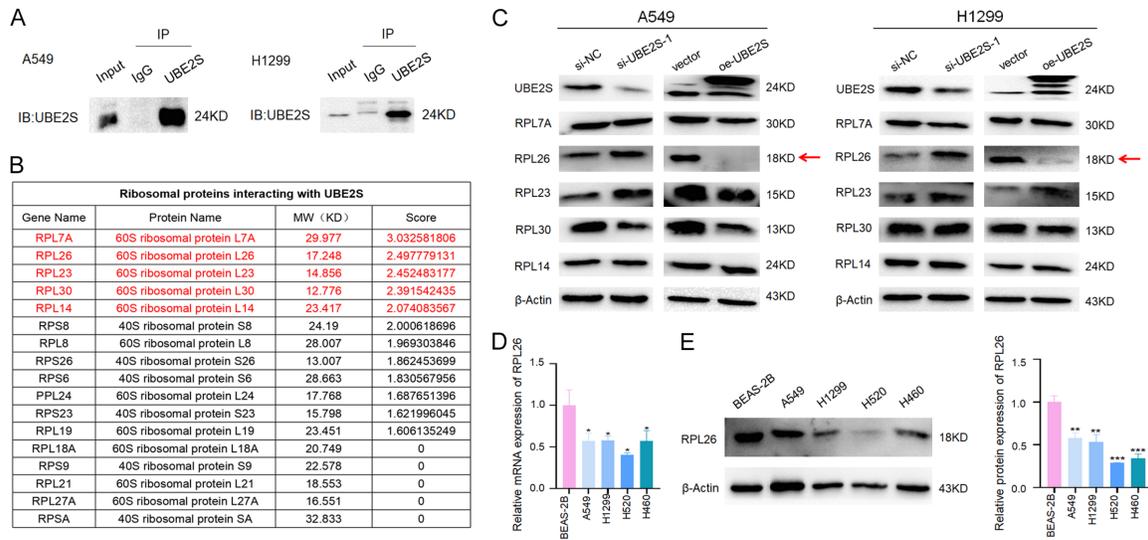
tor MG132, Western blot results showed that the downregulation of RPL26 protein was inhibited (**Figure 5E**), implying that the regulation of RPL26 by UBE2S was associated with the proteasome degradation pathway. Through the endogenous ubiquitination experiment in A549 cells, it was found that after the treatment of MG132 (20  $\mu$ M, 14 h), the ubiquitination of RPL26 was weakened by interference with UBE2S, while the RPL26 ubiquitination was enhanced by UBE2S overexpression (**Figure 5F**). We also used the lysosome inhibitor CHQ (25  $\mu$ M, 24 h) to analyze whether the RPL26 is regulated by lysosome degradation pathway. However, it was found that RPL26 was not degraded by lysosome pathway through UBE2S (**Figure S4**). Taken together, UBE2S negatively

regulates the protein level of RPL26 through ubiquitination modification.

### *UBE2S targets RPL26 for ubiquitination and degradation to promote NSCLC progression via upregulation of c-Myc*

The functional rescue experiments were performed to confirm whether the oncogenic role of UBE2S was dependent on ubiquitination degradation of RPL26. The co-transfection of pcDNA3.1 and pEGFP-C3 empty vector (vector group) was used as control group. The experimental groups were as follows: oe-UBE2S (pEGFP-C3+pcDNA3.1-3 $\times$ HA-UBE2S) transfection group; oe-RPL26 (pcDNA3.1+pEGFP-RPL26) transfection group; co-transfection of oe-UBE2S/RPL26 (pcDNA3.1-3 $\times$ HA-UBE2S+

## UBE2S-RPL26-c-Myc promotes NSCLC progression



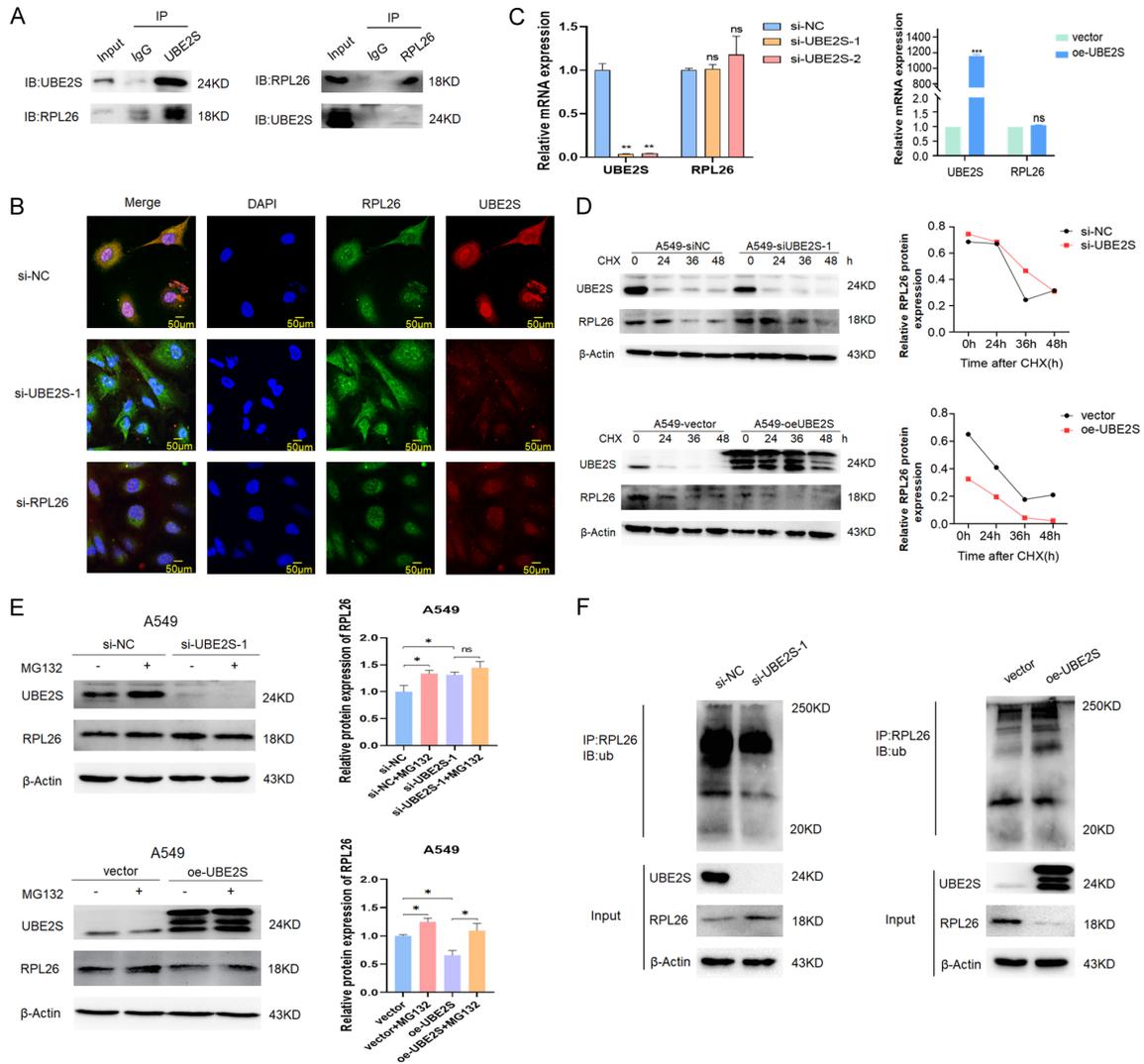
**Figure 4.** UBE2S downregulates RPL26 expression in NSCLC. **A.** The immunoprecipitation of UBE2S in A549 and H1299 cells; **B.** All ribosomal proteins in the mass spectrometry data are shown and the top five proteins were highlighted in red; **C.** The expression of selected top five ribosomal proteins in A549 and H1299 cells was analyzed by Western blot after UBE2S knockdown or overexpression; **D.** The mRNA level of RPL26 in lung cancer cell lines and BEAS-2B cell line were detected by RT-qPCR; **E.** The protein level of RPL26 in lung cancer cell lines and BEAS-2B cell line were detected by Western blot. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

pEGFP-RPL26) group. Western blot analysis verified that the transfection was successful (**Figure 6A**). The results of CCK8 assay showed that the cell proliferation ability of UBE2S overexpression group was enhanced, while that of RPL26 overexpression group was on the contrary. However, after co-expression of UBE2S and RPL26, the impact of UBE2S on proliferation was partially reversed (**Figure 6B**). Wound healing analysis showed that the effect of UBE2S on promoting migration was partially reversed after co-transfection of UBE2S and RPL26 (**Figure 6C**). Spheroid formation experiments also showed that exogenous co-overexpression of UBE2S and RPL26 significantly decreased the stemness ability of UBE2S overexpressing cells (**Figure 6D**). These results indicate that UBE2S plays an oncogenic role by degrading RPL26.

It was reported that more than a dozen of ribosomal proteins including RPL26 functions depending on MDM2-p53/p73 pathway and p53 independent (such as c-Myc) pathway in response to ribosomal stress and tumorigenesis [21-23]. Thus, here we detected the relationship of UBE2S, RPL26, p53, p73 and c-Myc proteins in A549 (p53 wild-type) and H1299 (p53 null) cells (**Figure 6E**). It was found that UBE2S downregulated p53 protein in A549

cells, which is consistent with a previous study [24]. But it had no significant impact on the expression of p73 protein in both A549 and H1299 cells. On the other hand, UBE2S obviously upregulated the expression of c-Myc in both H1299 and A549 cells, which suggested that the c-Myc dependent pathway may contribute to the oncogenic role of UBE2S in NSCLC. Subsequently, to examine whether RPL26 mediates the regulation of UBE2S on c-Myc protein, UBE2S and RPL26 plasmids were co-overexpressed in A549 and H1299 cells, and the expression of c-Myc was analyzed by Western blot. It was shown that overexpression of RPL26 alone inhibited the expression of c-Myc, which was contrary to the impact of overexpression of UBE2S alone on c-Myc. However, after co-transfection of UBE2S and RPL26 plasmids in A549 and H1299 cells, the upregulation of UBE2S on c-Myc protein was partially reversed by RPL26 (**Figure 6F**). In addition, in order to further verify whether the function of UBE2S rely on the degradation of RPL26, the double interference of UBE2S and RPL26 were performed to knockdown the two proteins. It was shown that the expression of c-Myc was downregulated after knocking down UBE2S alone, but the downregulation of c-Myc was partially reversed after double interference of

## UBE2S-RPL26-c-Myc promotes NSCLC progression



**Figure 5.** UBE2S downregulates RPL26 expression by ubiquitination-proteasome pathway in NSCLC. **A.** The interaction between UBE2S and RPL26 was detected by co-IP experiment in A549 cells; **B.** Immunofluorescence assay was used to detect the co-localization of UBE2S and RPL26 in A549 cells, Scale: 50 µm; **C.** The mRNA level of RPL26 was detected by RT-qPCR after RNA interference or overexpression of UBE2S in A549 cells; **D.** The A549 cells with 24 h of UBE2S knockdown or overexpression, were treated with 75 µM CHX for 24 h, 36 h, 48 h, respectively. And the protein level of RPL26 was detected by Western blot; **E.** The A549 cells with 48 h of UBE2S knockdown or overexpression, were treated with 20 µM MG132 for 14 h, and the protein level of RPL26 was detected by Western blot; **F.** The A549 cells with 48 h of UBE2S knockdown or overexpression, were treated with 20 µM MG132 for 14 h. The ubiquitination modification of RPL26 was detected by endogenous ubiquitination IP experiment. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; ns, no significant changes.

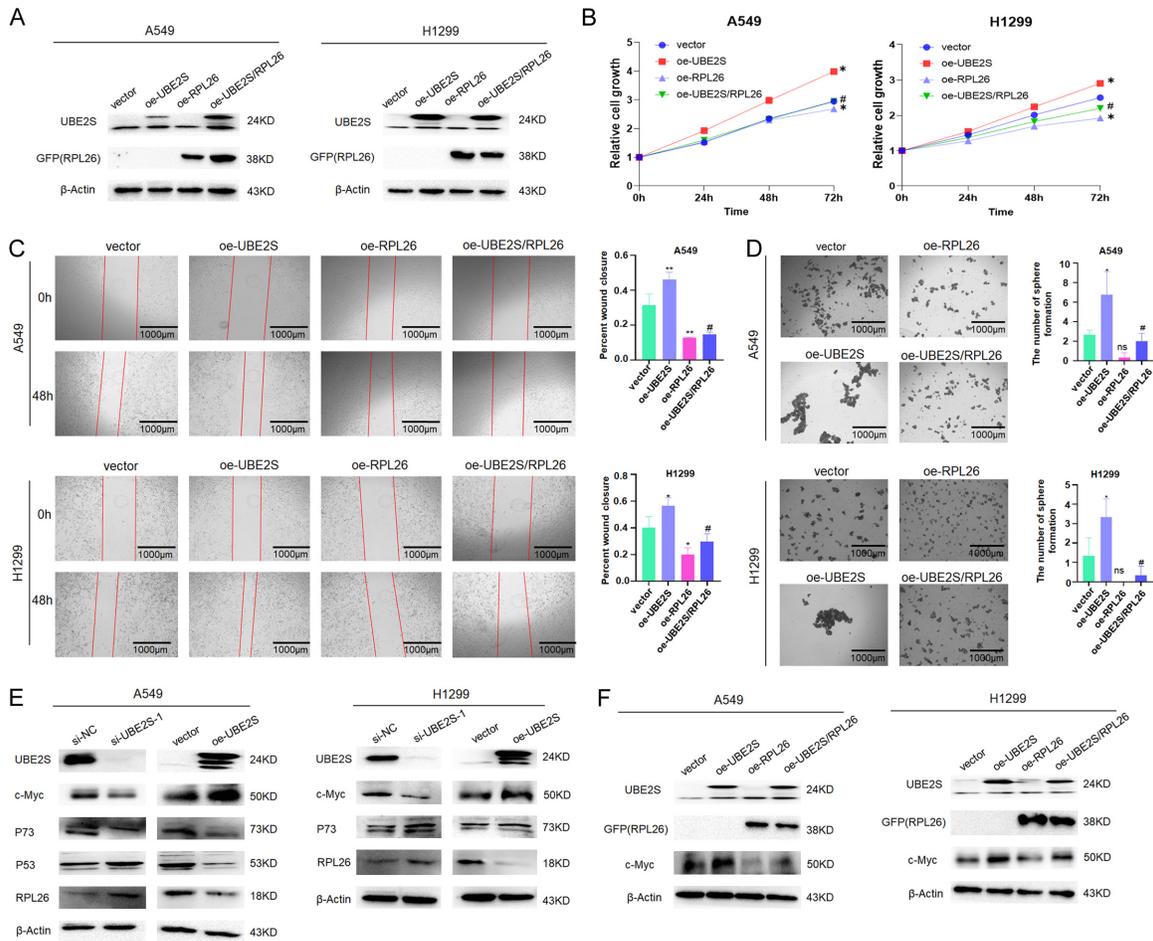
UBE2S and RPL26 in A549 and H1299 cells (Figure 7A). The results of CCK8 detection (Figure 7B), wound healing assay (Figure 7C) and sphere formation analysis (Figure 7D) indicated that the proliferation, migration and stemness of tumor cells were weakened after interfering with UBE2S alone, but double interference of UBE2S and RPL26 in NSCLC cells can partially reverse above phenomena

induced by si-UBE2S treatment alone. These data implied that UBE2S promotes tumor progression through ubiquitination degradation of RPL26 and upregulation of c-Myc pathway.

### *UBE2S increases tumor growth in NSCLC xenograft in vivo*

In order to verify the relationship of UBE2S, RPL26 and c-Myc in vivo, the A549 cell line

## UBE2S-RPL26-c-Myc promotes NSCLC progression



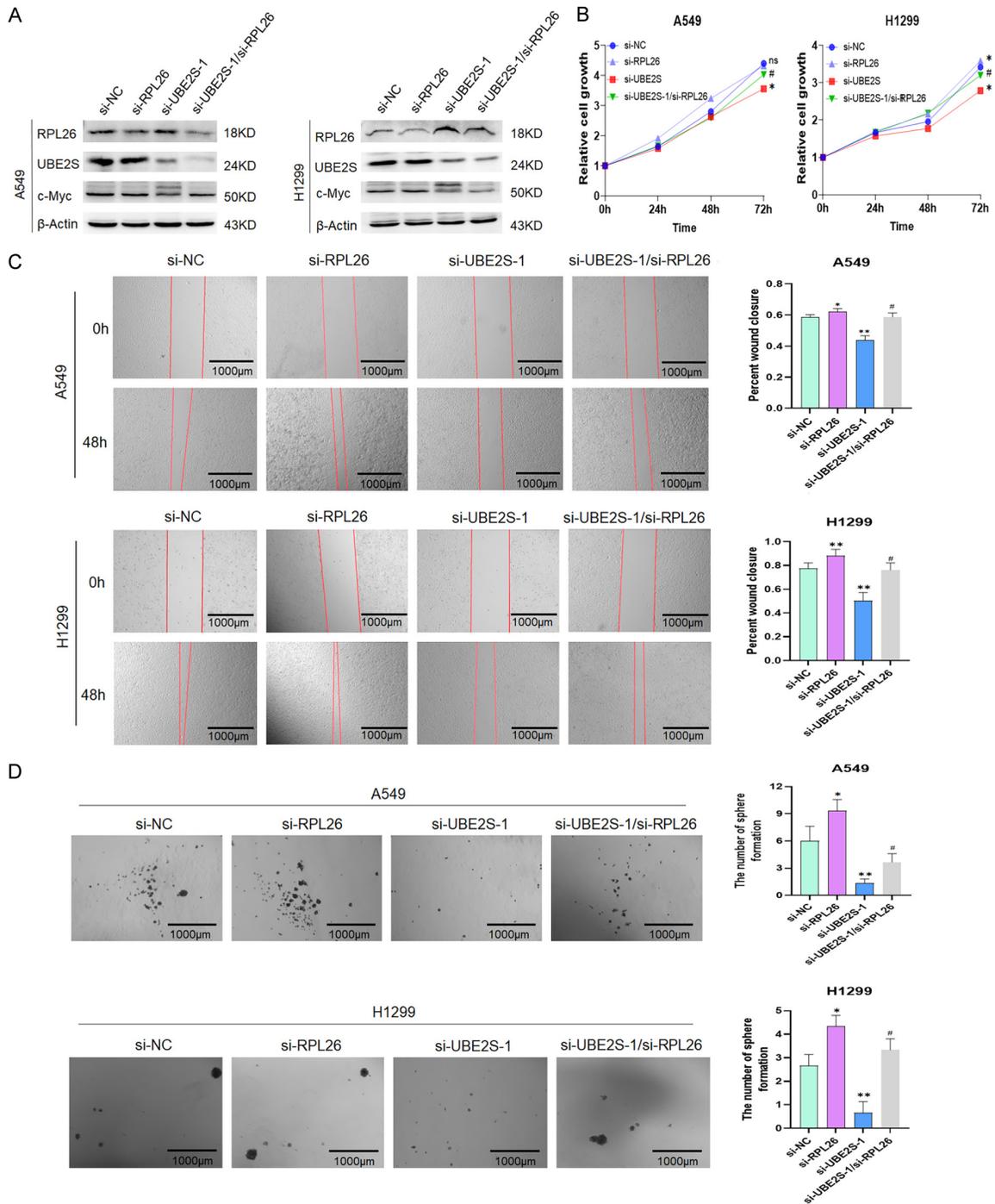
**Figure 6.** UBE2S-RPL26-c-Myc axis promotes the proliferation, migration and stemness of NSCLC. A. Western blot was used to detect the overexpression efficiency of UBE2S and RPL26 in A549 and H1299 cell lines; B. CCK8 assay was used to detect the proliferation of A549 and H1299 cells with overexpression of RPL26 alone or co-overexpression of RPL26/UBE2S; C. Wound healing assay was used to detect the migration of A549 and H1299 cells with overexpression of RPL26 alone or co-overexpression of RPL26/UBE2S, Scale: 1000  $\mu$ m; D. The spheroid formation experiment was performed to detect the stemness of A549 and H1299 cells with overexpression of RPL26 alone or co-overexpression of RPL26/UBE2S, Scale: 1000  $\mu$ m; E. After knockdown or overexpression of UBE2S for 72h, Western blot was used to detect the RPL26, p53, p73 and c-Myc protein levels; F. After RPL26 overexpression alone or co-transfection of RPL26/UBE2S for 72 h, Western blot was used to detect the c-Myc protein levels. \* $P < 0.05$  vs. vector; \*\* $P < 0.01$  vs. vector, # $P < 0.05$  vs. oe-UBE2S; ns, no significant changes.

stably knocking down UBE2S was established by infection with lentivirus sh-UBE2S-1 (Figure S5). Compared with sh-NC control group, the growth rate of xenograft tumors derived from sh-UBE2S-1 A549 cell group significantly decreased (Figure 8A-C). The IHC staining showed that knock down of UBE2S can suppress the c-Myc expression in mice after feeding of 32 days, whereas the expression of RPL26 were promoted (Figure 8D), suggesting that A549 cells with high UBE2S may have the capacity to proliferate in tumor xenografts.

## Discussion

Like most E2 enzymes, UBE2S recruits ubiquitin molecules and transfers them to substrate proteins through E3 ligases including APC/C, SAG, TRIM28 and RNF8 [25-27]. It's well known that in the regulation of cell cycle, UBE2S facilitates the exit of cells from the mitotic stage through the degradation of substrate proteins by APC/C during mitosis via the proteasome pathway [28, 29]. Studies have found that UBE2S exerts oncogenic activities in a variety of tumors [8], and several UBE2S substrates

## UBE2S-RPL26-c-Myc promotes NSCLC progression

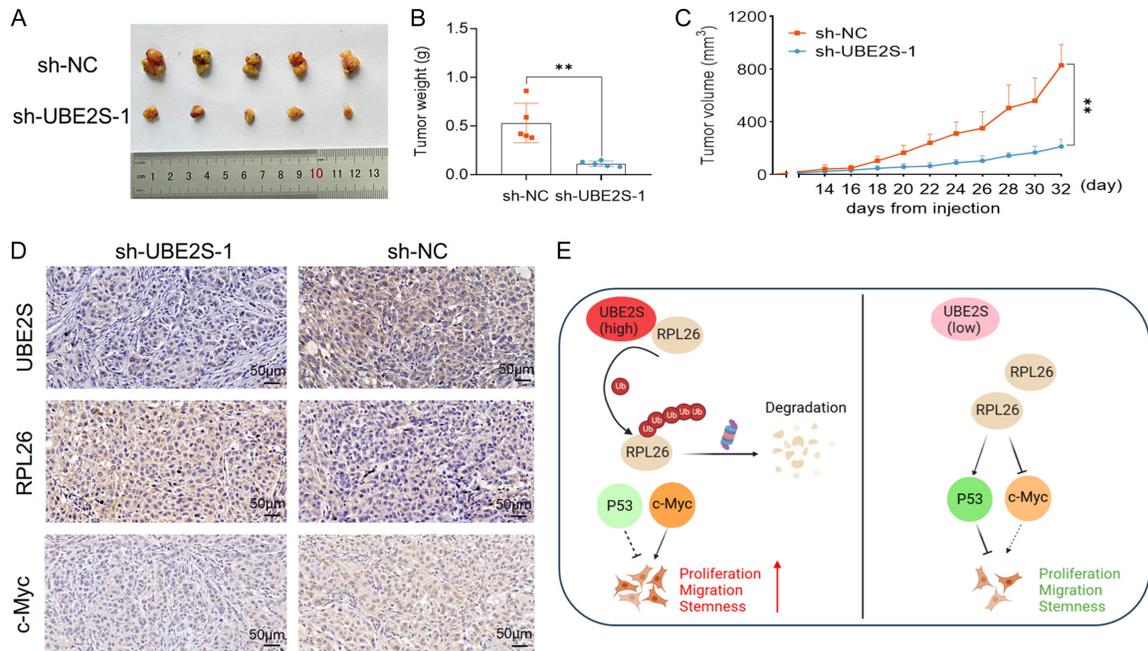


**Figure 7.** Knockdown of RPL26 reversed the proliferation, migration and stemness of NSCLC induced by UBE2S depletion. A. Western blot was used to detect the RNA interference efficiency of UBE2S and RPL26, and the c-Myc protein levels in A549 and H1299 cells; B. CCK8 assay was used to detect the proliferation of A549 and H1299 cells with knockdown of RPL26 or UBE2S alone, or co-knockdown of RPL26/UBE2S; C. Wound healing assay was used to detect the migration of A549 and H1299 cells with knockdown of RPL26 or UBE2S alone, or co-knockdown of RPL26/UBE2S. Scale: 1000  $\mu$ m; D. The spheroid formation ability was detected to analyze the stemness of A549 and H1299 cells with knockdown of RPL26 or UBE2S alone, or co-knockdown of RPL26/UBE2S. Scale: 1000  $\mu$ m. \* $P < 0.05$  vs. si-NC; \*\* $P < 0.01$  vs. si-NC; # $P < 0.05$  vs. si-UBE2S; ns, no significant changes.

such as P27 [25],  $\beta$ -TrCP1 [27], I $\kappa$ B $\alpha$  [30], VHL [31], TSC1 [32], PTEN [33],  $\beta$ -catenin [34] and

P53 [35] have been identified. To date, there are three teams have reported UBE2S's role in

## UBE2S-RPL26-c-Myc promotes NSCLC progression



**Figure 8.** UBE2S increases tumor growth in NSCLC xenograft in vivo. A. The photograph of NSCLC xenografts at the experimental end point; B. Tumor weights were measured at the experimental end point; C. Tumor volume was measured every 2 days after injection of tumor cells; D. Immunohistochemistry staining of UBE2S, RPL26 and c-Myc protein in NSCLC xenografts, Scale: 50  $\mu$ m; E. A working model of UBE2S in NSCLC (Created with BioRender.com). \*\* $P < 0.01$ .

NSCLC. Liu Z et al. observed that UBE2S can promote the proliferation and migration of A549 and H1299 cells, and speculated that p53 signaling pathway might be critical in the global molecular network of UBE2S [24]. Qin Y et al. found that upregulated UBE2S expression in NSCLC tissues significantly correlated with clinical progression (TNM III versus I+II), lymph node metastasis, and shorter survival time of the patients. Overexpressed UBE2S led to increased proliferation and migration of A549 cells, and upregulated expression of Wnt/ $\beta$ -catenin pathway, but the specific mechanism is not clear [36]. Using PC9 and H460 cells in combination with living zebrafish metastasis experiments, Ho JY et al. verified that UBE2S facilitated cancer cell migration by binding I $\kappa$ B $\alpha$  to activate NF- $\kappa$ B and epithelial-mesenchymal transition (EMT) signaling pathways [30]. Although these works have demonstrated the fundamental role of UBE2S in NSCLC, a more in-depth and accurate study on the molecular mechanism is necessary. In present study, we confirmed that high expression of UBE2S leads to the proliferation, migration as well as stemness of NSCLC cells in vitro and in vivo. Ribosomal protein RPL26 was identified

as the new substrate of UBE2S in NSCLC. At the molecular level, UBE2S degraded RPL26 protein through ubiquitin proteasome pathway. Further functional rescue experiments showed that the pro-oncogenic role of UBE2S on NSCLC could be achieved by ubiquitination degradation of RPL26, which caused upregulation of c-Myc pathway.

Compared with normal cells, highly proliferating cancer cells demand a huge amount of proteins and thus acquire increased protein synthesis. Ribosomal proteins (RPs) are known for playing an essential role in ribosome assembly, protein synthesis and translation. Over the past decades, the ribosome-independent functions of RPs have also been greatly reported in response to ribosomal stress and in maintaining normal cell homeostasis. Consistent with this notion is that numerous RPs including RPL5 [37], RPS13 [38], RPS6 [39], RPL11 [40] and RPS14 [41] etc., which possess oncogenic activity, have been found to be up-regulated at either mRNA or protein level in various human tumors. By contrast to the oncogenic functions of some RPs as briefed above, other ribosome-free RPs have been shown to play a role in sup-

pressing tumorigenesis by either activating tumor suppressors or inactivating oncoproteins [21]. So far, studies have shown that ribosomal proteins are involved in ribosomal stress and tumor progression mainly by two mechanisms. One is that ribosomal proteins participate in the regulation of MDM2-p53 or MDM2-p73 cascade as mediators, which can stabilize tumor suppressor gene p53/p73 and induce cell cycle arrest by binding to MDM2 and inhibiting MDM2 mediated p53/p73 ubiquitination and proteasome degradation [21]. On the other hand, RPs also directly inhibit oncogene c-Myc and regulate cell growth and proliferation in a p53 independent manner [22, 23]. In addition, RPs can be regulated by c-Myc or p53 during ribosomal biogenesis and protein synthesis as well.

RPL26 is considered as one of the most enchanting RPs because it not only interacts with MDM2, but also associates with p53 mRNA and enhances its translation [42]. Besides, p73, a p53 family tumor suppressor, is also controlled by RPL26 via protein stability and mRNA translation [43]. In pancreatic cancer, RPL26 was found to promote cell proliferation. However, in colorectal cancer, laryngeal squamous cell carcinoma and NSCLC, RPL26 could inhibit the proliferation of cancer cells by up regulating the expression of p53 and p73 [43-45]. This suggests that the function of RPL26 is in a context-dependent manner. Consistent with the previous study, we proved that RPL26 acted as tumor suppressor in NSCLC, and the expression and biological effect of RPL26 was reciprocal with that of UBE2S. However, which pathway contributes to the role of RPL26 in NSCLC is not fully cleared in previous study. In p53 wild-type cell line A549, we showed that UBE2S could down regulate p53. In a p53 null cell line H1299, overexpression of UBE2S results in the upregulation of c-Myc, suggesting that UBE2S can also play a carcinogenic role through the p53 independent (c-Myc) pathway in NSCLC (**Figure 8E**).

In summary, UBE2S can promote the ubiquitination of RPL26, thus upregulating the expression of c-Myc to enhance the proliferation, migration as well as stemness of NSCLC and exerts oncogenic activities. UBE2S could be a potential target for NSCLC treatment. Our research suggests that the UBE2S-RPL26-c-Myc axis is an underlying pathway in the patho-

genesis and progression of NSCLC, and it provide a new direction for the selection of therapeutic targets and the development of inhibitors for NSCLC.

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### Disclosure of conflict of interest

None.

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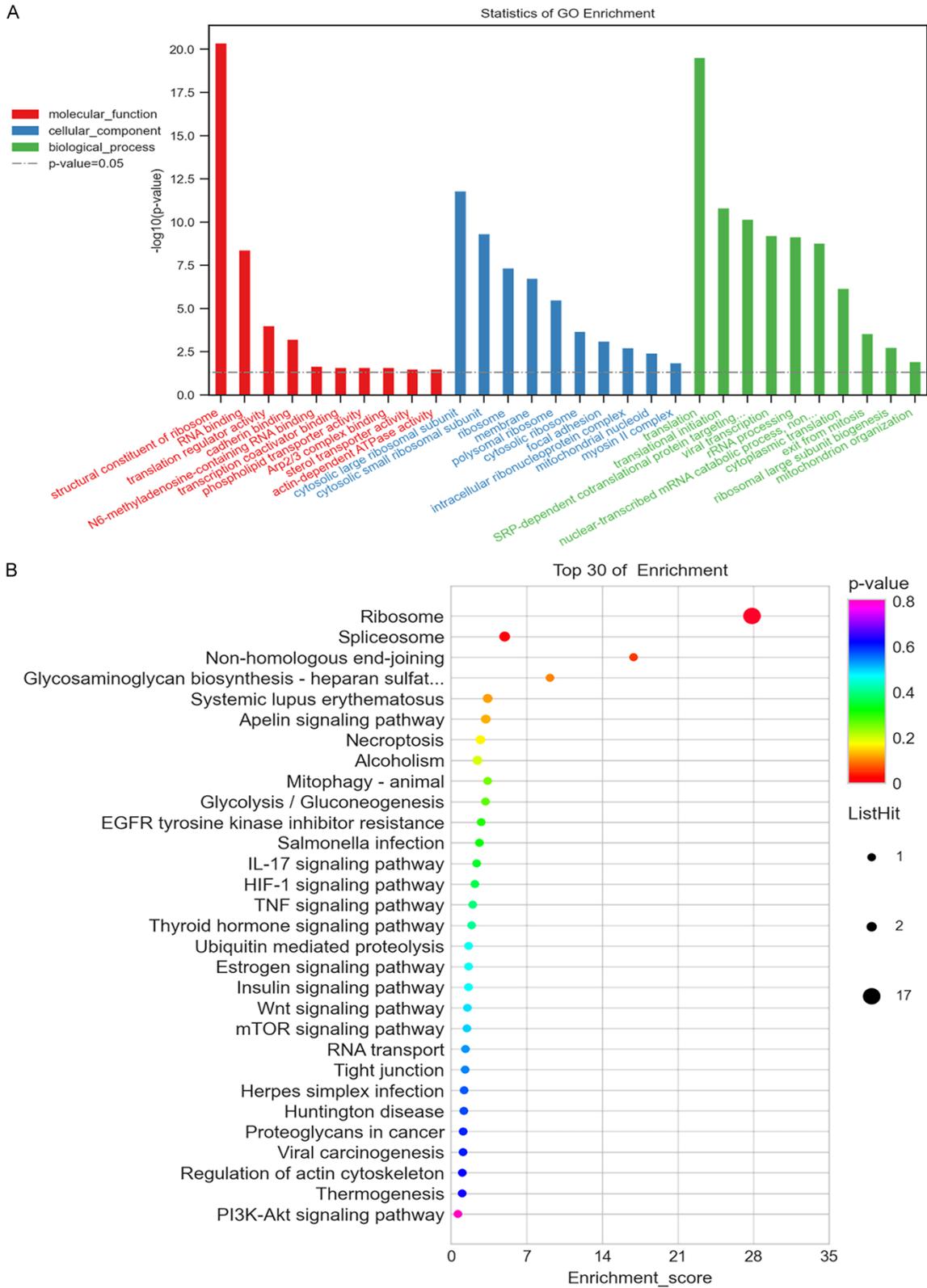
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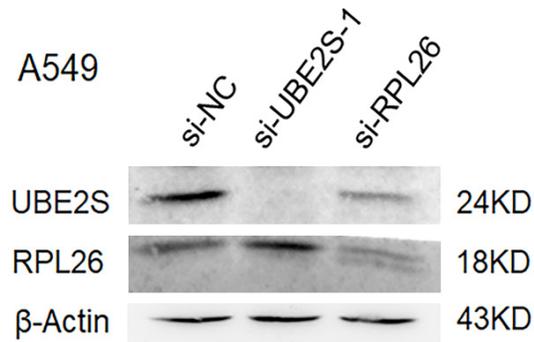
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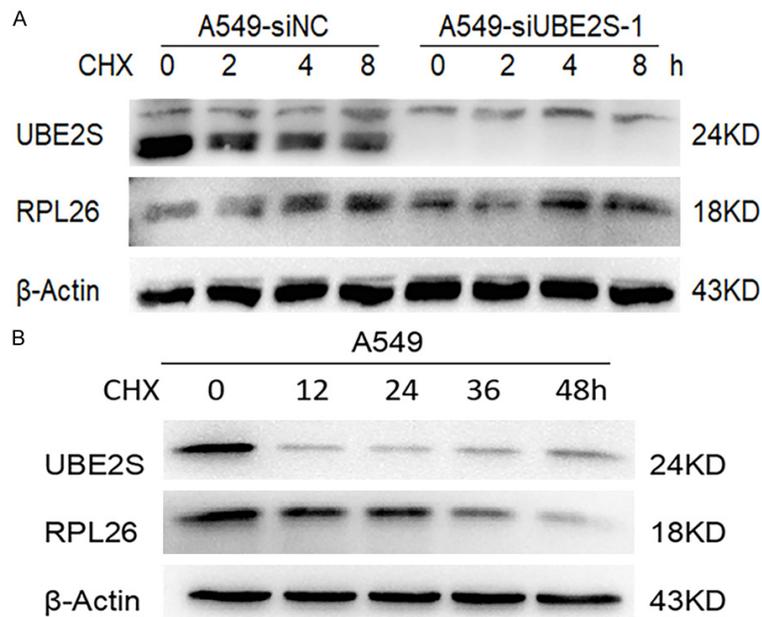


**Figure S1.** Molecular function and biological process analysis of interacting proteins with UBE2S in NSCLC cells by GO and KEGG analysis. GO (A) and KEGG (B) analysis showed that most interacting proteins of UBE2S are ribosomal proteins, and their molecular functions and biological processes are related to ribosomes biogenesis.

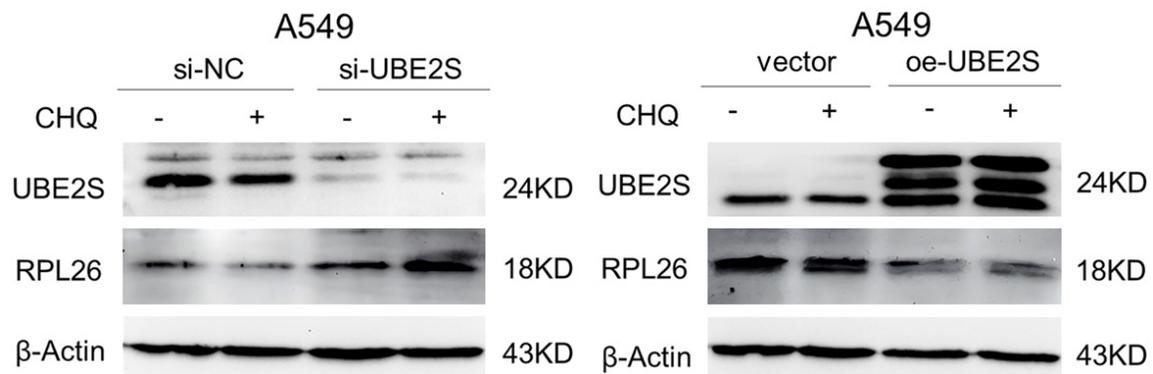
UBE2S-RPL26-c-Myc promotes NSCLC progression



**Figure S2.** The RNA interference efficiency of UBE2S and RPL26 in A549 cells by Western blot analysis.

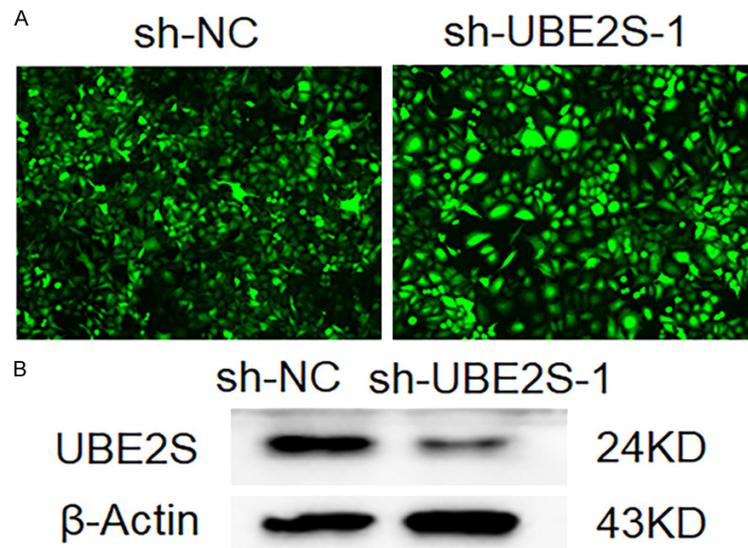


**Figure S3.** The protein expression analysis of RPL26 in A549 cells by Western blot. A. After knockdown of UBE2S for 48 h and treatment with CHX in A549 cells, Western blot was performed to detect the expression of RPL26 protein at the time point of 2 h, 4 h, 8 h, respectively. B. A549 cells with CHX treatment for 12 h, 24 h, 36 h and 48 h respectively, and the half-life of RPL26 protein was detected by Western blot.



**Figure S4.** The A549 cells with UBE2S knockdown or overexpression were treated with lysosome inhibitor CHQ (25 μM) for 24 h, and the protein level of RPL26 was detected by Western blot.

UBE2S-RPL26-c-Myc promotes NSCLC progression



**Figure S5.** Identification of A549 stable cell line with knockdown of UBE2S by lentiviral infection. A. Infection efficiency analysis of A549 stable cell line by fluorescence microscopy. B. Western blot analysis of UBE2S in infected cells.