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Original Article

LncRNA FEZF1-AS1 facilitates cisplatin resistance in non-small cell lung cancer through modulating the miR-32-5p-glutaminase axis

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Abstract: Non-small cell lung cancer (NSCLC) is one of the prevalent malignancies. Cisplatin (CDDP) is a conventional chemotherapeutic agent against NSCLC. However, inherent and acquired chemoresistance limited the effectiveness of cisplatin in treatment of NSCLC. This study aimed to investigate the roles and underlying mechanisms of lncRNA-FEZF1-AS1 in mediating cisplatin sensitivity in NSCLC. We found that FEZF1-AS1 levels were significantly higher in lung cancer patients and cell lines. Blocking FEZF1-AS1 sensitized lung cancer cells to cisplatin. Additionally, both glutamine metabolism and FEZF1-AS1 were significantly elevated in cisplatin resistant NSCLC cell lines, A549/CDDP R and SK-MES-1 CDDP/R. Analysis using bioinformatics, RNA pull-down assay and luciferase assay demonstrated that FEZF1-AS1 sponged miR-32-5p, which acted as a tumor suppressor in NSCLC. Glutaminase (GLS), a key enzyme in the glutamine metabolism, was predicted and validated as the direct target of miR-32-5p in NSCLC cells. Inhibiting glutamine metabolism or reducing glutamine supply effectively resensitized cisplatin-resistant cells. Furthermore, restoring miR-32-5p in FEZF1-AS1-overexpressing cisplatin resistant cells successfully overcame FEZF1-AS1-mediated cisplatin resistance by targeting GLS. These findings were further supported by *in vivo* xenograft mice experiments. This study uncovered the roles and molecular mechanisms of lncRNA FEZF1-AS1 in mediating cisplatin resistance in NSCLC, specifically through modulating the miR-32-5p-GLS axis, providing support for the development of new therapeutic approaches against chemoresistant lung cancer.

Keywords: Non-small cell lung cancer, cisplatin resistance, lncRNA-FEZF1-AS1, miR-32a-5p, glutamine metabolism, GLS

Introduction

Non-small cell lung cancer (NSCLC), known as one of the leading causes of cancer-related death, is divided into squamous cell carcinoma (SCC), adenocarcinoma and large cell carcinoma [1]. NSCLC is frequently diagnosed and leads to a poor prognosis and a low overall five-year survival rate [2]. In addition to surgical resection, standard treatments for NSCLC include chemotherapy and/or radiotherapy [3], especially for metastatic or advanced lung cancer [4]. Cisplatin (CDDP) is one of the most commonly used first-line chemotherapeutic agents for treatment of cancers through interacting with DNA to interfere with the formation of DNA adducts, leading to the activation of apoptosis in cancer cells [5]. Moreover, combination therapies have been widely applied to patients with metastatic NSCLC [6]. Despite the achieved effectiveness, a majority of patients develop cisplatin resistance, leading to chemotherapy failure and recurrence [6]. Currently, the precise molecular mechanisms for acquired CDDP resistance in NSCLC remain elusive. Therefore, there is an urgent need to investigate novel biomarkers and the underlying mechanisms for cisplatin resistance in order to further benefit the clinical treatment of lung cancer patients.

Long non-coding RNAs (lncRNAs) are a class of non-protein coding RNAs that are endogenously produced and have relatively large sizes (over 200 nucleotides in length) [7]. These RNAs do not undergo translation into protein [7]. Accumulating studies reported that lncRNAs

play vital roles in tumorigenesis and various processes, including growth, migration, apoptosis, metabolism, and chemoresistance [8], suggesting lncRNAs could serve as potential biomarkers and therapeutic targets for cancer. LncRNA-FEZF1-AS1 (FEZ family zinc finger 1 antisense RNA 1) is a recently discovered noncoding RNA which is positively associated with diverse cancers such as colorectal cancer [9], ovarian cancer [10], gastric cancer [11], prostate cancer [12] and lung cancer [13]. Increased expression of FEZF1-AS1 was detected in NSCLC tissue and associated with poor differentiation grade, lymph node metastasis, and advanced TNM stage [13], indicating FEZF1-AS1 is a potential biomarker for NSCLC diagnosis. Additionally, research has shown that lncRNA FEZF1-AS1 contributes to multi-drug resistance of gastric cancer [14], suggesting FEZF1-AS1 is tightly associated with drug resistance in lung cancer. However, the specific roles and molecular targets of FEZF1-AS1 in cisplatin-resistant NSCLC cells are still not fully understood.

Cancer cells exhibit abnormal cellular metabolism rates, including elevated glucose and glutamine metabolism, to support cell proliferation [15]. Glutamine metabolism is crucial for providing energy and building blocks for cancer cell progression and is considered a hallmark of cancer [16]. There is increasing evidence indicating that malignant tumor cells have a dependency on glutamine [17]. In addition, glutaminase (GLS) is an important enzyme involved in catalyzing glutaminolysis [18]. Therefore, the observation of overexpressed GLS in various cancer cells suggests that blocking GLS-mediated glutamine metabolism could be an effective approach against NSCLC.

In this study, we identified that FEZF1-AS1 is significantly upregulated in both lung cancer tissues and cells. Additionally, we established and characterized cisplatin-resistant NSCLC cell lines, A549/CDDP R and SK-MES-1 CDDP R. We will investigate the functions and molecular targets of FEZF1-AS1 in these cisplatin-resistant NSCLC cells. Furthermore, we aim to identify miRNA targets that are crucial in the FEZF1-AS1-mediated glutamine metabolism and cisplatin resistance. This study will contribute to our understanding of the molecular pathways involved in CDDP resistance and the development of novel therapeutic approaches for chemoresistant lung cancer.

Materials and methods

NSCLC patient tissues collection

A total of fifty NSCLC patients were enrolled in this study. Fresh lung tumor specimens and their corresponding normal adjacent lung tissues were collected from the Second Affiliated Hospital, Zhejiang University School of Medicine from June 2017 to May 2021. Patients did not receive other chemo- or radiotherapies prior to surgery. This study was approved by the Institutional Review Board of Second Affiliated Hospital, Zhejiang University School of Medicine. The enrolled patients were diagnosed as NSCLC by histological or cytological confirmation. After dissection, tissues were frozen in liquid nitrogen immediately and stored at -80°C until use.

Cell culture

Human lung cancer cell lines, A549, H520, H1650, SK-MES-1 and H1703 as well as normal lung epithelial cell line BEAS-2B were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Invitrogen, CA, USA), which was supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 mg/ml) (Invitrogen, CA, USA). All cells were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C. Cisplatin was purchased from Sigma-Aldrich (Shanghai, China) and dissolved in PBS to make a 5 mM stock.

Establishment of cisplatin resistant NSCLC cell line

The generation of cisplatin resistant lung cancer cell line was performed according to previous reports [19]. Briefly, A549 cells were continuously exposed to gradually elevated concentrations of cisplatin (2-50 μM) for four months. Survival cells were pooled and collected for downstream experiments. The cisplatin-resistant A549 cells were re-selected every six passages or four months. After culturing, freezing, or storage for a period of six passages or four months, the cisplatin-resistant cells were treated again with a consistent concentration

of cisplatin to eliminate any cells that may have lost their resistance during this period. The IC50s of A549 parental and CDDP resistant cells were 6.34 μM and 31.66 μM, respectively. The drug resistant indexs (DRIs) of A549 parental and CDDP resistant cells were 0.104 and 0.565, respectively.

Cell transfection

The siRNA, miRNA or plasmid DNA was transfected into lung cancer cells using the Lipofectamine 2000 transfection reagent (Thermofisher, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, cells were seeded in 6-well plates (2×10^5/well) and cultured overnight. The siRNAs against LDHA and FEZF1-AS1 were synthesized by Hanbio (Shanghai, China). LDHA overexpressing plasmid and control plasmid were purchased from Origene.com. miR-32-5p precursor and control miRNA were purchased from GenePharma Inc. (Shanghai, China). siRNA or miRNA was transfected at 50 nM for 48 hours. Plasmid DNA was transfected at 1 μg/ml for 48 hours.

RNA isolation and qRT-PCR

Total RNAs were isolated using the TRIzol reagent (Thermo Fisher Scientific, Shanghai, China). RNA samples were examined by a Nanodrop 2000 Spectrophotometers (Invitrogen, Carlsbad, CA, USA). RNA sample (1 μg) was reverse transcribed into cDNA using the miScript reverse transcription kit (Bio-Rad Laboratories, Hercules, CA, USA) and PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) for detections of miRNA and mRNA expressions, respectively. The qRT-PCR reactions were performed using the SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Shanghai, China). PCR reactions were set as follows: 95°C for 5 min followed by 36 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 40 s, and an extension for 10 min at 72°C. β-actin and U6 were used for an internal control. Primer sequences were as follows: FEZF1-AS1: Forward: 5'-TTAGGAGGCTTGTTCTGTGT-3', Reverse: 5'-GCGCAGGTACTTAAGAAAGA-3'; GLS: Forward: 5'-TTCCAGAAGGCACAGACATGGTTG-3', Reverse: 5'-GCCAGTGTCGCAGCCATCAC-3'; β-actin: Forward: 5'-CTGAGAGGGAAATCGTGCGT-3', Reverse: 5'-CCACAGGATTCCATACCCAAGA-3'; miR-32-5p: Forward: 5'-TATTGCACATTACTAAGCCTT-3', Reverse: 5'-GAATACCTCGGACCCTGC-3'; U6: Forward: 5'-CTCGCTTCGGCAGCACA-3', Reverse: 5'-AACGCTTCACGAATTTGCGT-3'. The relative gene expression was analyzed by the $2^{-\Delta\Delta Ct}$ method. Experiments were performed in triplicate and repeated three times.

RNA pull-down assay

Scramble, sense or antisense lncRNA FEZF1-AS1 DNA oligomers was biotin-labeled from RiboBio Co., Ltd. (Guangzhou, China). Lung cancer cell lysates were incubated with the above probes for 1 hour. Then, the Streptavidin-coupled agarose beads (ThermoFisher, Shanghai, China) were added into the lysates for 1 h. After washing the beads, the amounts of miR-32-5p from the pull-downed RNA-RNA complexes were examined by qRT-PCR. Experiments were repeated three times.

RNA immunoprecipitation (RIP)

To assess whether FEZF1-AS associates with the miR-32-5p-mediated RISC complex, RIP assays were performed using anti-Ago2 antibody (Thermofisher, USA) and the Magna RIP-TM RNA-binding protein immunoprecipitation kit (Millipore, USA) according to the manufacturer's protocols. Lung cancer cells were lysed and incubated with anti-Ago2 antibodies for overnight at 4°C. After washing, protein A magnetic beads were added and incubated for 4 hours. The coprecipitated RNAs were washed out from beads. The amount of FEZF1-AS1 from the complex was detected by qRT-PCR.

Luciferase assay

Lung cancer cells were co-transfected with control miRNA or miR-32-5p and the luciferase reporter vector containing wild-type (WT) or binding sites mutant (Mut) FEZF1-AS1 or 3'-UTR of GLS as indicated. The luciferase activity was measured using the Dual-Luciferase Assay Kit (Promega Corporation, Madison, WI, USA). Experiments were performed in triplicate and repeated three times.

Bioinformatics analysis

The predictions of the lncRNA-miRNA and miRNA-mRNA interaction were performed from the online non-coding RNA service, starBase. The correlations between lncRNA-miRNA and miR-

NA-mRNA were analyzed from starBase and Pearson's correlation coefficient analysis.

Measurements of glutamine metabolism

The glutamine metabolism rate was determined by glutamine uptake and glutaminase activity assays using the Glutamine Assay kit (Colorimetric) (#ab197011, Abcam, Cambridge, MA, USA) and the PicoProbe™ Glutaminase (GLS) Activity Assay Kit (#K455-100, Biovision Inc, Milpitas, CA, USA) according to the manufacturer's protocols. Assays were conducted in triplicate and repeated three times.

Cell viability assay (MTT)

The cell viability of lung cancer cells in response to cisplatin treatments was determined by MTT assay. Briefly, cells (8×10^3) were seeded in 96-well plates and cultured for overnight. Post treatments with cisplatin (range from 0.25 to 32 μM) for 48 h, MTT reagent [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to each well and incubated for 4 hrs. Dimethylsulphoxide (DMSO) was then added and incubated for 1 hr at 37°C. Absorbance was recorded at 595 nm. Experiments were performed in triplicate and repeated three times.

Clonogenic assay

The responses of NSCLC cells to cisplatin treatment were determined using the clonogenic assay. Cells (200 cells/well) were seeded in 6 cm dishes and cultured for 24 hrs. After continually treatment with cisplatin for 9-14 days. Survival colonies were fixed with 4% PFA and stained with crystal violet (0.05% w/v) for 5 min. After washing with PBS, the colonies were examined under bright field microscope. Colonies consisting of 100 cells or more were counted. Experiments were performed in triplicate.

Cell apoptosis assay

The cell apoptosis rates of NSCLC cells to cisplatin treatment were determined using Annexin V apoptosis assay using the Annexin V-FITC/PI apoptosis kit (ThermoFisher, Shanghai, China) according to the manufacturer's instructions. NSCLC cells were centrifuged and washed with cold PBS. The FITC-Annexin V (5 μL) and 1 μL PI working solution (100 μg/mL) from the kit were added into cells and incubated for 15 min at room temperature with light protection. Fluorescence was examined by a FACScan flow cytometer (FACS Calibur™, BD Biosciences, CA, USA). Experiments were performed in triplicate and repeated three times.

In vivo xenograft experiments

Animal protocols were approved by the Ethics Committee of Second Affiliated Hospital, Zhejiang University School of Medicine and experiments were performed according to the European Communities Council Directive of 24 November 1986 (86/609/EEC). Thirty male BALB/c nude mice (4-6 weeks) were subcutaneously injected with 549 CDDP R cells (8×10^6). After tumors developed, mice were randomly separated into four groups (10 mice each group) and treated with: a) Saline control; b) sh lncFEZF1-AS1; c) cisplatin (5 mg/kg intraperitoneal (i.p.), 2 times/wk); d) sh lncFEZF1-AS1 plus cisplatin (5 mg/kg, i.p., 2 times/wk). Survival rate was monitored for seven weeks. Tumor volumes were calculated by V (mm^3) = $1/2ab^2$.

Western blot

Proteins were isolated from lung cancer cells using RIPA buffer (ThermoFisher Scientific, Shanghai, China) supplied with 1× protease inhibitor cocktail (ThermoFisher Scientific, Shanghai, China). Lysates were incubated for 15 min on ice and centrifuged at 10,000 g for 10 min at 4°C. Protein concentrations were measured using the bicinchoninic acid (BCA) assay. Protein (40 μg) from each sample was loaded and fractionated on 10% SDS-PAGE gels followed by transferring to a PVDF membrane (ThermoFisher Scientific, Shanghai, China). Membranes were blocked by 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) at room temperature for 1 hr. Membranes were incubated with primary antibodies (1:1000) at 4°C for overnight. After washing by TBST, membranes were incubated with a secondary horseradish peroxidase (HRP)-labelled antibody (1:3000) for 1 h at room temperature. Rabbit anti-β-actin and anti-GLS antibodies were purchased from Cell Signaling Technology (#56750 and #4970, Danvers, MA, USA). Protein bands were detected and visualized using SuperSignal

West Pico enhanced chemiluminescence substrate (Pierce, IL, USA).

Statistical analysis

Statistical significance was evaluated using the software Prism 7.0 (GraphPad Software, La Jolla, CA, USA). Data is graphically represented as mean ± SD and repeated three times. The difference between two experimental groups was examined by Student's t-test. The differences among three or more groups were analyzed by one-way ANOVA. Differences were considered to be statistically significant where $P<0.05$.

Results

FEZF1-AS1 is upregulated and promotes cisplatin resistance in NSCLC

Accumulating studies revealed oncogenic roles of lncRNA FEZF1-AS1 in various types of cancers [9-14]. Moreover, bioinformatics analysis using data from the TCGA cancer database illustrated that FEZF1-AS1 was significantly upregulated in multiple cancers, including lung tumors (Figure S1A-C), indicating FEZF1-AS1 is positively associated with progressions of NSCLC. To evaluate the biological roles of FEZF1-AS1 in non-small cell lung cancer, we validated the expressions of FEZF1-AS1 in human lung tumor tissues and matched normal lung tissues. Results from qRT-PCR demonstrated that FEZF1-AS1 was significantly increased in lung tumor specimens (**Figure 1A**). Kaplan-Meier survival analysis illustrated that lung cancer patients with higher FEZF1-AS1 expression were associated with lower survival rate ($P<0.01$) (**Figure 1B**), suggesting FEZF1-AS1 could potentially be a prognostic marker for lung cancer patients. In addition, the expressions of FEZF1-AS1 were consistently upregulated in human five lung cancer cell lines, A549, H520, SK-MES-1, H1703 and H1650 compared to normal lung epithelial cells BEAS-2B (**Figure 1C**). To investigate the effects of FEZF1-AS1 on chemosensitivity, FEZF1-AS1 was knocked down by siRNA in A549 and SKMES-1 cells (Figure S2). Expectedly, lung cancer cells with lower FEZF1-AS1 expressions displayed remarkedly increased sensitivity to cisplatin as shown by cell viability assay (**Figure 1D**, **1E**). Conversely, overexpression of FEZF1-AS1 significantly attenuated the cisplatin sensitivity of lung cancer cells (Figure S3A, S3B). The original IC50s of A549 and SK-MES-1 cells were 6.15 μM and 2.88 μM, respectively. Silencing FEZF1-AS1 effectively decreased the IC50s of A549 and SKMES-1 cells to 1.81 μM and 0.78 μM (**Figure 1D**, **1E**). Consistently, results from clonogenic assay showed NSCLC cells with lower FEZF1-AS1 had lower survival capacities under cisplatin treatments (**Figure 1F**). Taken together, these results uncovered an oncogenic role of FEZF1-AS1, which promotes cisplatin resistance in NSCLC.

Inhibition of FEZF1-AS1 re-sensitizes CDDP resistant NSCLC cells

To investigate the molecular mechanisms of the FEZF1-AS1-mediated cisplatin sensitivity, we generated *in vitro* cisplatin resistant cell line (A549/CDDP R) by treating A549 parental with gradually increased concentrations of cisplatin. Survival cells were collected and pooled for downstream experiments. Cisplatin resistance was validated by cell viability assay and other drug resistant markers (Figure S4A-C). Apparently, A549/CDDP R cells could tolerate higher concentrations of cisplatin (**Figure 2A**, **2B**). The IC50 of A549/CDDP R cells increased to 31.66 μM, which was around 5-fold higher than that from A549 parental cells (**Figure 2A**). Expectedly, FEZF1-AS1 was detected to be significantly upregulated in CDDP resistant cells (**Figure 2C**), suggesting blocking FEZF1-AS1 could be an effective approach against chemoresistant NSCLC. Clinically, high FEZF1-AS1 expression was associated with cisplatin resistance in lung cancer patients (Figure S5). Statistical analysis revealed that out of the 50 lung cancer patients, 12 had low FEZF1-AS1 expression while 38 had high FEZF1-AS1 expression. Among the patients with low FEZF1-AS1 expression, the majority (11 cases) were sensitive to cisplatin while only 1 patient was resistant to it (Figure S5). In contrast, among the patients with high FEZF1-AS1 expression, 6 were sensitive to cisplatin while the majority of them (32 cases) were cisplatin resistant (Figure S5). These results demonstrated that FEZF1-AS1 was positively correlated to cisplatin resistance in lung cancer patient. Subsequently, FEZF1-AS1 was silenced in A549/CDDP R cells (**Figure 2D**). Cell viability assay and apoptosis assay showed knockdown FEZF1-AS1 effectively re-sensitized A549/

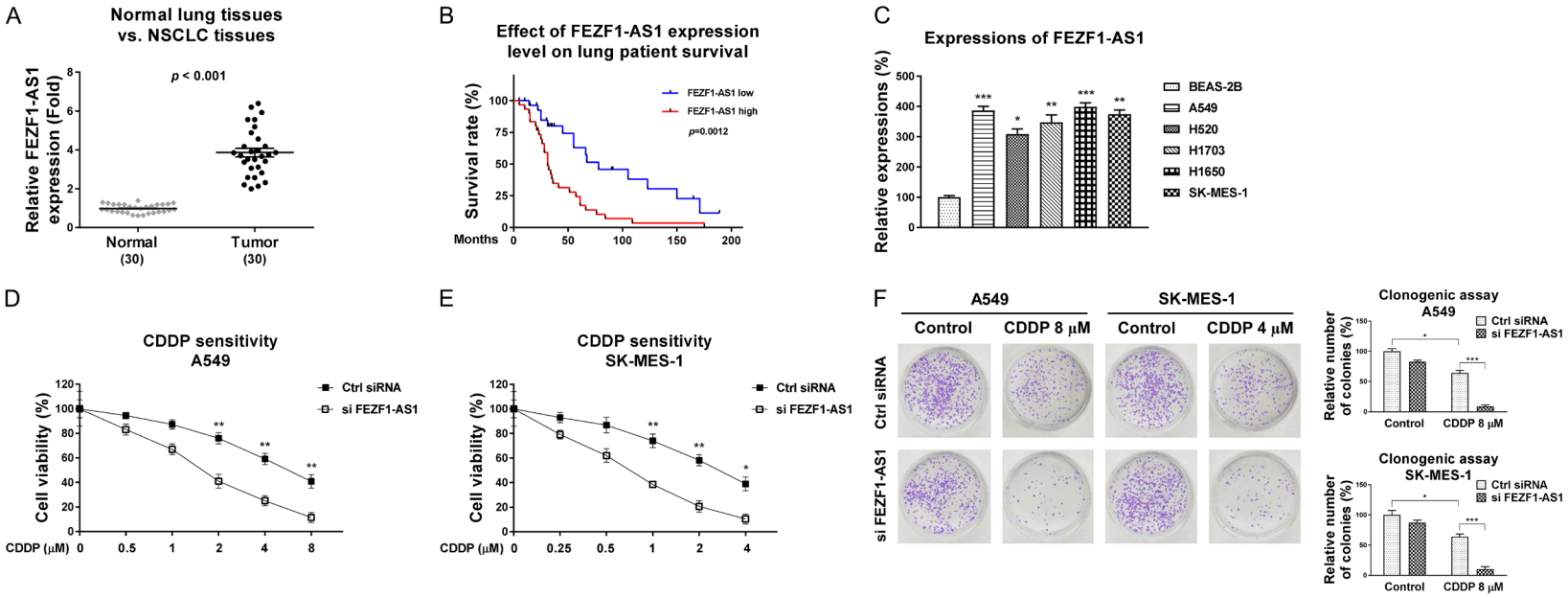
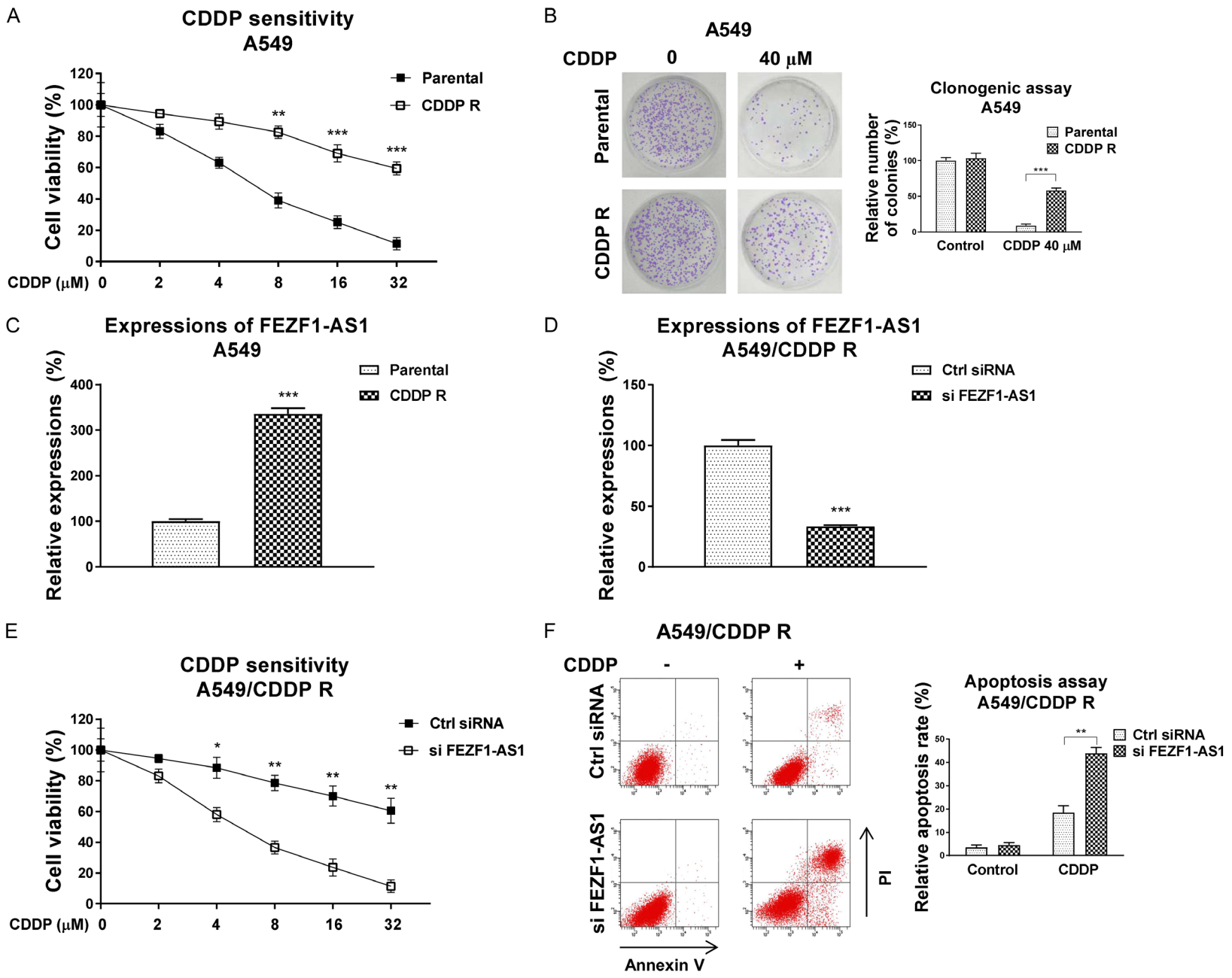


Figure 1. FEZF1-AS1 is positively associated with NSCLC and cisplatin resistance. (A) Expressions of FEZF1-AS1 in human normal and tumorous lung tissues. (B) Kaplan-Meier survival curve illustrated survival rates of lung cancer patients with higher or lower FEZF1-AS1 expression. (C) Expressions of FEZF1-AS1 in normal and tumorous lung cancer cell lines. (D) A549 and (E) SK-MES-1 cells without or with FEZF1-AS1 knockdown were treated with cisplatin at the indicated concentrations. Drug responses of cells were determined by cell viability assay and (F) clonogenic assay. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.


A
CDDP sensitivity
A549
Cell viability (%)
Parental
CDDP R
CDDP (μM) 0 2 4 8 16 32
B
A549
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Parental
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Clonogenic assay
A549
Relative number of colonies (%)
Control
CDDP 40 μM
C
Expressions of FEZF1-AS1
A549
Relative expressions (%)
D
Expressions of FEZF1-AS1
A549/CDDP R
Ctrl siRNA
si FEZF1-AS1
E
CDDP sensitivity
A549/CDDP R
F
A549/CDDP R
CDDP - +
Annexin V
PI
Apoptosis assay
A549/CDDP R
Relative apoptosis rate (%)
Control
CDDP

Figure 2. Inhibition of FEZF1-AS1 re-sensitizes CDDP resistant NSCLC cells. (A) Validation of cisplatin resistance in A549/CDDP R cells by cell viability assay and (B) clonogenic assay. (C) Expressions of FEZF1-AS1 in A549 parental and CDDP R cells. (D) FEZF1-AS1 was knocked-down in A549 parental and CDDP R cells. (E) A549/CDDP R cells were treated with cisplatin at the indicated concentrations. Cell viability and (F) apoptosis assay were performed. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

CDDP resistant cells to cisplatin (**Figure 2E**, **2F**), suggesting FEZF1-AS1 could serve as a therapeutic target for CDDP resistant lung cancer.

FEZF1-AS1 sponges miR-32-5p in NSCLC

In view of the functions of FEZF1-AS1 in regulating cisplatin resistance and glutamine metabolism of lung cancers, we evaluated the downstream molecular targets of FEZF1-A1. Since lncRNAs function as sponge of miRNAs to downregulate their expressions [20], the potential target miRNAs analyzed from starBase indicated that miR-32-5p contains putative binding sites of FEZF1-AS1 (**Figure 3A**). Expectedly, expressions of FEZF1-AS1 and miR-32-5p was detected to be negatively correlated in lung cancer tissues (**Figure 3B**). To further validate this association, FEZF1-AS1 was overexpressed in NSCLC cells. A549 and SK-MES-1 cells with higher FEZF1-AS1 expression showed suppressed miR-32-5p expression (**Figure 3C**). To test whether FEZF1-AS1 could directly bind to seed region of miR-32-5p, RNA pull-down assay and luciferase assay were conducted. qRT-PCR results showed the miR-32-5p was only enriched in the antisense FEZF1-AS1 precipitated RNA complex in NSCLC cells compared with control probe and sense FEZF1-AS1 probe (**Figure 3D**). In addition, RIP (RNA-binding protein immunoprecipitation) assay was performed using lung cancer cells extracts. Results revealed that FEZF1-AS1 binds directly to Ago2-associated RNA-induced silencing complex mediated by miRNA (**Figure 3E**). Furthermore, overexpression of miR-32-5p significantly elevated the FEZF1-AS1 level which pulled down by Ago2 complexes (**Figure 3F**), indicating FEZE1-AS1 binds with miR-32-5p in lung cancer cells. Luciferase assay further verified that co-transfection of miR-32-5p and WT-FEZF1-AS1 significantly blocked the luciferase activity of NSCLC cells compared with that from co-transfection with miR-32-5p plus the binding site mutant FEZF1-AS1 (**Figure 3G**, **3H**). Taken together, the above results validated FEZF1-AS1 suppressed miR-32-5p by direct association with miR-32-5p to form a ceRNA network.

miR-32-5p sensitizes lung cancer cells to cisplatin

Given the results mentioned above, which indicated that FEZF1-AS1 promotes resistance to cisplatin and decreases the expression of miR-32-5p, we hypothesized that miR-32-5p functions as a tumor suppressor molecule to make lung cancer cells more responsive to cisplatin. qRT-PCR results demonstrated that miR-32-5p was significantly downregulated in NSCLC tissues compared with normal tissues (**Figure 4A**). Consistently, we observed a noticeable suppression of miR-32-5p in NSCLC cells compared to normal lung epithelial cells (**Figure 4B**). miR-32-5p was detected to be downregulated in cisplatin resistant lung cancer cells (**Figure 4C**). Furthermore, low miR-32-5p was positively associated with cisplatin resistance in lung cancer patients (Figure S6). We then evaluated the effects of miR-32-5p on the sensitivity of lung cancer cells to cisplatin. The overexpression of miR-32-5p substantially decreased the survival rate of A549/CDDP R cells when treated with cisplatin (**Figure 4D**), suggesting that miR-32-5p plays a role in sensitizing lung cancer cells to chemotherapy and counteracting chemoresistance.

Cisplatin resistant lung cancer cells exhibit increased glutamine metabolism

Studies have revealed that the dysregulated cellular metabolism of cancer cells contributed to abnormal tumor growth and drug resistance [15-18]. Glutamine metabolism is essential for providing major energy and building blocks to cancer cells. Therefore, we examined the glutamine metabolism of CDDP resistant lung cancer cells. As expected, glutamine uptake (**Figure 5A**) and glutaminase activity (**Figure 5B**) were significantly higher in A549/CDDP R cells compared to parental cells. Additionally, cisplatin resistant lung cancer cells were more sensitive to cisplatin when supplied with low levels of glutamine compared to normal cell culture conditions (**Figure 5C**). Consistently, blocking glutamine metabolism through silencing glutaminase synergistically inhibited the

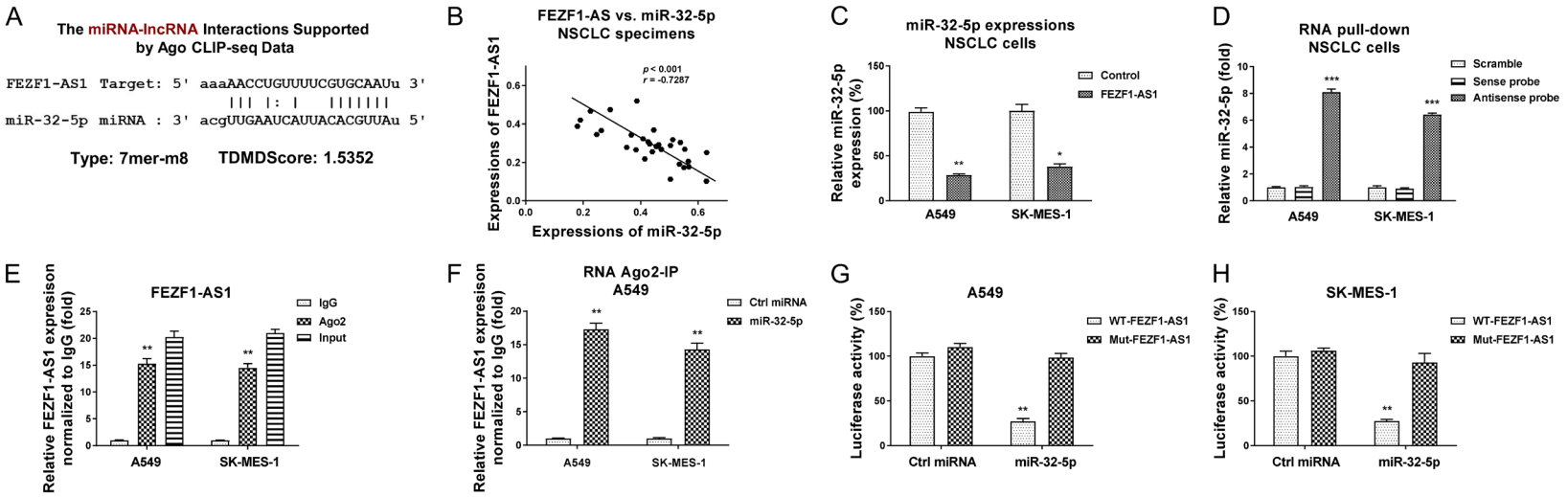

Figure 3. FEZF1-AS1 sponges miR-32-5p. (A) Prediction of the FEZF1-AS1 and miR-32-5p association from starBase. (B) Negative correlation between FEZF1-AS1 and miR-32-5p was shown in NSCLC tissues by Pearson correlation coefficient analysis. (C) miR-32-5p expressions in NSCLC cells with control or FEZF1-AS1 transfection. (D) RNA pull-down assay was performed in NSCLC cells incubated with biotin-labeled scramble, sense or antisense FEZF1-AS1 probe. Enrichment of miR-32-5p was examined by qRT-PCR. (E) Anti-Ago2 RIP was performed in lung cancer cells and relative expression of FEZF1-AS1 in the pulldown complex was detected by qRT-PCR. (F) Anti-Ago2 RIP was performed in A549 and SK-MES-1 cells with or without miR-32-5p overexpression. Relative expression of FEZF1-AS1 in the pulldown complex was detected by qRT-PCR. (G) Luciferase assay was performed in A549 and (H) SK-MES-1 cells by co-transfection of control miRNA or miR-32-5p with WT-FEZF1-AS1 or ut-FEZF1-AS1. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

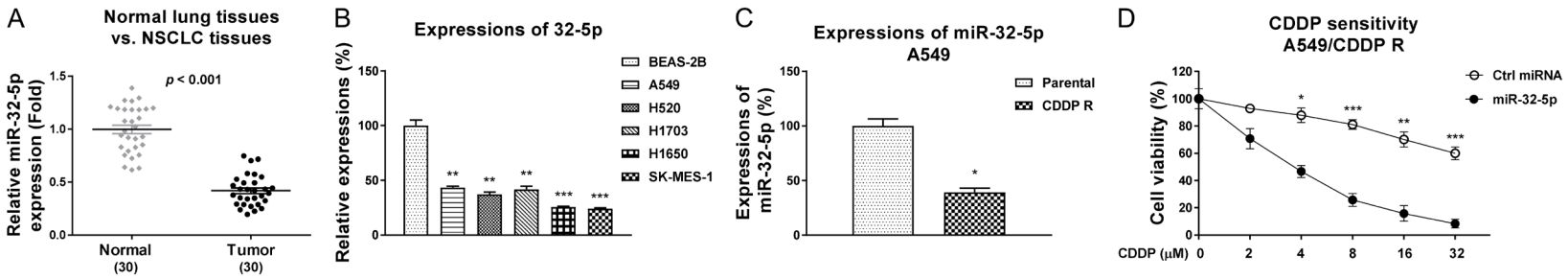

Figure 4. miR-32-5p sensitizes cisplatin resistant lung cancer cells. A. Expressions of miR-32-5p in human normal and tumorous lung tissues. B. Expressions of miR-32-5p in normal and tumorous lung cancer cell lines. C. miR-32-5p was high-expressed in A549/CDDP R cells. D. A549/CDDP R cells without or with miR-32-5p overexpression were treated with cisplatin at the indicated concentrations. Drug responses of cells were determined by cell viability assay. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

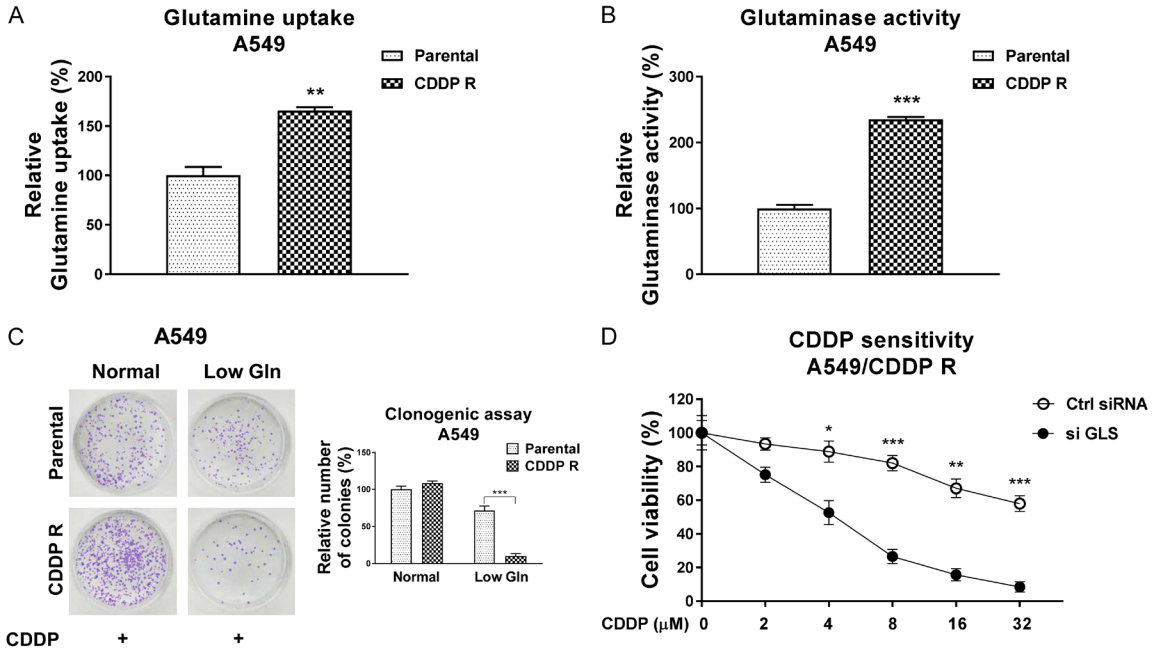

Figure 5. Glutamine metabolism is elevated in cisplatin resistant lung cancer cells. (A) The glutamine uptake and (B) glutaminase activity were examined in A549 parental and cisplatin resistant cells. (C) Cisplatin resistant cells were cultured under normal and low glutamine conditions, cells were treated with control or cisplatin. Cell survival rate was evaluated by clonogenic assay. (D) Cisplatin resistant cells without or with GLS silencing were treated cisplatin for 48 hours. Cell viability was determined by MTT assay. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

viability of A549/CDDP R cells when treated with cisplatin (**Figure 5D**). In summary, the above results demonstrate a positive correlation between glutamine metabolism and the FEZF1-AS1-mediated cisplatin sensitivity of lung cancer cells.

FEZF1-AS1 and miR-32-5p inversely regulate glutamine metabolism

We then assessed the functions of FEZF1-AS1 and miR-32-5p in regulating glutamine metabolism. Expectedly, silencing FEZF1-AS1 effectively blocked the glutamine uptake and glutaminase activity of NSCLC cells (**Figure 6A**, **6B**). On the other way, overexpression of miR-32-5p rendered similar phenotypes on A549 and SK-MES-1 cells that the glutamine uptake and glutaminase activity were significantly inhibited by miR-32-5p (**Figure 6C**, **6D**).

GLS is directly targeted by miR-32-5p in NSCLC cells

Previous studies have revealed that microRNAs inhibited expressions of their target mRNAs through binding to the 3'UTR of target mRNAs [21]. We identified potential targets of miR-32-5p in lung cancer cells by bioinformatic analysis from the TargetScan databases. Interestingly, we noticed that the 3'UTR of GLS, which is known to be involved in glutamine metabolism and is positively correlated with the progression of lung cancer [17], contains putative miR-32-5p binding sites (**Figure 7A**). Notably, GLS was significantly upregulated in lung cancer tissues and cell lines compared with their normal counterparts (**Figure 7B**, **7C**). In addition, GLS was apparently elevated in cisplatin resistant cells compared with parental cells (**Figure 7D**). To assess the clinical correlation of the potential miR-32-5p-GLS interaction, Pearson's correlation coefficient analysis was performed. The miR-32-5p expression was negatively correlated with GLS mRNA expression in NSCLC tissues from both TCGA database (Figure S7A, S7B) and qRT-PCR analysis (**Figure 7E**). Consequently, blocking GLS effectively sensitized A549 and SK-MES-1 cells to cisplatin (**Figure 7F**, **7G**). To test whether miR-32-5p could target GLS, we transfected control miRNAs or the miR-32-5p into A549 and SK-MES-1 cells. Overexpression of miR-32-5p significantly suppressed the protein expression of GLS (**Figure 7H**, **7I**). To verify whether miR-32-5p directly

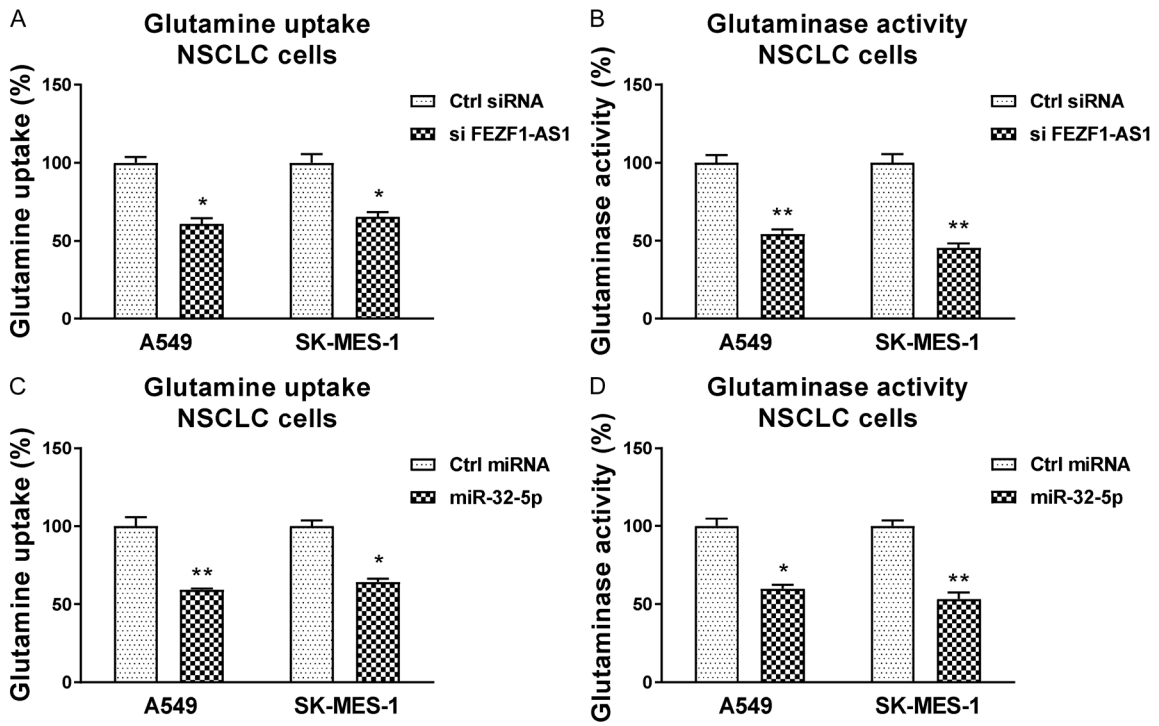

Figure 6. FEZF1-AS1 and miR-32-5p inversely regulate glutamine metabolism of lung cancer cells. (A) A549 and SK-MES-1 cells were transfected with control siRNA or FEZF1-AS1 siRNA. The glutamine uptake and (B) glutaminase activity were measured. (C) A549 and SK-MES-1 cells were transfected with control miRNA or miR-32-5p. The glutamine uptake and (D) glutaminase activity were measured. *, $P<0.05$; **, $P<0.01$.

binds to the 3'UTR of GLS mRNA, luciferase reporter assay was performed by co-transfection of control miRNA or miR-32-5p with WT-GLS 3'UTR or binding site mutant GLS 3'UTR (Mut-GLS) into NSCLC cells. Expected results demonstrated that miR-32-5p effectively blocked the luciferase activity of vector containing the WT-GLS 3'-UTR compared with that from the binding site mutant GLS 3'UTR (**Figure 7J**, **7K**). Taken together, these data consistently demonstrated GLS is a direct target of miR-32-5p in NSCLC.

miR-32-5p re-sensitizes cisplatin resistant cells through targeting GLS

We next asked whether the miR-32-5p-mediated cisplatin sensitization was through direct targeting GLS. Consequently, rescue experiments were performed by co-transfection of control miRNA, miR-32-5p alone or plus GLS into A549/CDDP R cells (**Figure 8A**). Western blot results showed overexpression of GLS into miR-32-5p overexpressing A549/CDDP R cells successfully recovered the GLS expression (**Figure 8A**). Furthermore, A549/CDDP R cells with restoration of GLS displayed rescued glutamine metabolism phenotypes (**Figure 8B**, **8C**). Cell viability assay and apoptosis assay consistently demonstrated that recovery of GLS in miR-32-5p overexpressing A549/CDDP R cells de-sensitized lung cancer cells to cisplatin (**Figure 8D**). Summarily, these rescue experiments validated that miR-32-5p sensitizes cisplatin resistant lung cancer cells by direct targeting GLS.

The FEZF1-AS1-miR-32-5p-GLS axis contributes to CDDP resistance in NSCLC cells in vitro and in vivo

Finally, the biological roles of the FEZF1-AS1-miR-32-5p-GLS axis were evaluated in cisplatin resistant lung cancer cells. Bioinformatics analysis from the non-coding RNA database starBase and qRT-PCR results from NSCLC patients indicated a significantly positive correlation between FEZF1-AS1 and GLS mRNA expressions in NSCLC patients (Figure S8A, S8B). Thus, A549/CDDP R cells were co-transfected with control plasmid, FEZF1-AS1 overexpression vector alone or combined with miR-32-5p.

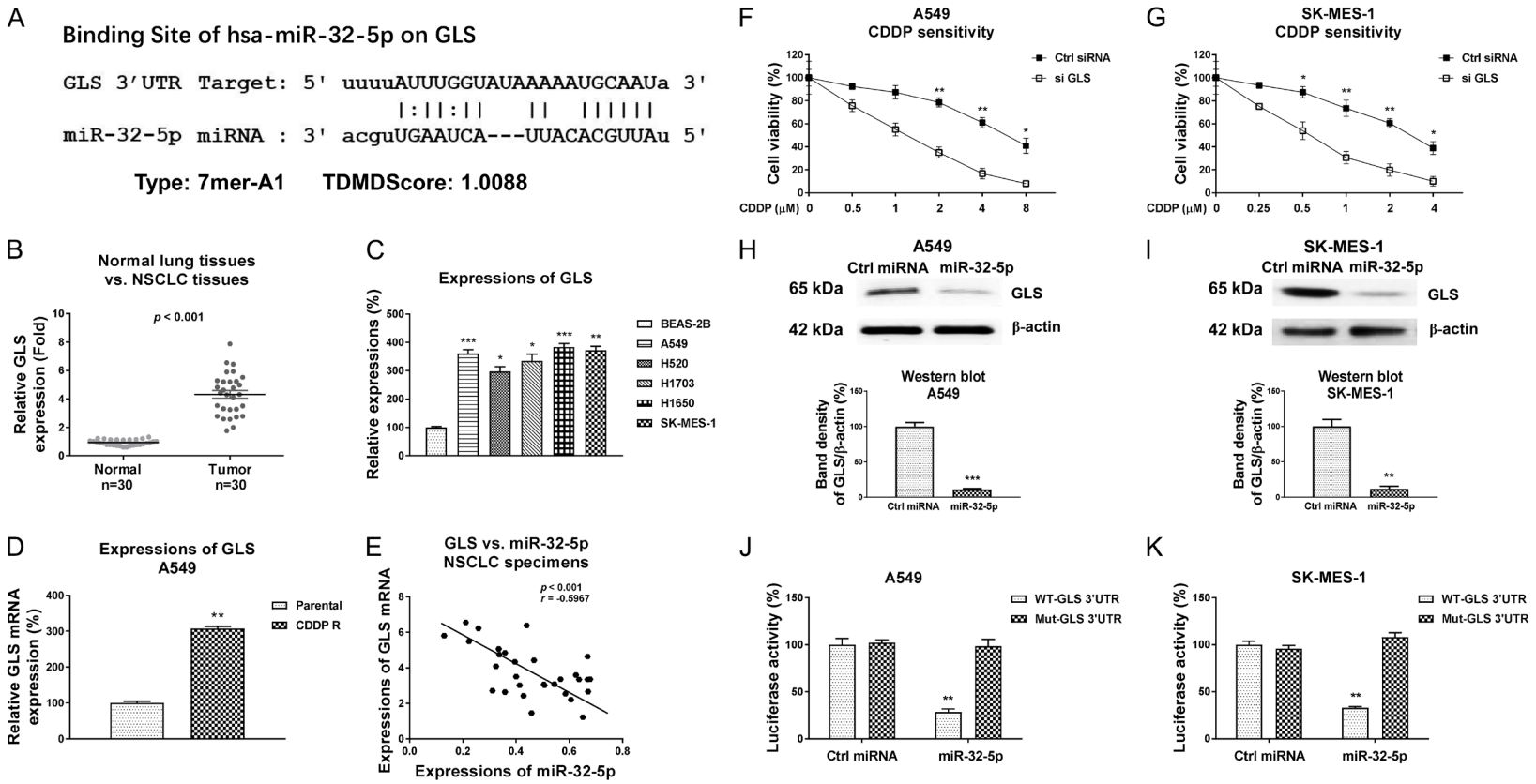

Figure 7. miR-32-5p directly targets GLS in NSCLC cells. (A) Prediction of the binding of miR-32-5p on 3'UTR of GLS mRNA from starBase. (B) Expressions of GLS in human normal and tumorous lung tissues. (C) Expressions of GLS in normal and tumorous lung cancer cell lines. (D) Expressions of LDHA in A549 parental and CDDP R cells. (E) Negative correlation between GLS and miR-32-5p was shown in NSCLC tissues by Pearson correlation coefficient analysis. (F) A549 and (G) SK-MES-1 cells were transfected with control siRNA or si GLS, followed by treatment with cisplatin. Cell viability was examined by MTT assay. (H) A549 and (I) SK-MES-1 cells were transfected with control miRNA or miR-32-5p for 48 hours. Protein expression of GLS was examined by Western blot assay. (J, K) Luciferase assay was performed in A549 and SK-MES-1 cells by co-transfection of control miRNA or miR-32-5p with WT-GLS 3'UTR or Mut-GLS 3'UTR. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

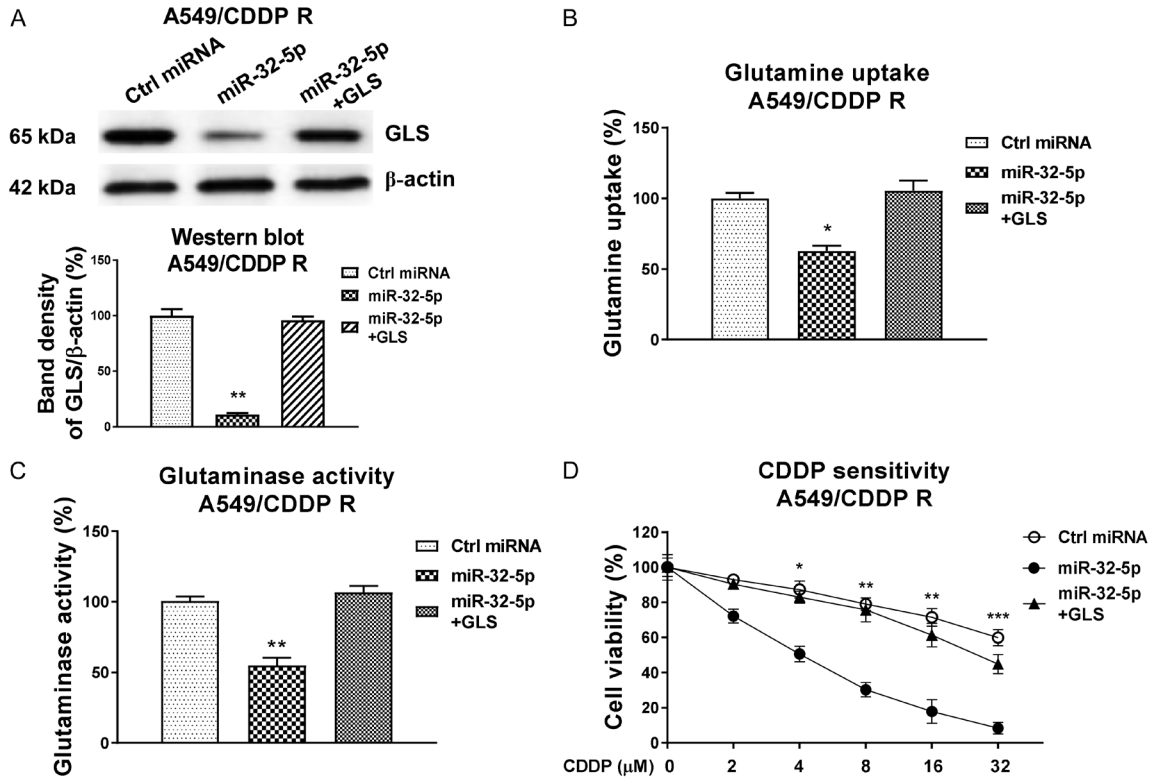

Figure 8. Recovery of GLS rescues cisplatin resistance of NSCLC cells. (A) A549 cisplatin resistant cells were transfected with control miRNA, miR-32-5p alone or plus GLS, expressions of GLS were examined by Western blot. (B) The glutamine uptake and (C) glutaminase activity were examined from the above transfected cells. (D) The transfected A549/CDDP R cells were treated with cisplatin, cell responses to drug were determined by cell viability assay. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Overexpression of FEZF1-AS1 significantly inhibited miR-32-5p expression and upregulated GLS expression. This regulation was further overcome by miR-32-5p rescue (**Figure 9A**, **9B**). Consistently, A549/CDDP R cells with co-transfection of FEZF1-AS1 plus miR-32-5p successfully decreased the glutamine uptake (**Figure 9C**) and GLS activity (**Figure 9D**) compared with FEZF1-AS1 transfection alone. Expectedly, recovery of miR-32-5p in FEZF1-AS1-overexpressing A549/CDDP R cells effectively re-sensitized cisplatin resistant cells from cell viability assay and cell apoptosis assay (**Figure 9E**, **9F**).

To further validate these *in vitro* results, an in vivo xenograft mice model was applied. A549 CDDP R cells were subcutaneously injected into nude mice. After tumors developed, mice were randomly separated into three groups (n=10) and treated with a) Saline control; b) sh FEZF1-AS1; c) cisplatin alone; d) FEZF1-AS1 knockdown plus cisplatin. Mice survival rates and tumor sizes were recorded. Expected results from **Figure 10A** illustrated that the majority of mice which were treated with control, sh FEZF1-AS1 or cisplatin alone died within seven weeks. Mice treated with sh FEZF1-AS1 plus cisplatin obtained a remarkedly prolonged survival rate (**Figure 10A**) and reduced tumor sizes (**Figure 10B**, **10C**) compared with mice from other treatment groups. In summary, these *in vivo* results validated that FEZF1-AS1-promoted cisplatin resistance was through modulating the miR-32-5p-GLS axis (**Figure 10D**), indicating the combination of FEZF1-AS1 silencing with cisplatin could synergistically attenuate lung tumor growth.

Discussion

NSCLC is one of the most common malignant tumors [1, 2]. Surgical resection is the standard treatment for NSCLC patients in the early stages [3]. Additionally, chemotherapy and/or radiotherapy are the primary strategies for lung cancer patients in advanced or metastatic stages

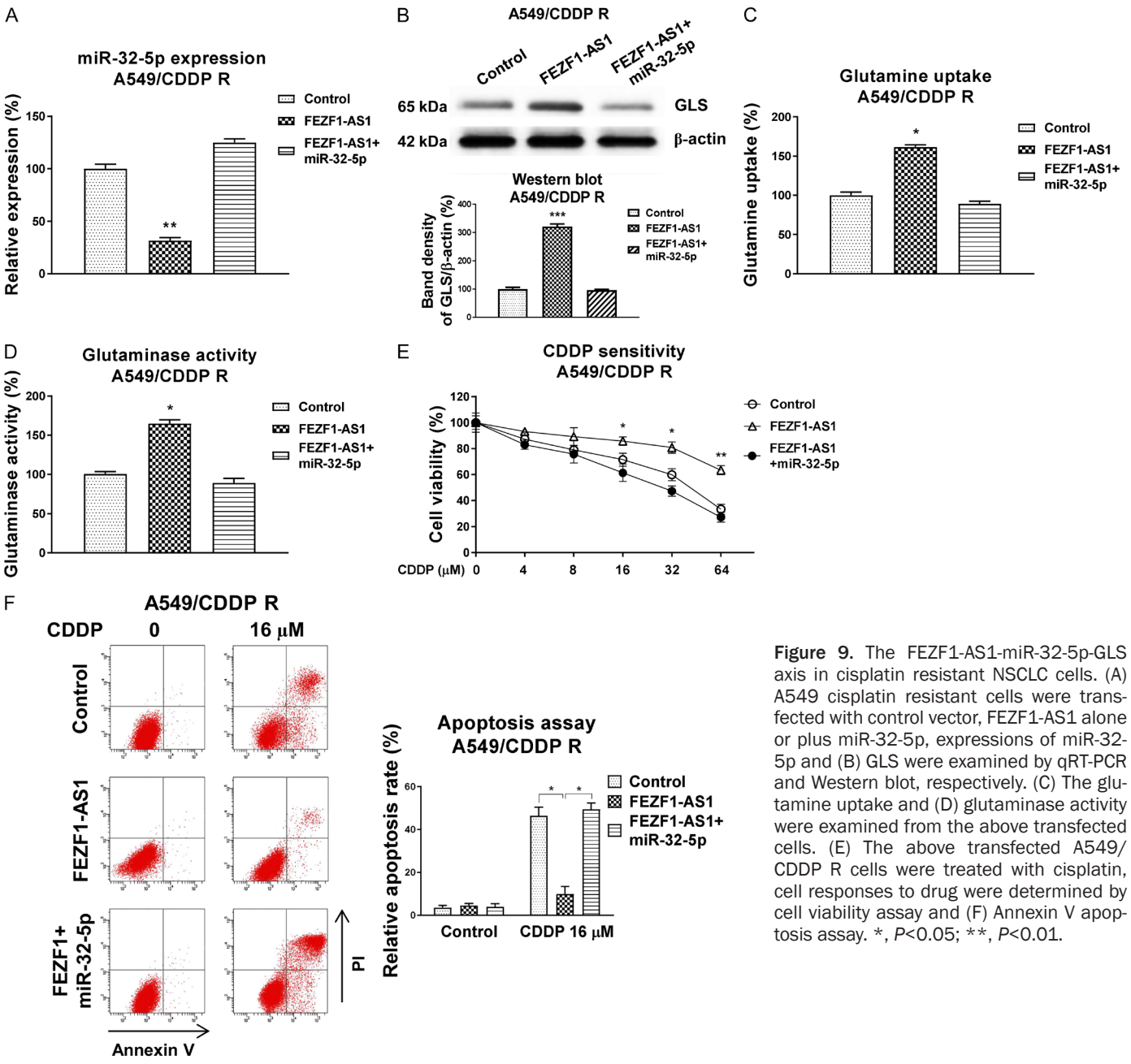

Figure 9. The FEZF1-AS1-miR-32-5p-GLS axis in cisplatin resistant NSCLC cells. (A) A549 cisplatin resistant cells were transfected with control vector, FEZF1-AS1 alone or plus miR-32-5p, expressions of miR-32-5p and (B) GLS were examined by qRT-PCR and Western blot, respectively. (C) The glutamine uptake and (D) glutaminase activity were examined from the above transfected cells. (E) The above transfected A549/CDDP R cells were treated with cisplatin, cell responses to drug were determined by cell viability assay and (F) Annexin V apoptosis assay. *, $P<0.05$; **, $P<0.01$.

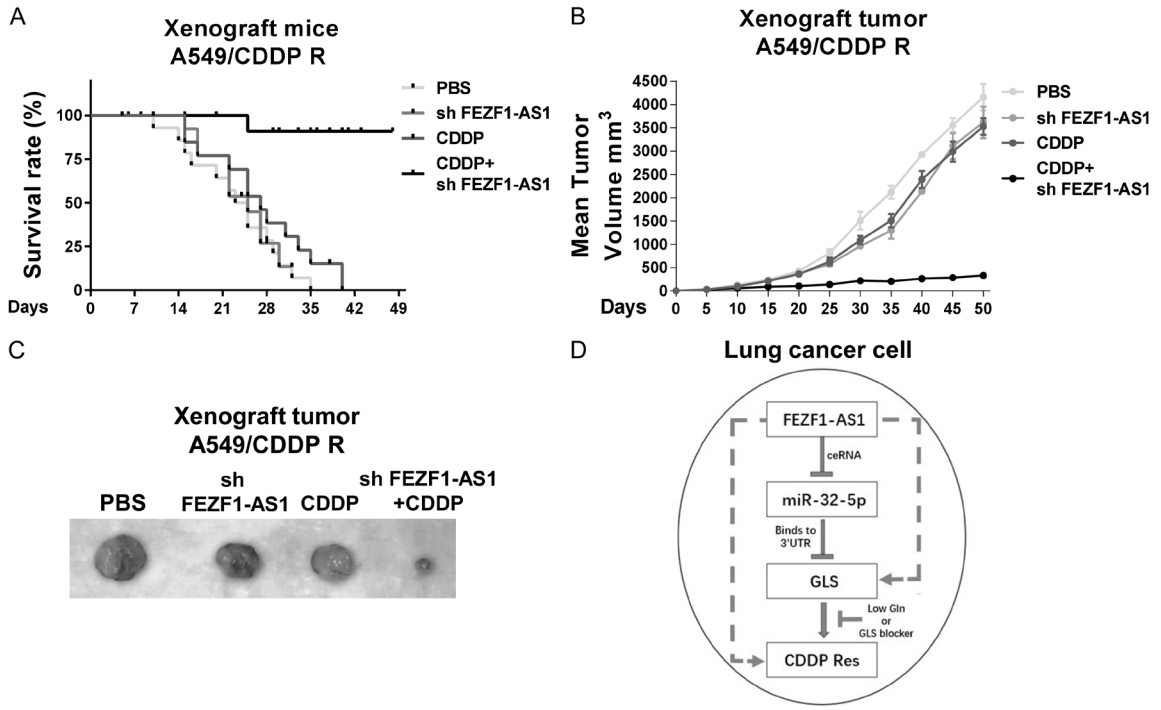

Figure 10. *In vivo* xenograft mice experiments. (A) A549 cisplatin resistant cells were subcutaneously injected into nude mice. Mice were randomly grouped (n=10) and treated with PBS control, cisplatin alone, sh FEZF1-AS1 alone or plus cisplatin. Mice survival rates and (B, C) tumor sizes were recorded and analyzed. (D) Flowchart shows the molecular mechanism of this study.

[3]. Cisplatin is an effective chemotherapeutic agent that interacts with purine bases in DNA, inducing DNA damage and apoptosis of cancer cells [5]. However, studies have reported that a large percentage of NSCLC patients develop cisplatin resistance, limiting the clinical applications of chemotherapy. Therefore, the investigation of novel and effective strategies to overcome chemoresistance is an urgent task in the treatment of NSCLC. In this study, we demonstrated the involvement of lncRNA/miRNA-mediated cisplatin resistance in NSCLC. Our results, obtained from clinical patient specimens and a drug-resistant cell line in vitro, showed for the first time that lncRNA FEZF1-AS1 is positively associated with cisplatin resistance in lung cancer, consistent with previous studies that revealed oncogenic roles of FEZF1-AS1 in NSCLC [13, 14]. Moreover, blocking FEZF1-AS1 effectively resensitized cisplatin-resistant lung cancer cells, suggesting that FEZF1-AS1 may be a potential therapeutic target for lung cancer.

A number of studies have indicated that long non-coding RNAs (lncRNAs) function by sponging their target microRNAs (miRNAs) to form a competing endogenous RNA (ceRNA) network, resulting in the de-repression of downstream targets of miRNAs [20]. Bioinformatics analysis indicated that FEZF1-AS1 contains binding sites for miR-32-5p, and this RNA-RNA complex was further verified through RNA pull-down and luciferase assays. Other studies have reported that miR-32-5p plays tumor suppressive roles in multiple cancers [22-24]. In this study, we found that miR-32-5p is negatively associated with cisplatin resistance in lung cancer, as it was significantly downregulated in cisplatin-resistant lung cancer cells. Additionally, we identified GLS as a target of miR-32-5p in lung cancer cells through bioinformatics analysis and luciferase reporter assays, which showed that miR-32-5p directly targets the 3'UTR of GLS. The biological roles of the miR-32-5p-GLS association were further validated through rescue experiments, which showed that the re-expression of GLS in miR-32-5p overexpressing lung cancer cells successfully rescued cisplatin resistance. For the first time, we reported this miRNA/mRNA association, which was specific in NSCLC cells.

Reprogramming cellular metabolism, such as glutamine metabolism, is recognized as a key characteristic of cancer cells that contributes to rapid proliferation and selective advantages against chemotherapy [15]. Additionally, blocking glutamine metabolism has been shown to effectively inhibit tumor progression and chemoresistance [17]. In this study, we consistently observed elevated glutamine metabolism in cisplatin-resistant lung cancer cells through glutamine uptake and GLS activity assays. Importantly, we found that cisplatin-resistant cells displayed increased sensitivity to cisplatin under conditions of low glutamine supply compared to normal conditions, suggesting that blocking glutamine metabolism is an effective strategy for overcoming cisplatin resistance in NSCLC cells. In addition to A549 CDDP R cells, we established another cisplatin resistance cell line from SK-MES-1 cells (Figure S9A). Consistent with A549 CDDP R cells, FESZF1-AS1 and GLS were upregulated and miR-32-5p was downregulated in SK-MES-1 CDDP R cells (Figure S9B-D). The glutamine metabolism rate was significantly elevated in SK-MES-1 CDDP R cells (Figure S9E). Silencing FEZF1-AS1 or GLS and overexpression of miR-32-5p effectively sensitized SK-MES-1 CDDP R cells to cisplatin (Figure S9F-H). Results from two cisplatin resistant lung cancer cell lines consolidated the above *in vitro* results. Finally, we performed mechanism rescue experiments that recovery of miR-32-5p in FEZF1-AS1 overexpressing CDDP resistant lung cancer cells successfully re-suppressed the GLS and glutamine metabolism, leading to the re-sensitization of cisplatin resistance lung cancer cells to cisplatin. Importantly, results from *in vitro* study were further validated through *in vivo* xenograft experiments. Initially, we examined the expression of lncRNA FEZF1-AS1, miR-32-5p, and GLS in various lung cancer cell lines to expand the scope of our findings to broad our results/conclusions to all lung cancer cell lines. Subsequently, we conducted all functional assays using two lung cancer cell lines, in order to prevent irreproducibility of results in other lung cancer cell lines.

This study has demonstrated an FEZF1-AS1-miR-32-5p-GLS axis in regulating cisplatin resistance in lung cancer. Previous studies have reported the oncogenic role of FEZF1-AS1 [9-14] and the tumor-suppressive role of miR-32-5p in various cancers [22-24]. GLS, a downstream molecule of this axis, has been reported to have critical roles in modulating cancer cell proliferation [25], migration and invasion [26]. Therefore, the FEZF1-AS1-miR-32-5p-GLS axis may play essential roles in regulating diverse lung cancer progressions. However, it is important to acknowledge the limitations of this study, as the present data does not rule out the possibility of other potential miRNA targets of FEZF1-AS1 being involved in regulating cisplatin resistance in lung cancer cells. Recent research has shown that FEZF1-AS1 can target multiple miRNAs, including miR-1254 [27], miR-363 [28], and miR-632 [29]. Consistent with miR-32-5p, these FEZF1-AS1-sponged miRNAs functioned as tumor-suppressive molecules in various cancers and targeted specific mRNAs. We admit that FEZF1-AS1 might target other miRNAs to regulate chemoresistance in lung cancer. For instance, miR-363, which was sponged by FEZF1-AS1, directly targeted SphK2 [30]. SphK2 was further revealed to contribute to 5-Fu resistance in colon cancer cells [31] and gefitinib resistance in lung cancer cells [32]. Thus, FEZF1-AS1 might promote cisplatin resistance of lung cancer through the miR-363-SphK2 pathway. In this study, through *in vitro* and *in vivo* experiments, identified a novel target of FEZF1-AS1, miR-32-5p, and integrated the FEZF1-AS1-miR-32-5p-GLS axis with cisplatin resistance in lung cancer.

In summary, this study unveiled the biological roles and molecular mechanisms of the lncRNA-FEZF1-AS1-promoted cisplatin resistance of NSCLC cells through sponging miR-32-5p as a ceRNA to regulate GLS expression and glutamine metabolism. These conclusions will be benefit to the development of novel therapeutic approaches against chemoresistant lung cancer.

Disclosure of conflict of interest

None.

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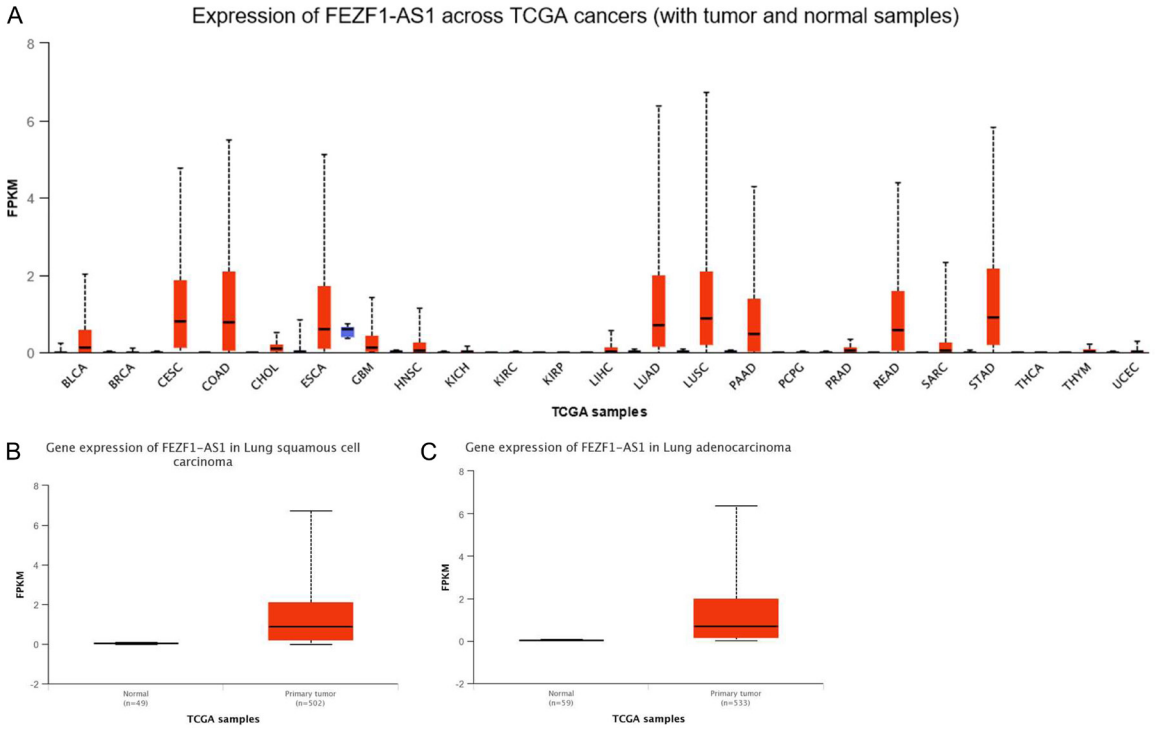

Figure S1. Expression of FEZF1-AS1 in NSCLC and other cancers across TCGA cancers (with normal and tumor specimens) analyzed from http://ualcan.path.uab.edu. A. Expression of FEZF1-AS1 across TCGA cancers (with normal and tumor specimens). B. Expression of FEZF1-AS1 in normal and lung squamous cell carcinoma. C. Expression of FEZF1-AS1 in normal and lung adenocarcinoma.

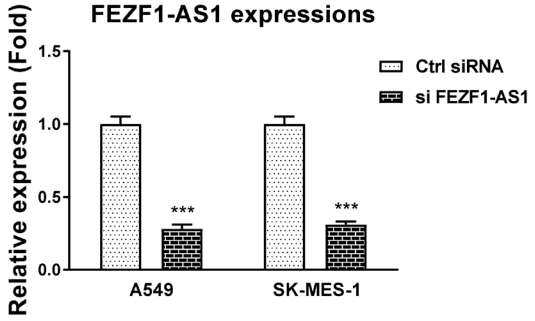

Figure S2. FEZF1-AS1 was knocked down in A549 and SK-MES-1 cells.

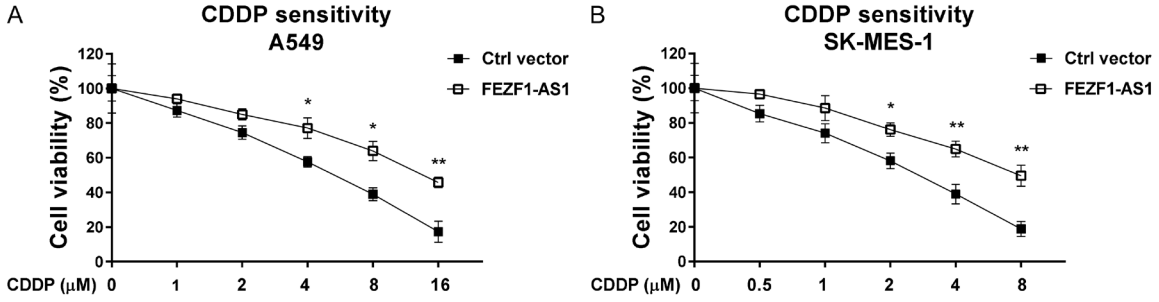

Figure S3. Overexpression of FEZF1-AS1 attenuates the cisplatin sensitivity of lung cancer cells. (A) A549 and (B) SK-MES-1 cells were transfected with control vector or FEZF1-AS1 overexpression vector for 48 hours, followed by treatment with cisplatin at the indicated concentrations. Cell viability was examined by MTT assay. *, $P<0.05$; **, $P<0.01$.

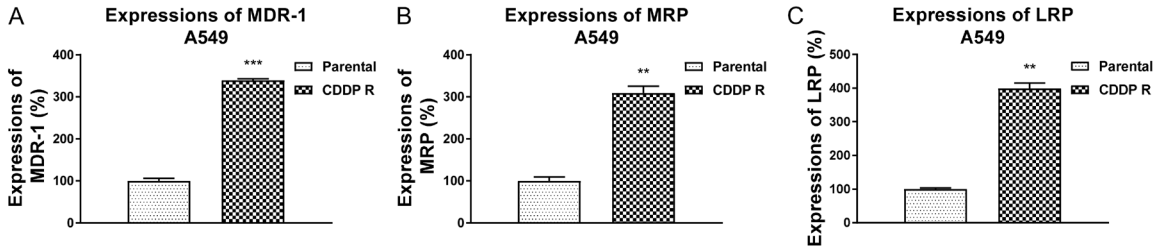

Figure S4. Drug resistance markers expressions in A549 cisplatin resistant cells.

	FEZF1-AS1 Low (n=12)	FEZF1-AS1 High (n=38)
CDDP Sensitive (n=17)	11 (64.7%)	6 (35.3%)
CDDP Resistant (n=33)	1 (3%)	32 (97%)

Figure S5. Statistical analysis of the correlation between FEZF1-AS1 expression and cisplatin resistance in lung cancer patients.

	miR-32-5p Low (n=36)	miR-32-5p High (n=14)
CDDP Sensitive (n=17)	5 (29.4%)	13 (70.6%)
CDDP Resistant (n=33)	31 (93.9%)	2 (6.1%)

Figure S6. Statistical analysis of the correlation between miR-32-5p expression and cisplatin resistance in lung cancer patients.

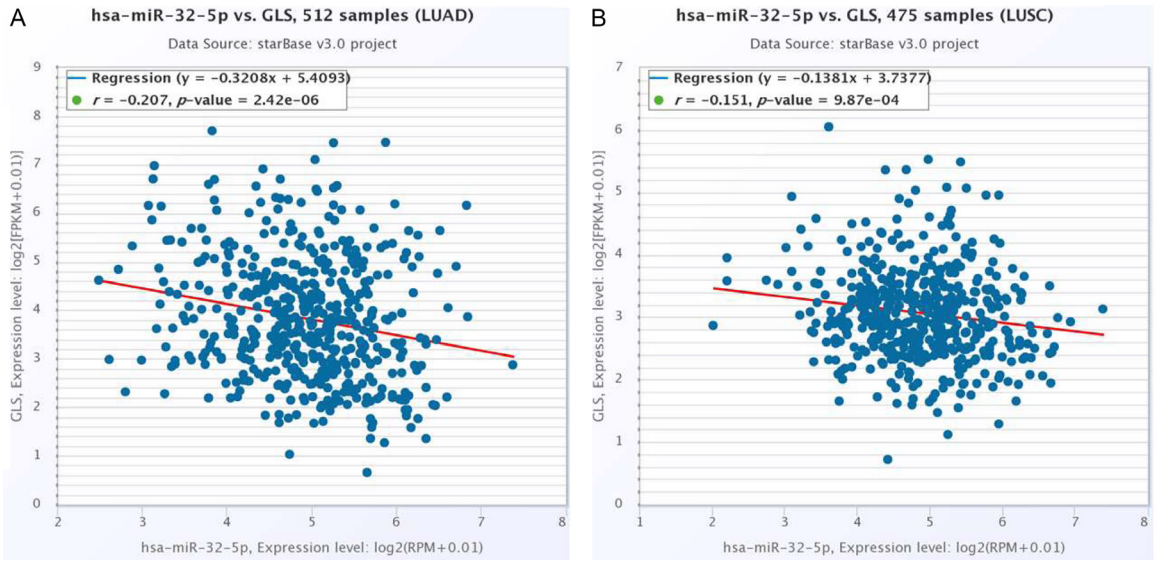

Figure S7. Correlations between miR-32-5p and GLS in (A) lung adenocarcinoma and (B) lung squamous cell carcinoma analyzed from starBase.

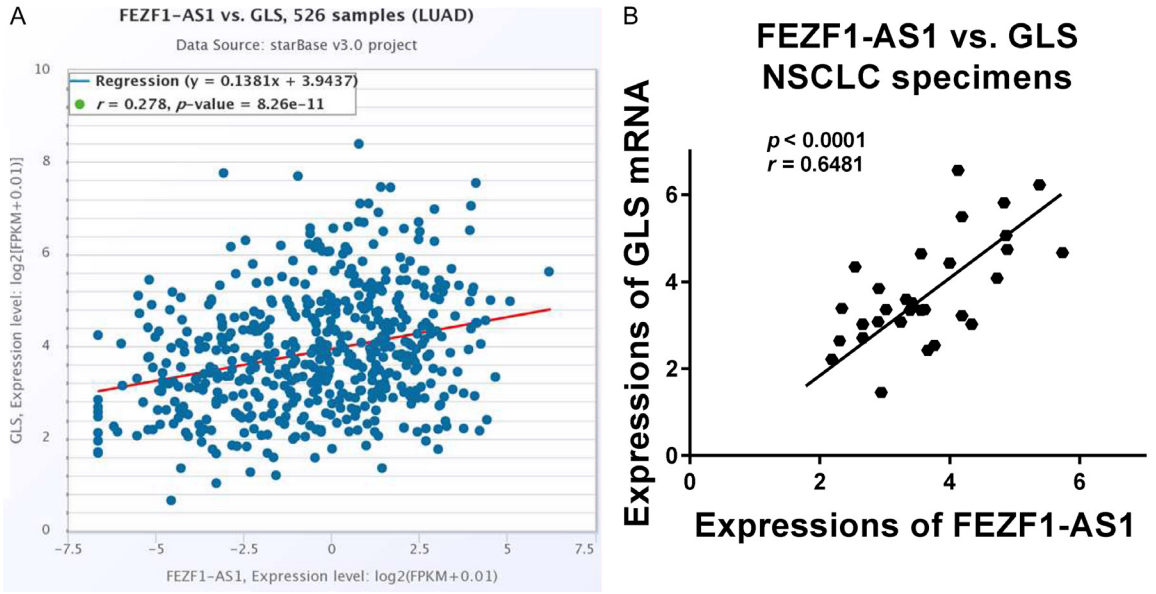

Figure S8. Correlations between FEZF1-AS1 and GLS in lung cancer analyzed from (A) starBase and (B) NSCLC patients.

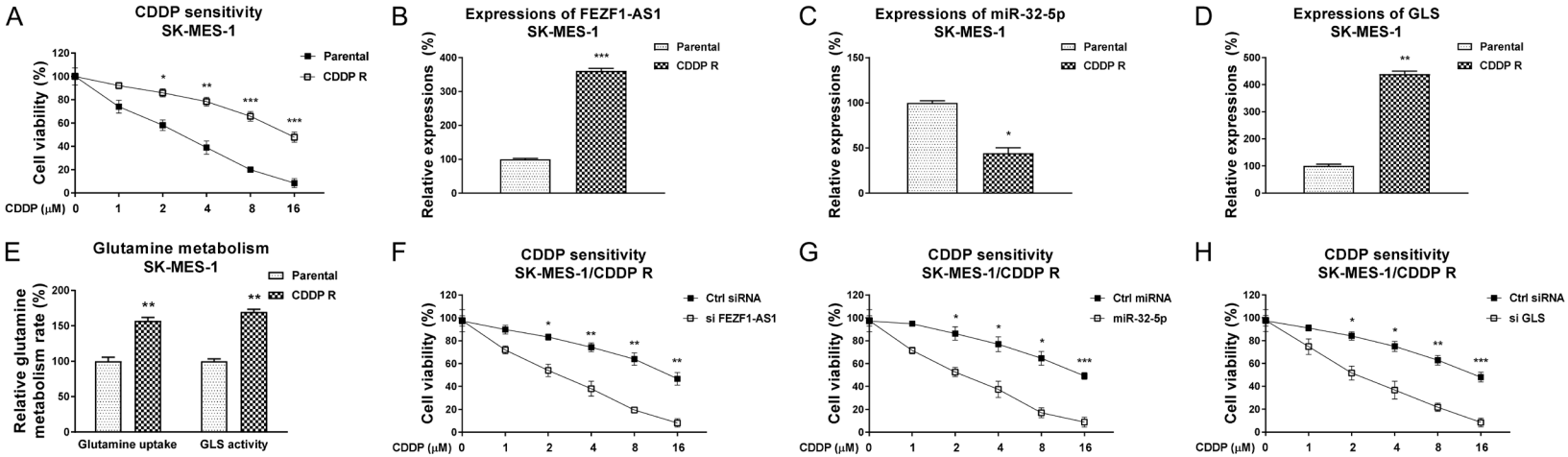

Figure S9. Phenotypes in SK-MES-1 cisplatin resistant cells. (A) Validation of cisplatin resistance in SK-MES-1/CDDP R cells by cell viability assay. (B) Expressions of FEZF1-AS1, (C) miR-32-5p and (D) GLS in SK-MES-1 parental and cisplatin resistant cells. (E) Glutamine uptake and GLS activity were examined in SK-MES-1 parental and cisplatin resistant cells. (F-H) SK-MES-1 cisplatin resistant cells were transfected with control or siFEZF1-AS1, miR-32-5p or siGLS, followed by treatments with cisplatin. Cell viabilities in response to cisplatin were detected by MTT assay. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.