

Original Research

LINC00520 Regulates VDAC1 Oligomerization to Promote Cisplatin Resistance in Lung Adenocarcinoma

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Abstract

Background: Platinum-based chemotherapy remains the primary treatment for lung adenocarcinoma (LUAD), although its efficacy is frequently limited by acquired cisplatin resistance. The involvement of long non-coding RNAs (lncRNAs) in this resistance is not well understood. **Methods:** Differentially expressed lncRNAs were identified through bioinformatics analysis. LINC00520 expression was further validated in LUAD clinical specimens and cisplatin (CDDP)-resistant A549 cells. MS2-tagged RNA affinity purification (MS2-TRAP) coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to screen for LINC00520-interacting proteins, which were subsequently validated by RNA immunoprecipitation (RIP). Functional assays, including Cell Counting Kit-8 (CCK-8), colony formation, and apoptosis analysis, were conducted. Voltage-dependent anion-selective channel protein 1 (VDAC1) oligomerization and mitochondrial apoptosis were assessed by western blotting and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) staining. *In vivo* xenograft models were used to corroborate key findings. **Results:** LINC00520 was markedly upregulated in LUAD tissues and A549/CDDP-resistant cells, and elevated expression correlated with poor prognosis. Functionally, LINC00520 promoted proliferation, suppressed apoptosis, and enhanced cisplatin resistance, whereas its knock-down restored chemosensitivity. Mechanistically, LINC00520 directly bound VDAC1 and inhibited its oligomerization, thereby suppressing mitochondrial apoptosis. *In vivo*, LINC00520 silencing suppressed tumor growth and partially restored cisplatin sensitivity. **Conclusions:** LINC00520 drives cisplatin resistance in LUAD by modulating VDAC1-mediated mitochondrial apoptosis, suggesting that LINC00520 may represent a potential prognostic biomarker and therapeutic target for reversing cisplatin resistance in LUAD.

Keywords: lung adenocarcinoma; LINC00520; VDAC1; oligomerization; cisplatin; drug resistance

1. Introduction

Lung cancer remains the top cause of cancer-related fatalities around the world, with lung adenocarcinoma (LUAD) comprising over half of all cases [1,2,3]. Even with progress in targeted and immune therapies, platinum-based chemotherapy, especially cisplatin, remains central to LUAD treatment [4]. Cisplatin (CDDP) exerts its anti-tumor activity mainly by forming DNA adducts that trigger apoptotic cell death [5]. Nevertheless, the frequent emergence of acquired resistance severely compromises its clinical efficacy, posing a major barrier to improving patient outcomes in LUAD [6,7].

Long noncoding RNAs (lncRNAs) have emerged as pivotal regulators of tumor biology, with growing evidence implicating them in chemoresistance through diverse mechanisms, including epigenetic remodeling, transcriptional

control, and post-transcriptional regulation [8,9,10]. Prior studies indicate that modulating specific lncRNAs can alter the cisplatin response of lung cancer cells. For example, suppression of Lymphocyte antigen 6K antisense RNA 1 sensitizes resistant xenografts to cisplatin by interfering with mitotic progression [11]. Similarly, LINC00665 has been shown to enhance cisplatin resistance in LUAD via Enhancer of zeste homolog 2-mediated downregulation of cyclin-dependent kinase inhibitor 1C [12]. Despite these findings, the involvement of lncRNAs in cisplatin resistance in LUAD remains incompletely understood, warranting further investigation.

Through bioinformatics analysis, we identified LINC00520 as being overexpressed in both the cisplatin-resistant A549 cell line (A549/CDDP) and LUAD tissues. LINC00520 is 2092 bp in length and located on chro-



mosome 14q22.3. Its upregulation has been reported in breast cancer, where knockdown of this lncRNA reduces metastatic and invasive capabilities [13]. Elevated LINC00520 levels have also been detected in various malignancies, including melanoma, nasopharyngeal carcinoma, and glioma, pointing to a potential oncogenic role [14,15,16]. In non-small cell lung cancer, existing evidence suggests that LINC00520 promotes disease progression via the miR-1252-5p/FOXR2 axes, with its transcription being directly activated by Specificity protein 1 [17,18]. Nevertheless, whether LINC00520 contributes to cisplatin resistance specifically in LUAD remains to be determined.

In this study, we investigated the functional role of LINC00520 in LUAD cisplatin resistance and explored the underlying molecular mechanisms. We found that LINC00520 is significantly upregulated in cisplatin-resistant LUAD cells and tumor tissues. Functionally, LINC00520 promotes cisplatin resistance both *in vitro* and *in vivo* by directly binding to voltage-dependent anion-selective channel protein 1 (VDAC1), thereby inhibiting VDAC1 oligomerization and suppressing mitochondria-mediated apoptosis. Collectively, these results identify LINC00520 as an essential factor in chemotherapy resistance and suggest its potential as a prognostic marker and therapeutic target to address cisplatin resistance in LUAD.

2. Materials and Methods

2.1 Bioinformatics Analysis

To identify lncRNAs associated with cisplatin resistance in LUAD, two datasets, GSE43493 and GSE108214, were retrieved from the Gene Expression Omnibus (GEO) (National Center for Biotechnology Information, Bethesda, MD, USA) database, which contain lncRNA expression profiles of A549 and cisplatin-resistant A549 (A549/CDDP) cells [19,20,21]. Differential expression of lncRNAs was assessed using the limma package in R and Student's *t*-test, with thresholds of $|\log_2(\text{fold-change})| \geq 1$ and $p < 0.05$. Visualization was done using volcano plots created with the ggplot2 package (version 3.5.1; Posit Software, PBC, Boston, MA, USA) [22,23]. Overexpressed lncRNAs in LUAD were screened from the LncCAR database (Sun Yat-sen University, Guangzhou, China) [24] using criteria of $\log_2(\text{fold-change}) \geq 0.6$ and $p < 0.05$. Coding potential was predicted using LNCipedia (version 5.2; Center for Medical Genetics, Ghent University, Ghent, Belgium) [25]. Survival analysis for LINC00520 was performed using clinical data from the TCGA-LUAD and GSE50081 cohorts [26]. From the Genomics of Drug Sensitivity in Cancer (GDSC) (release 8.5; Wellcome Sanger Institute, Cambridge, UK) database, the half-maximal inhibitory concentration (IC₅₀) of cisplatin in LUAD cell lines was retrieved [27].

2.2 Cell Culture and Establishment of A549/CDDP Cell Line

BEAS-2B, A549, H1975, and CALU-3 cells were grown in RPMI-1640, while HEK293T cells were maintained in DMEM (both from Gibco, Waltham, MA, USA); all media contained 10% fetal bovine serum and 1% penicillin/streptomycin (New Cell & Molecular Biotech, Suzhou, China). The cell lines were sourced from the Cancer Research Institute's cell bank at Central South University (Changsha, China) [28,29]. The cisplatin-resistant A549 subline (A549/CDDP) was established by exposing parental A549 cells to stepwise increasing concentrations of cisplatin (Qilu Pharmaceutical, Shandong, China), starting from the IC₅₀ (5 μ M). The induction process lasted 180 days, during which cells were subjected to repeated 24- to 72-hour drug exposure cycles. All experiments were performed using cells at passages 3–10. Each cell line was authenticated by short tandem repeat (STR) profiling and tested negative for mycoplasma contamination.

2.3 Clinical Tissue Samples

Paired frozen LUAD tissues and adjacent non-cancerous lung tissues were collected from 30 patients who had received no prior radiotherapy or chemotherapy. All surgeries were performed at the Hunan Cancer Hospital (Changsha, China). Histopathological examination confirmed the diagnosis of each specimen. The Ethics Committee of Hunan Cancer Hospital approved this study (Ethical lot number: KYJJ-2023-004), and all patients involved provided written informed consent. Clinicopathological characteristics of the cohort are summarized in Table 1.

2.4 RNA Extraction and RT-qPCR Analysis

Total RNA was extracted using TRIzol reagent from Thermo Fisher Scientific, based in Waltham, MA, USA. The reverse transcription process utilized a random primer kit from Vazyme Biotech (Nanjing, China). Using SYBR Green qPCR Master Mix (RiboBio, Guangzhou, China), gene expression was quantified on a real-time PCR cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). β -Actin acted as the reference control, and the $2^{-\Delta\Delta C_t}$ was employed to calculate relative transcript levels. The sequences for primers are listed in Table 2.

2.5 Plasmid Construction and Transfection

The full-length human LINC00520 sequence (NCBI transcript ID: NR_026796.1) was amplified by high-fidelity PCR (Eppendorf SE, Hamburg, Germany) and cloned into pCDH-puro and pcDNA3.1-12xMS2 vectors for overexpression. For knockdown, shRNAs targeting LINC00520 were synthesized (Vigene Biosciences, China) and inserted into the pLKO.1-puro vector. All plasmids were delivered into cells using Lipofectamine 3000 reagent (Thermo Fisher Scientific). The shRNA sequences are provided in Table 2.

Table 1. Clinical information of 30 enrolled patients.

Serial number	Gender	Age	TNM stage	Clinical stage
1	Male	69	T1aN0M0	Ia
2	Female	61	T1aN0M0	Ia
3	Female	66	T2N2M0	IIIa
4	Female	65	T1cN0M0	Ia
5	Female	63	T1bN0M0	Ia
6	Female	71	T1cN0M0	Ia
7	Male	64	T3N2M0	IIIb
8	Female	47	T1aN0M0	Ia
9	Male	71	T2bN0M0	IIa
10	Male	61	T2aN0M0	Ib
11	Male	40	T1cN1M0	IIb
12	Male	54	T2bN0M0	IIa
13	Female	63	T1bN0M0	Ia
14	Female	47	T3N1M0	IIIa
15	Female	52	T1cN0M0	Ia
16	Male	42	T2aN1M0	IIa
17	Male	41	T2aN2M0	IIIa
18	Female	49	T1bN0M0	Ia
19	Female	51	T1aN0M0	Ia
20	Female	60	T1bN0M0	Ia
21	Female	70	T1aN0M0	Ia
22	Male	53	T3N1M0	IIIa
23	Female	38	T2bN0M0	IIa
24	Female	51	T1cN1M0	IIb
25	Male	56	T3N2M0	IIIb
26	Male	60	T3N1M0	IIIa
27	Male	58	T1bN0M0	Ia
28	Female	69	T3N2M0	IIIb
29	Female	56	T1bN2M0	IIIa
30	Male	62	T3N2M0	IIIb

TNM: Tumor-Node-Metastasis (according to the American Joint Committee on Cancer [AJCC] 8th edition).

2.6 Generation of Stable Cell Lines and Pharmacological Inhibition

Lentiviral particles for LINC00520 overexpression (pCDH-LINC00520-puro) or knockdown (pLKO.1-shLINC00520-puro) were generated in HEK293T cells using the packaging plasmids pMD2.G and psPAX2. The resulting lentiviral particles were used to transduce A549 and A549/CDDP cells. Stable transductants were selected with puromycin (10 µg/mL) yielding the following lines: A549-Vector, A549-LINC00520, A549/CDDP-shCTR, and A549/CDDP-shLINC00520. VBIT4, a VDAC1 oligomerization inhibitor (Topscience, Shanghai, China), was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) and used at a concentration of 2 µM [30]. For single-agent treatment, cells were incubated with VBIT4 alone for 48 h. For combination experiments, cells were pretreated with 2 µM VBIT4 for 2 h, followed by co-culture with or without 10 µM cisplatin for an additional 48 h. Control cells were treated with an equivalent volume of DMSO for the same duration.

2.7 Cell Viability, Proliferation, and Colony Formation Assays

Cell viability was evaluated using the Cell Counting Kit-8 (CCK-8) assay (New Cell & Molecular Biotech). For IC₅₀ determination, cells were seeded in 96-well plates at 1000 cells per well and exposed to escalating concentrations of cisplatin (0–48 µM) for 48 h. Using GraphPad Prism 7 (version 7.00; GraphPad Software, Boston, MA, USA), IC₅₀ values were computed via nonlinear regression analysis. Cells were exposed to 10 µM cisplatin for proliferation studies, with CCK-8 assays carried out daily from the start until day 4. For colony formation, cells were placed in 6-well plates with 3000 cells per well and kept in a medium with 10 µM cisplatin for 14 days. The colonies were counted using ImageJ software (version 1.54g; U.S. National Institutes of Health, Bethesda, MD, USA) after they were fixed and stained with crystal violet.

2.8 Apoptosis and Mitochondrial Membrane Potential (MMP) Detection

After cisplatin treatment (10 µM, 48 h), apoptosis was assessed by flow cytometry using Annexin V-FITC/PI staining (MultiSciences Biotech, Hangzhou, China). Specifically, cells were first gated on the dot plot with FSC on the x-axis and SSC on the y-axis to exclude cell debris and doublets. Within the selected cell population, the RedFL: APC channel was assigned to the x-axis and the BluFL: PI channel to the y-axis; cells were then distributed across four quadrants. Quadrant boundaries were set based on the blank group without any treatment. The quadrant gating parameters defined from the blank group were then automatically applied to all other groups. MMP was evaluated with the 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) staining kit (MultiSciences), and the red/green fluorescence intensity ratio was measured via flow cytometry. Data analysis was performed using FlowJo software (version 10.8.1; FlowJo, LLC, Ashland, OR, USA).

2.9 MS2-Tagged RNA Affinity Purification (MS2-TRAP) and LC-MS/MS

MS2-TRAP was performed to identify proteins interacting with LINC00520. Both pcDNA3.1-LINC00520-12xMS2 and pcDNA3.1-MS2-Flag were used to co-transfect A549 cells. After 48 h, cells were crosslinked with UV light (362 nm, 30 min) and lysed. Immunoprecipitation was performed using Protein A/G magnetic beads (Bimake, Houston, TX, USA) that had been pre-incubated with anti-Flag antibody (Thermo Fisher Scientific) or normal mouse IgG (Millipore, Burlington, MA, USA). Captured proteins were resolved by SDS-PAGE, followed by in-gel tryptic digestion and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (Thermo Fisher Scientific). Raw data were processed using Proteome Discoverer (version 2.4; Thermo Fisher Scientific), and pathway en-

Table 2. The list of sequences for primers and shRNA.

Gene name	Primers
<i>β-actin</i> -F	5'-CTCCATCCTGGCCTCGCTGT-3'
<i>β-actin</i> -R	5'-GCTGTACACCTTCACCGTTCC-3'
<i>LINC00520</i> -F	5'-GCTCCTCCTTCACCTTCT-3'
<i>LINC00520</i> -R	5'-TCCTTGCCACCAATCTC-3'
<i>LINC00648</i> -F	5'-GGCATTAGAGGACGAG-3'
<i>LINC00648</i> -R	5'-ACTGTGGGTGGGTTAGA-3'
<i>FLJ42351</i> -F	5'-CCTTATCTTTCCAACACTG-3'
<i>FLJ42351</i> -R	5'-TACTTGCTGGCATCCTG-3'
<i>SPIN3</i> -F	5'-AGTGGGAGTCCGATGTGGG-3'
<i>SPIN3</i> -R	5'-AATCAGCGGGCGAGCAG-3'
<i>HOTAIRM1</i> -F	5'-ATTCTGCCTCTATTACCA-3'
<i>HOTAIRM1</i> -R	5'-AAATGACTAGGGCTTCTA-3'
<i>PCED1B-AS1</i> -F	5'-TTCTTCCAATCCGTTCT-3'
<i>PCED1B-AS1</i> -R	5'-TCTTCAGGCTTTATCCC-3'
<i>TP53TG1</i> -F	5'-TTTACCCAACTTCTCGC-3'
<i>TP53TG1</i> -R	5'-TGTCTTCTCATTCTTCTCCCT-3'
<i>ABCB1</i> -F	5'-AGTTGAGTGGTGGGCAGAAG-3'
<i>ABCB1</i> -R	5'-ACCACTGCTTCGCTTTCTGT-3'
<i>ABCC1</i> -F	5'-AGCCAGTGAAGCAAGATA-3'
<i>ABCC1</i> -R	5'-TTTGGTATGAGGCTTGCTGTA-3'
<i>LRP</i> -F	5'-GAGCAGTTCACAGTGTGTCC-3'
<i>LRP</i> -R	5'-GCGTGACGACAGAAACCGAAA-3'
<i>shLINC00520-1</i>	5'-GAATAGTAGTAGTTAACAATGCTCGAGCATTGTTAACTACTACTATTCTTTTG-3'
<i>shLINC00520-2</i>	5'-GGACTGCAATTCACAAGAACAACCTCGAGTGTCTTGTGAATTGCAGTCCTTTTG-3'
<i>shCTR</i>	5'-TTCTCCGAACGTGTACGTTTCAAGAGAACGTGACACGTTTCGGAGAATTTTT-3'

richment was analyzed using the clusterProfiler R package based on KEGG pathways (release 119.0; Kanehisa Laboratories, Institute for Chemical Research, Kyoto University, Kyoto, Japan), using a false discovery rate (FDR) threshold of <0.05.

2.10 Western Blot Analysis

Following cisplatin treatment (10 μ M, 48 h), total protein and mitochondrial protein fractions were isolated using cell lysis buffer (Beyotime, Shanghai, China) and the Minute Mitochondria Isolation Kit (Invent Biotech, Beijing, China), respectively. Proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked and then incubated with rabbit primary antibodies against VDAC1 (monoclonal, 81538-1-RR; 1:1000), cytochrome c (polyclonal, 10993-1-AP; 1:1000), GAPDH (polyclonal, 10494-1-AP; 1:1000), and COX IV (polyclonal, 11242-1-AP; 1:1000), all of which were obtained from Proteintech (Wuhan, China). After washing, the blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (polyclonal, SA00001-2; 1:10,000 Proteintech, Wuhan, China). Immunoreactive signals were visualized using the Bio-Rad ChemiDoc XRS imaging system (Bio-Rad Laboratories, Hercules, CA, USA). For VDAC1 oligomer detection, samples were not boiled, and reducing

agents (β -mercaptoethanol or DTT) were omitted from the loading buffer. Prior to SDS-PAGE, crosslinking was performed with 1 mM disuccinimidyl suberate for 30 min at room temperature.

2.11 RNA Immunoprecipitation (RIP) Assay

Lysates of A549 cells were incubated with protein A/G magnetic beads pre-coated with either 5 μ g of anti-VDAC1 antibody or 5 μ g of control IgG (monoclonal, 98136-1-RR; Proteintech, Wuhan, China). Following overnight incubation at 4 $^{\circ}$ C, RNA was extracted from the immunoprecipitated complexes. Enrichment of LINC00520 was then assessed by RT-qPCR and confirmed by agarose gel electrophoresis.

2.12 RNA Fluorescence In Situ Hybridization (FISH) Assay and Immunocytochemistry (ICC) Assays

For FISH, cells were fixed with 4% paraformaldehyde, permeabilized, and hybridized with digoxigenin-labeled LINC00520 probes (Sangon Biotech, Shanghai, China). Hybridization signals were detected using an anti-digoxigenin antibody (ab420, Abcam, Shanghai, China) followed by a YF 594-conjugated secondary antibody (SA00013-3, Proteintech, Wuhan, China). The LINC00520 probes are a pool containing three probes: LINC00520 probe-1, 5'-CAAATAGCACTCCTCTGCTTCTATAAA

ATTTGTGAAATTC-3'; LINC00520 probe-2, 5'-TCCTT TAAGGTAGAGACTTTGAACAAATTGTTGAAATCT A-3'; LINC00520 probe-3, 5'-TGAGGTCCAAGTCACA AGAAGTCCAAGGCACAAGAAGCT-3'.

For ICC, cells were incubated with anti-VDAC1 antibody (81538-1-RR, 1:200), followed by YF 488-conjugated secondary antibody (Polyclonal, SA00013-2, 1:1000, Proteintech, Wuhan, China). Nuclei were counterstained with 4',6-diamidino-2-phenylindole. Images were acquired using confocal microscope Ultra-View Vox (PerkinElmer, Inc., Waltham, MA, USA). For colocalization analysis, the red (LINC00520) and green (VDAC1) fluorescence signals were overlaid using ImageJ software. The degree of colocalization was measured by determining Pearson's correlation coefficient from at least three randomly chosen fields.

2.13 *In Vivo* Xenograft Model

All animal studies received approval from the Ethics Committee at Hunan Cancer Hospital (GZ2023-012). Four-week-old female BALB/c nude mice ($n = 64$) were purchased from Hunan SJA Laboratory Animals (Changsha, China). To establish subcutaneous xenografts, 3×10^6 cells from each of the following four groups were injected into the right posterior neck of the nude mice: A549-Vector (control), A549-LINC00520 (overexpression), A549/CDDP-shCTR (resistant control), and A549/CDDP-shLINC00520 (LINC00520 knockdown). For each cell line, mice were randomly divided into two treatment groups ($n = 8$ per group): one received cisplatin (2.5 mg/kg) and the other received an equal volume of normal saline, injected intraperitoneally twice weekly [31]. When tumors reached approximately 5 mm in diameter, treatment was initiated. Throughout the treatment period, no significant body weight loss or overt signs of toxicity (e.g., lethargy, ruffled fur, or decreased activity) were observed in any of the experimental groups. Tumor volume was measured weekly by investigators blinded to the group allocation using the formula: $\text{volume} = (\text{length} \times \text{width}^2) / 2$. At six weeks post-injection, all animals were euthanized by intraperitoneal injection of sodium pentobarbital at a dose of 150 mg/kg body weight (delivered as ~60 μL per 20 g mouse from a 50 mg/mL solution). Death was confirmed by respiratory and cardiac arrest, followed by cervical dislocation as a secondary method. Tumors were excised, weighed, and processed for histology. All animal procedures were performed in accordance with the ARRIVE guidelines, and the ARRIVE 2.0 Essential 10 checklist is provided as **Supplementary Material**.

2.14 *Histological and Immunohistochemical (IHC) Analysis*

Tumor tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned into 4- μm thick slices. Hematoxylin and eosin (H&E) staining was performed for

morphological assessment. For IHC, sections were incubated with a primary antibody against Ki-67 (84192-4-RR, Proteintech, Wuhan, China), followed by detection using a HRP-conjugated secondary antibody (SA00001-2, Proteintech, Wuhan, China) and diaminobenzidine (DAB) substrate. Immunohistochemical staining was evaluated using a semi-quantitative scoring system that incorporated both the percentage of positive cells and the intensity of immunoreactivity. The percentage of positive cells was assigned a score from 0 to 4 (0, $\leq 5\%$; 1, 6–25%; 2, 26–50%; 3, 51–75%; 4, $\geq 76\%$), and the staining intensity was scored from 0 to 3 (0, negative; 1, weak; 2, moderate; 3, strong). The final score (range 0–12) was obtained by multiplying these two scores. To minimize inter-observer variability, all slides were scored independently by two investigators blinded to experimental outcomes, and discrepancies were resolved by consensus.

2.15 *Statistical Analysis*

All experiments were carried out in triplicate, with at least two biological replicates. Data are expressed as mean $\pm$ standard deviation (SD). Normality of continuous variables was assessed using the Shapiro–Wilk test (with a significance threshold of $\alpha = 0.05$), and homogeneity of variances among groups was evaluated using Levene's test ($\alpha = 0.05$). For data that met both normality and variance homogeneity assumptions, group comparisons were performed using Student's *t*-test (for two groups) or one-way analysis of variance (ANOVA) followed by Tukey's post hoc test (for three or more groups). When the assumptions of normality or variance homogeneity were violated, the corresponding non-parametric alternatives were applied: the Mann–Whitney U test for two-group comparisons and the Kruskal–Wallis test followed by Dunn's post hoc test for multiple-group comparisons. All statistical analyses were conducted using GraphPad Prism 7. All reported *p*-values are exact values from these analyses, unless denoted as $p < 0.0001$ for values beyond the software's display precision.

3. Results

3.1 *Identifying lncRNAs Related to Cisplatin Resistance in LUAD Cells*

To identify lncRNAs implicated in cisplatin resistance in LUAD, we re-analyzed the GSE43493 and GSE108214 datasets, which profile lncRNA expression in parental A549 and cisplatin-resistant A549 (A549/CDDP) cells. A total of 117 lncRNAs were found to be upregulated in A549/CDDP cells. Among these, 12 were also overexpressed in LUAD tissues according to the LncAR database. After filtering based on non-coding potential from the LNCipedia database, seven lncRNAs with no predicted coding capacity were selected: LINC00520, LINC00648, FLJ42351, SPIN3, HOTAIRM1, PCED1B-AS1, and TP53TG1 (Fig. 1A–C).

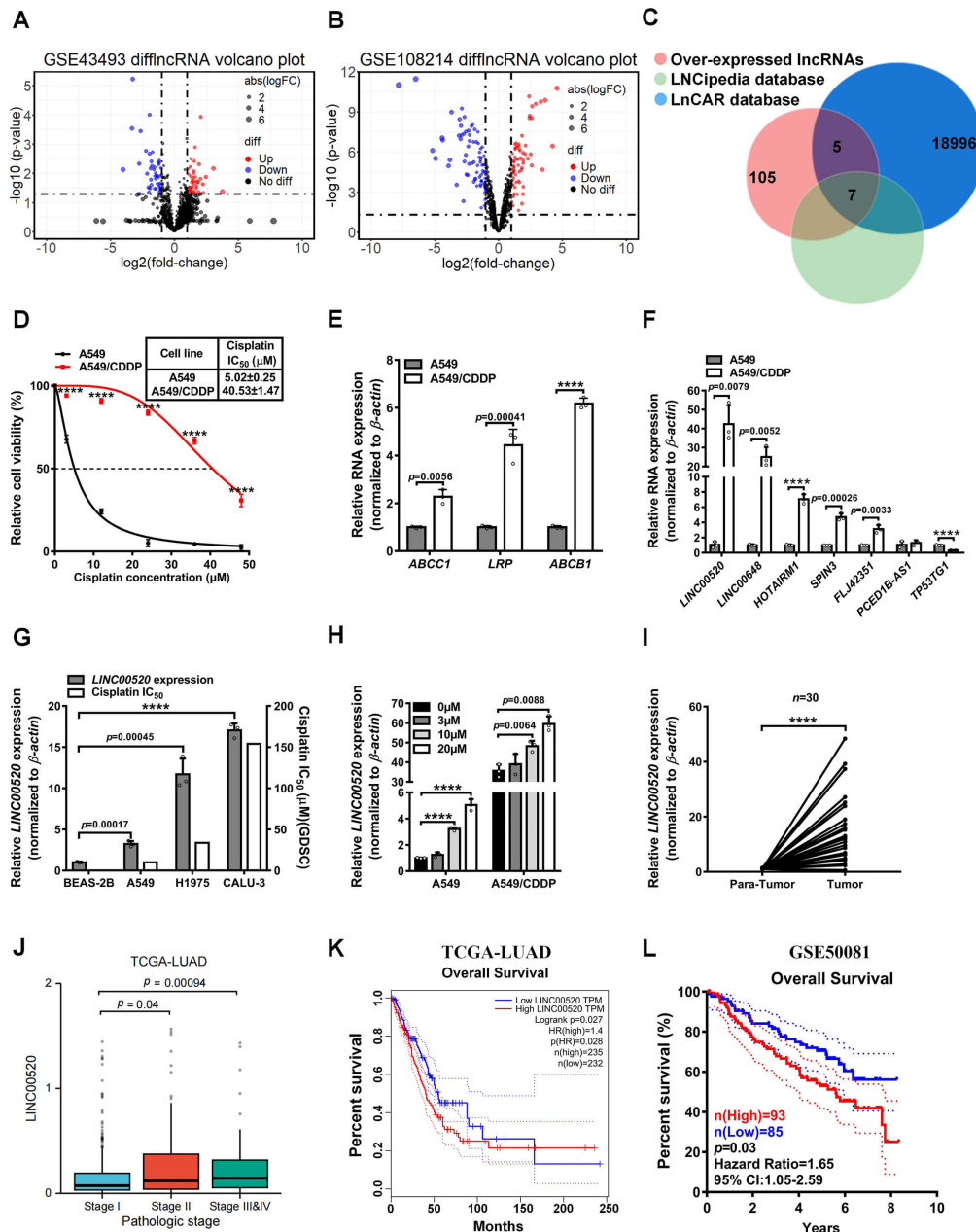


Fig. 1. LINC00520 expression is closely associated with cisplatin resistance in LUAD. (A,B) Volcano plots depicting differential lncRNA expression in the GSE43493 (A) and GSE108214 (B) datasets. (C) Venn diagram of the lncRNA profiling. (D) IC₅₀ of cisplatin in parental A549 and cisplatin-resistant A549 (A549/CDDP) cells. (E) RT-qPCR analysis of three established drug resistance markers—ABCB1, ABCC1, and LRP—in A549 and A549/CDDP cells. (F) RT-qPCR validation of the seven candidate lncRNAs in A549 and A549/CDDP cells. (G) The relationship between LINC00520 expression and cisplatin sensitivity in various LUAD cell lines, as indicated by GDSC database data. (H) LINC00520 expression levels in A549 and A549/CDDP cells following treatment with escalating concentrations of cisplatin (0, 3, 10, and 20 μ M), as measured by RT-qPCR. (I) LINC00520 expression in 30 paired LUAD tumor and adjacent non-tumor tissues, determined by RT-qPCR. (J) LINC00520 expression levels according to tumor stage in the TCGA-LUAD cohort. (K) Kaplan-Meier survival curves for 467 LUAD patients from the TCGA cohort, stratified by high versus low LINC00520 expression. (L) Validation of the association between LINC00520 expression and patient prognosis using the GSE50081 dataset. Data are presented as mean $\pm$ SD. ****, $p < 0.0001$; exact p -values for other comparisons are indicated in the figure. ABCB1, ATP-binding cassette subfamily B member 1; ABCC1, ATP-binding cassette subfamily C member 1; LRP, lung resistance protein; LUAD, lung adenocarcinoma; lncRNA, long non-coding RNA; CDDP, clinical specimens and cisplatin; RT-qPCR, Reverse transcription quantitative polymerase chain reaction; GDSC, Genomics of Drug Sensitivity in Cancer; TCGA, The Cancer Genome Atlas; SD, standard deviation.

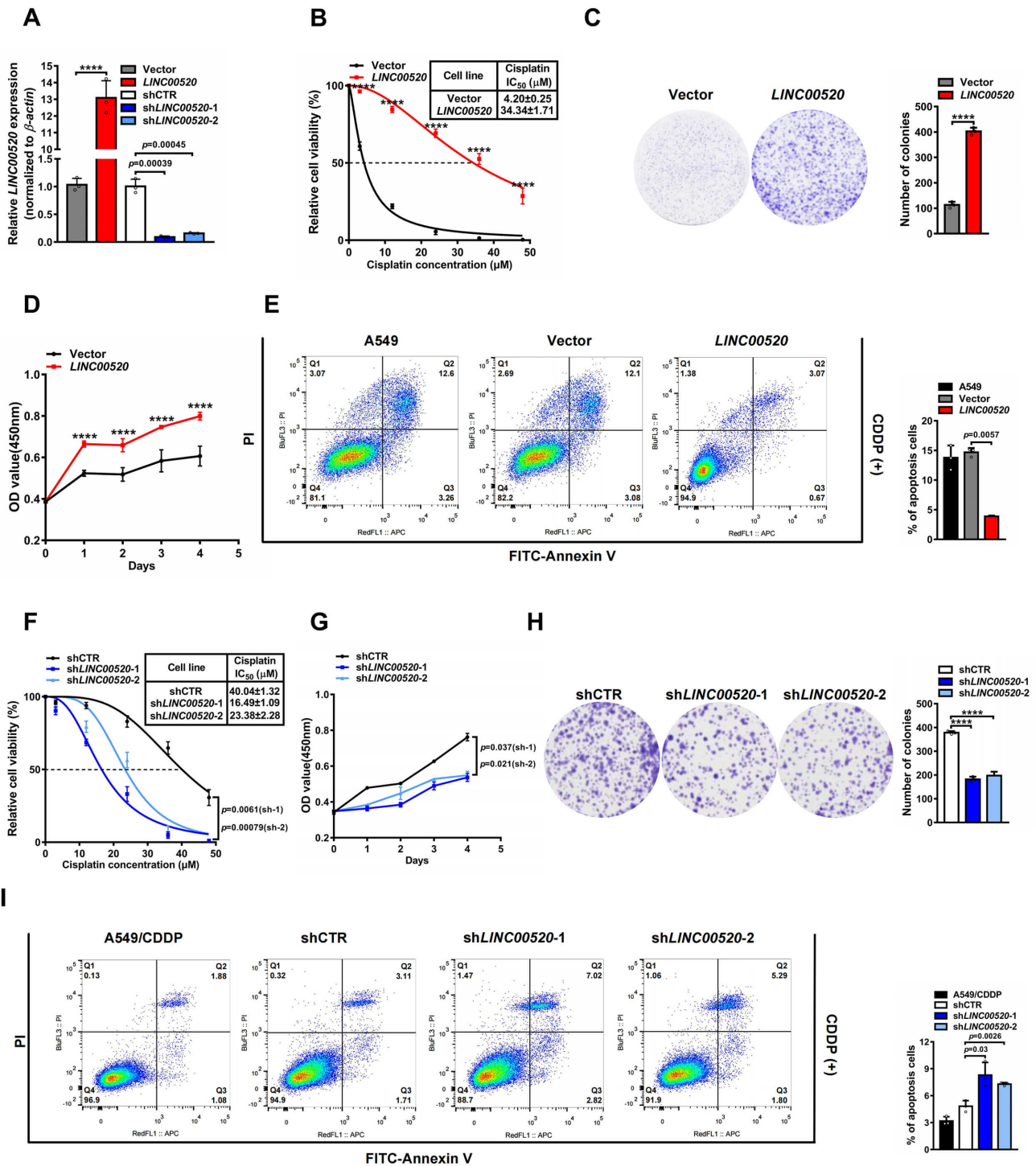


Fig. 2. LINC00520 overexpression reduces cisplatin sensitivity in LUAD cells. (A) RT-qPCR validation of LINC00520 overexpression efficiency in A549 cells and knockdown efficiency in A549/CDDP cells. (B) Cisplatin IC_{50} values in A549-Vector versus A549-LINC00520 cells. (C–E) Effects of LINC00520 overexpression on colony formation (C), cell proliferation (D), and apoptosis (E) in A549 cells treated with cisplatin. Proliferation and colony formation were assessed using CCK-8 and colony formation assays, respectively. Apoptosis was measured by annexin-V/PI double-staining followed by flow cytometry. (F) Cisplatin IC_{50} values in A549/CDDP-shCTR versus A549/CDDP-shLINC00520 cells. (G–I) Effects of LINC00520 knockdown on cell proliferation (G), colony formation (H), and apoptosis (I) in A549/CDDP cells treated with cisplatin. Assays were performed as described in (C–E). Data are presented as mean $\pm$ SD. ****, $p < 0.0001$; exact p -values for other comparisons are indicated in the figure. CCK-8, Cell Counting Kit-8; PI, Propidium Iodide.

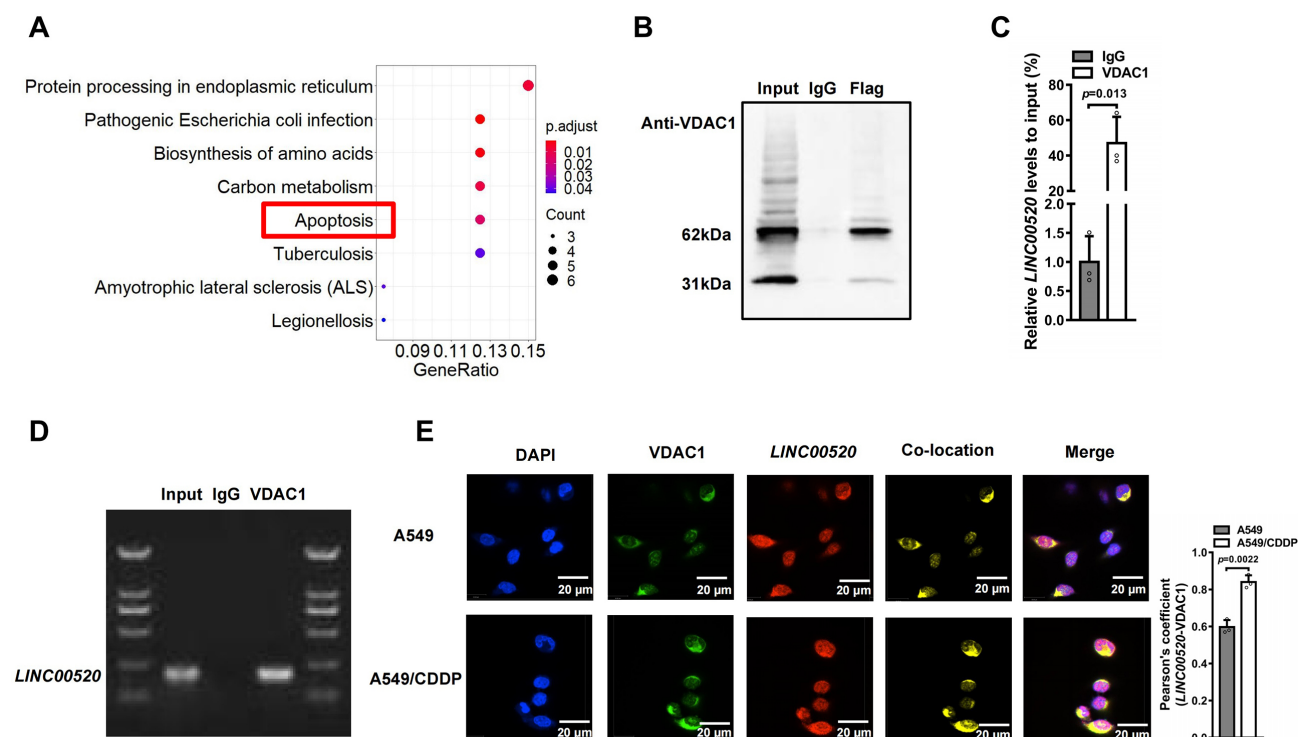


Fig. 3. LINC00520 directly binds to VDAC1. (A) KEGG pathway analysis of the 72 putative LINC00520-interacting proteins identified by MS2-TRAP combined with LC-MS/MS. (B) Validation of the interaction between LINC00520 and VDAC1 by MS2-TRAP followed by western blotting. (C) RIP assay followed by RT-qPCR confirming the interaction between VDAC1 and LINC00520. Data are presented as the mean $\pm$ SD. (D) Agarose gel electrophoresis analysis of RIP-PCR products. (E) Subcellular colocalization of LINC00520 (red) and VDAC1 (green) in A549 and A549/CDDP cells assessed by FISH coupled with immunofluorescence. Nuclei were counterstained with DAPI (blue). Colocalization was quantified by calculating Pearson's correlation coefficient based on at least three randomly selected fields of view per group. Data are presented as mean $\pm$ SD. Scale bar = 20 μ m. KEGG, Kyoto Encyclopedia of Genes and Genomes; MS2-TRAP, MS2-tagged RNA affinity purification; RIP, RNA immunoprecipitation; RT-qPCR, Reverse transcription quantitative polymerase chain reaction; FISH, fluorescence *in situ* hybridization; DAPI, 4',6-diamidino-2-phenylindole.

To experimentally validate the bioinformatics findings, we established the A549/CDDP cell line. The IC_{50} of cisplatin was 5.02 ± 0.25 μ M for A549 cells and 40.53 ± 1.47 μ M for A549/CDDP cells, yielding a resistance index of 8.07 ± 0.11 (Fig. 1D). Compared to the parental A549 cells, the A549/CDDP cells displayed significantly elevated levels of the drug resistance markers ATP-binding cassette subfamily B member 1 (ABCB1), ATP-binding cassette subfamily C member 1 (ABCC1), and lung resistance protein (LRP), consistent with their acquired resistant phenotype (Fig. 1E). RT-qPCR analysis revealed that LINC00520, LINC00648, HOTAIRM1, SPIN3, and FLJ42351 were significantly upregulated in A549/CDDP cells, whereas PCED1B-AS1 and TP53TG1 showed no significant change or decreased expression. Notably, LINC00520 exhibited the most pronounced increase, with levels approximately 40-fold higher in A549/CDDP cells than in A549 cells (Fig. 1F). When compared with the normal bronchial epithelial cell line BEAS-2B, LINC00520 expression progressively increased across LUAD cell lines,

including A549, H1975, and CALU-3. Consistently, cisplatin IC_{50} values obtained from the GDSC database showed a similar upward trend in these cell lines (Fig. 1G). Furthermore, LINC00520 expression increased in both A549 cells and A549/CDDP cells following short-term exposure to increasing cisplatin concentrations (Fig. 1H).

Analysis of 30 paired LUAD tissue samples showed that LINC00520 expression was higher in tumor tissues compared to adjacent non-tumor tissues (Fig. 1I). Consistently, data from the TCGA-LUAD cohort showed a significant positive association between LINC00520 levels and advanced tumor stage (Fig. 1J). Kaplan-Meier survival analysis indicated that elevated LINC00520 expression correlated with poor prognosis in LUAD patients, a finding validated using the GSE50081 dataset (Fig. 1K,L). Based on these observations, LINC00520 was selected for subsequent functional and mechanistic studies.

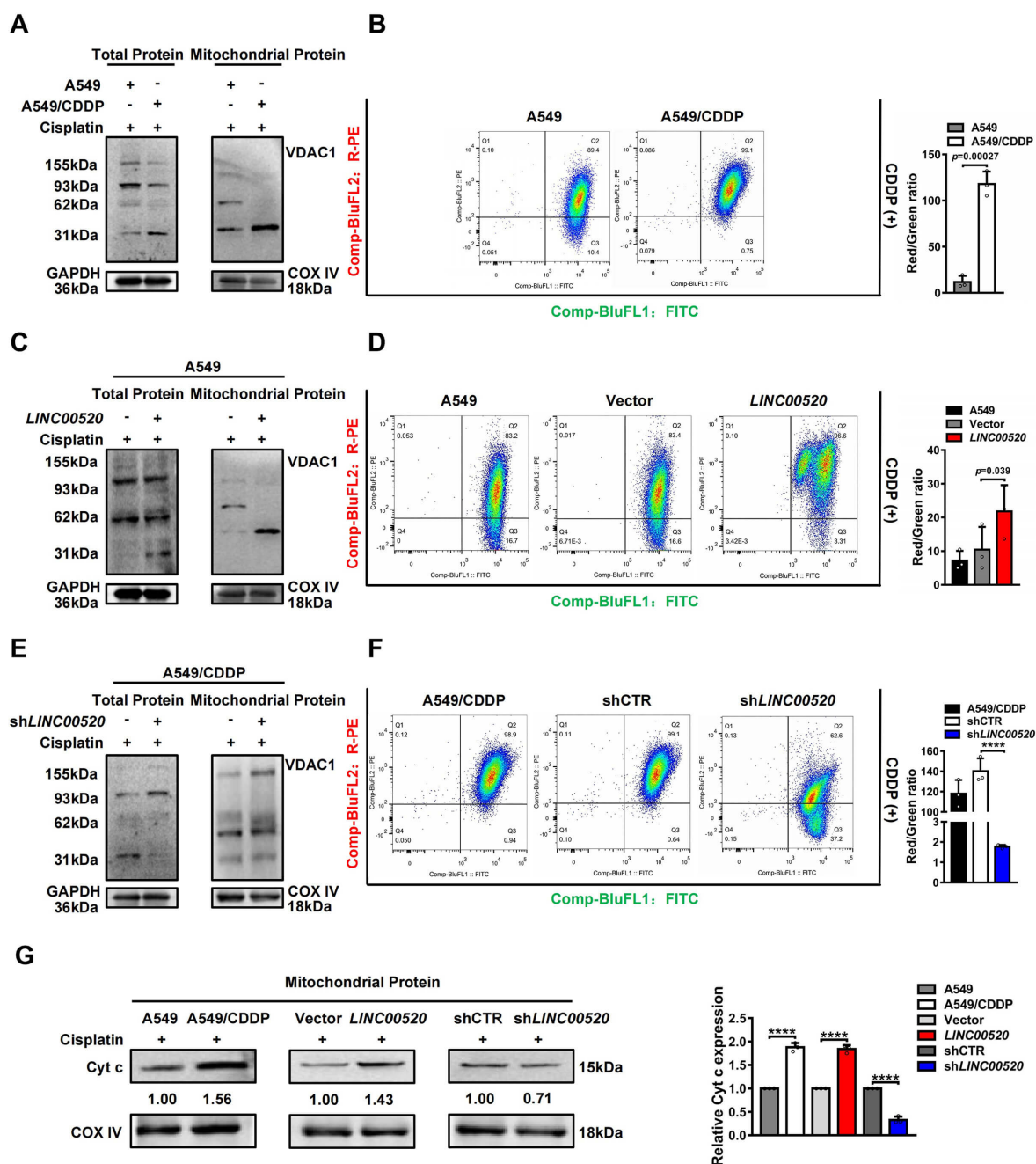


Fig. 4. LINC00520 suppresses VDAC1 oligomerization and mitochondrial apoptosis. (A) Western blot analysis of VDAC1 oligomerization in total cellular and mitochondrial fractions from A549 and A549/CDDP cells following cisplatin treatment. GAPDH and COX IV served as loading controls for total and mitochondrial fractions, respectively. (B) MMP assessed by JC-1 staining in A549 and A549/CDDP cells after cisplatin treatment. (C) VDAC1 oligomerization detected by western blotting in total and mitochondrial fractions from A549-Vector and A549-LINC00520 cells treated with cisplatin. GAPDH and COX IV served as reference proteins for total and mitochondrial fractions, respectively. (D) MMP measured by JC-1 staining in A549-Vector and A549-LINC00520 cells following cisplatin exposure. (E) Western blot analysis comparing VDAC1 oligomerization in total and mitochondrial fractions between A549/CDDP-shCTR and A549/CDDP-shLINC00520 cells following cisplatin treatment. GAPDH and COX IV served as loading controls, respectively. (F) MMP evaluated by JC-1 staining in A549/CDDP-shCTR and A549/CDDP-shLINC00520 cells after cisplatin treatment. (G) Mitochondrial Cyt c levels detected by western blotting in the indicated cell lines following cisplatin treatment. COX IV was used as the loading control. Data are presented as mean $\pm$ SD. ****, $p < 0.0001$; exact p -values for other comparisons are indicated in the figure. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; COX, Cytochrome C Oxidase; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; MMP, mitochondrial membrane potential; Cyt c, cytochrome c.

3.2 LINC00520 Overexpression Reduces Cisplatin Sensitivity in LUAD Cells

To investigate the functional role of LINC00520 in cisplatin sensitivity, LINC00520 was overexpressed in A549 cells and knocked down in A549/CDDP cells. The efficiency of these manipulations was confirmed by RT-qPCR (Fig. 2A). CCK-8 assays showed that cisplatin IC₅₀ was markedly higher in A549-LINC00520 cells ($34.34 \pm 1.71 \mu\text{M}$) than in control cells ($4.20 \pm 0.25 \mu\text{M}$) (Fig. 2B). Consistently, LINC00520 overexpression enhanced cell proliferation and colony formation upon cisplatin treatment, while flow cytometry revealed a substantial decrease in apoptosis (Fig. 2C–E). Conversely, LINC00520 knockdown in A549/CDDP cells resulted in a reduced cisplatin IC₅₀ ($16.49 \pm 1.09 \mu\text{M}$) compared with control cells ($40.04 \pm 1.32 \mu\text{M}$) (Fig. 2F). Furthermore, downregulation of LINC00520 attenuated proliferation and colony formation in the presence of cisplatin and increased apoptosis (Fig. 2G–I). Collectively, these findings indicate that elevated LINC00520 expression promotes cisplatin resistance in LUAD cells.

3.3 LINC00520 Binds to VDAC1 to Suppress Oligomerization and Mitochondrial Apoptosis

To investigate the mechanism underlying LINC00520-mediated cisplatin resistance, proteomic profiling was performed using MS2-TRAP combined with LC-MS/MS. A total of 72 potential LINC00520-binding proteins were identified. KEGG pathway analysis revealed enrichment in pathways associated with the endoplasmic reticulum, amino acid biosynthesis, and apoptosis (Fig. 3A). Among the apoptosis-associated proteins, VDAC1 was selected for further investigation due to its well-established role in regulating cisplatin-induced apoptosis through the mitochondrial pathway [32,33]. The interaction between LINC00520 and VDAC1 was validated by MS2-TRAP followed by western blotting (Fig. 3B). RIP assays further confirmed direct binding of VDAC1 to LINC00520 (Fig. 3C,D). Moreover, FISH coupled with immunofluorescence revealed subcellular colocalization of LINC00520 and VDAC1 in both A549 and A549/CDDP cells, with notably stronger signals in the A549/CDDP cells (Fig. 3E). Collectively, these results demonstrate that LINC00520 directly interacts with VDAC1 in LUAD cells.

The assembly of VDAC into higher-order complexes drives mitochondrial apoptosis, serving as a common pathway triggered by various pro-apoptotic signals [34,35,36]. In line with the resistant phenotype, A549/CDDP cells exhibited increased VDAC1 monomer levels and decreased oligomeric forms in both total cell lysates and mitochondrial fractions relative to parental A549 cells (Fig. 4A). Moreover, JC-1 staining revealed that MMP was higher in A549/CDDP cells than in A549 cells (Fig. 4B). Consistently, following cisplatin treatment, A549-LINC00520 cells showed reduced VDAC1 oligomerization, increased

VDAC1 monomer levels (in both total and mitochondrial fractions), and elevated MMP compared with control A549 cells (Fig. 4C,D). Conversely, knockdown of LINC00520 in A549/CDDP cells enhanced VDAC1 oligomerization, decreased monomer levels in both total and mitochondrial fractions, and reduced MMP (Fig. 4E,F). Furthermore, mitochondrial cytochrome c (Cyt c) levels were higher in A549/CDDP cells than in A549 cells. Consistent with this observation, LINC00520 overexpression in A549 cells increased mitochondrial Cyt c levels, whereas LINC00520 knockdown in A549/CDDP cells decreased them (Fig. 4G). Collectively, these findings suggest that LINC00520 suppresses VDAC1 oligomerization, thereby inhibiting mitochondrial apoptosis.

3.4 LINC00520 Promotes Cisplatin Resistance in LUAD Cells by Blocking VDAC1 Oligomerization

To determine whether LINC00520 confers cisplatin resistance specifically through VDAC1 oligomerization, we employed VBIT4, a pharmacological inhibitor of VDAC1 oligomerization [30]. In A549 cells treated with cisplatin in the presence of VBIT4, we observed an elevated MMP and a reduced apoptosis rate compared with cells treated with cisplatin alone (Fig. 5A,B). Furthermore, the cisplatin IC₅₀ was markedly higher in A549-VBIT4 cells ($42.00 \pm 0.85 \mu\text{M}$) than in DMSO-treated controls ($8.01 \pm 0.15 \mu\text{M}$) (Fig. 5C). Consistently, VBIT4 treatment enhanced cell proliferation and colony formation under cisplatin exposure (Fig. 5D,E). These results demonstrate that inhibition of VDAC1 oligomerization promotes cisplatin resistance in A549 cells.

To further validate that LINC00520 functions through VDAC1 oligomerization, we treated A549/CDDP-shLINC00520 cells with VBIT4 in the presence of cisplatin. The increased VDAC1 oligomerization induced by LINC00520 knockdown was attenuated upon VBIT4 treatment (Fig. 6A). Notably, the cisplatin IC₅₀, which decreased from $39.39 \pm 1.76 \mu\text{M}$ to $15.02 \pm 1.08 \mu\text{M}$ following LINC00520 knockdown, was restored to $43.57 \pm 1.21 \mu\text{M}$ upon VBIT4 addition (Fig. 6B). Consistently, the reductions in proliferation and colony formation caused by LINC00520 knockdown were reversed by VBIT4 treatment (Fig. 6C,D). Moreover, VBIT4 treatment suppressed apoptosis and elevated MMP in A549/CDDP-shLINC00520-VBIT4 cells compared with control cells (Fig. 6E,F). These rescue experiments demonstrate that LINC00520 mediates cisplatin resistance in LUAD cells by suppressing VDAC1 oligomerization, thereby inhibiting mitochondrial apoptosis.

3.5 LINC00520 Promotes Tumor Growth and Confers Cisplatin Resistance in LUAD Xenograft Models

To further validate the role of LINC00520 in cisplatin resistance *in vivo*, we established subcutaneous tumor xenograft models using A549 cells with stable LINC00520

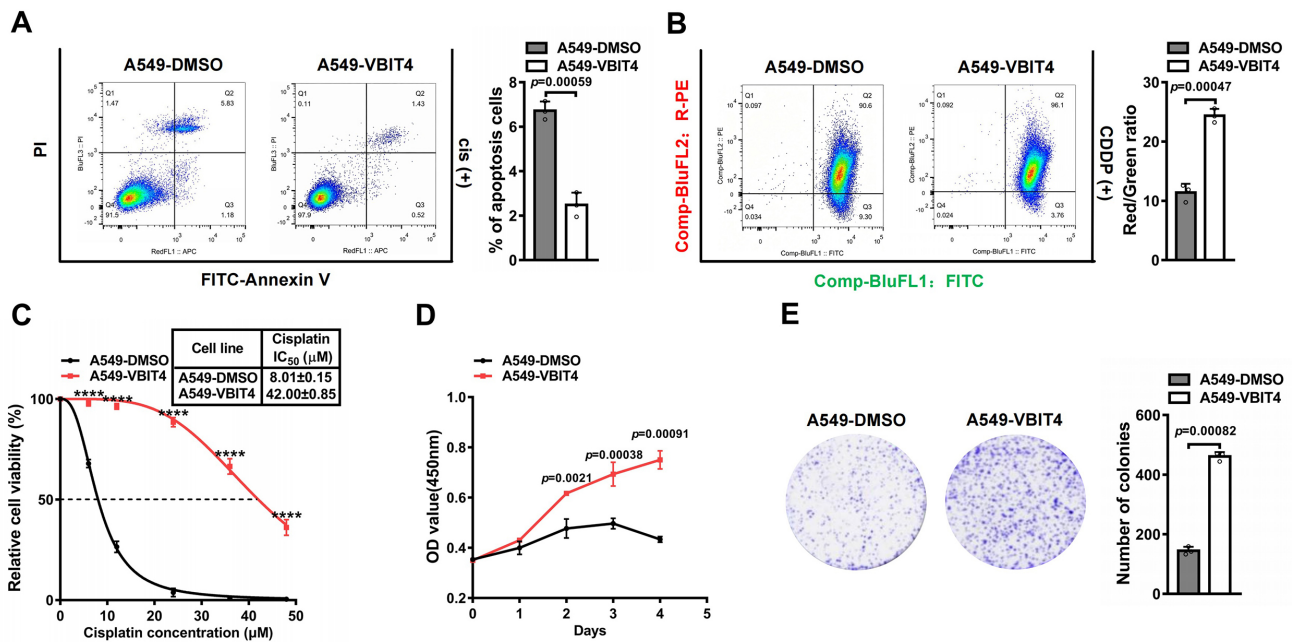


Fig. 5. Pharmacological inhibition of VDAC1 oligomerization recapitulates cisplatin resistance in A549 cells. (A,B) Apoptosis rate and MMP in A549-DMSO and A549-VBIT4 cells. (C) Cisplatin IC_{50} values in A549-DMSO versus A549-VBIT4 cells. (D,E) Cell proliferation (D) and colony formation (E) in A549-DMSO and A549-VBIT4 cells treated with cisplatin. Data are presented as mean $\pm$ SD. ****, $p < 0.0001$; exact p -values for other comparisons are indicated in the figure. DMSO, dimethyl sulfoxide.

overexpression or knockdown. LINC00520 overexpression significantly promoted tumor growth and conferred cisplatin resistance. Following six weeks of cisplatin administration, tumors in the A549-LINC00520 group remained substantially larger, with a final weight of 0.24 ± 0.10 g, compared with 0.05 ± 0.02 g in the control group (Fig. 7A–C). Conversely, LINC00520 knockdown attenuated cisplatin resistance. In mice bearing A549/CDDP-shLINC00520 xenografts, cisplatin treatment led to markedly smaller tumors (final weight 0.02 ± 0.01 g) relative to the control group (0.14 ± 0.03 g) (Fig. 7D–F). IHC analysis revealed that Ki-67 expression was significantly increased in the LINC00520 overexpression group, whereas it was markedly reduced in the knockdown group, consistent with the proliferation data (Fig. 7G,H). Collectively, these findings demonstrate that LINC00520 promotes tumor proliferation and confers cisplatin resistance in LUAD *in vivo*.

4. Discussion

In this study, we identified LINC00520 as a previously unrecognized driver of cisplatin resistance in LUAD. LINC00520 is markedly overexpressed in cisplatin-resistant LUAD cells and clinical tissues, and its high expression is associated with a negative prognosis for patients. Mechanistically, LINC00520 directly binds to VDAC1, inhibiting its oligomerization and thereby suppressing cisplatin-induced mitochondrial apoptosis. Pharmacological inhibition of VDAC1 oligomerization using

VBIT4 reversed the cisplatin-sensitive phenotype induced by LINC00520 knockdown. Consistently, xenograft models confirmed that LINC00520 overexpression promotes tumor growth and attenuates cisplatin efficacy, whereas its knockdown sensitizes tumors to cisplatin. Collectively, these findings establish LINC00520 as a critical regulator of cisplatin resistance in LUAD and emphasize its potential as a prognostic marker and treatment target.

VDAC1, a channel protein residing on the outer mitochondrial membrane, functions as a central gatekeeper of apoptosis [37,38]. Under physiological conditions, VDAC1 predominantly exists as monomers and dimers that allow controlled metabolite flux [39]. Upon apoptotic stimulation, VDAC1 self-assembles into higher-order oligomers, which facilitate mitochondrial permeability transition pore opening and cytochrome c release, subsequently triggering the caspase cascade [40]. In our study, cisplatin-resistant A549/CDDP cells exhibited reduced VDAC1 oligomerization, preserved mitochondrial membrane potential, and increased mitochondrial cytochrome c retention. LINC00520 overexpression in sensitive A549 cells recapitulated this resistant phenotype, while LINC00520 knockdown in resistant cells restored VDAC1 oligomerization and sensitized cells to cisplatin. MS2-TRAP assays further revealed that LINC00520 preferentially associates with VDAC1 dimers, suggesting that LINC00520 acts as a molecular “brake” that stabilizes VDAC1 in lower-order forms, thereby preventing the assembly of pro-apoptotic higher-order oligomers. The pre-

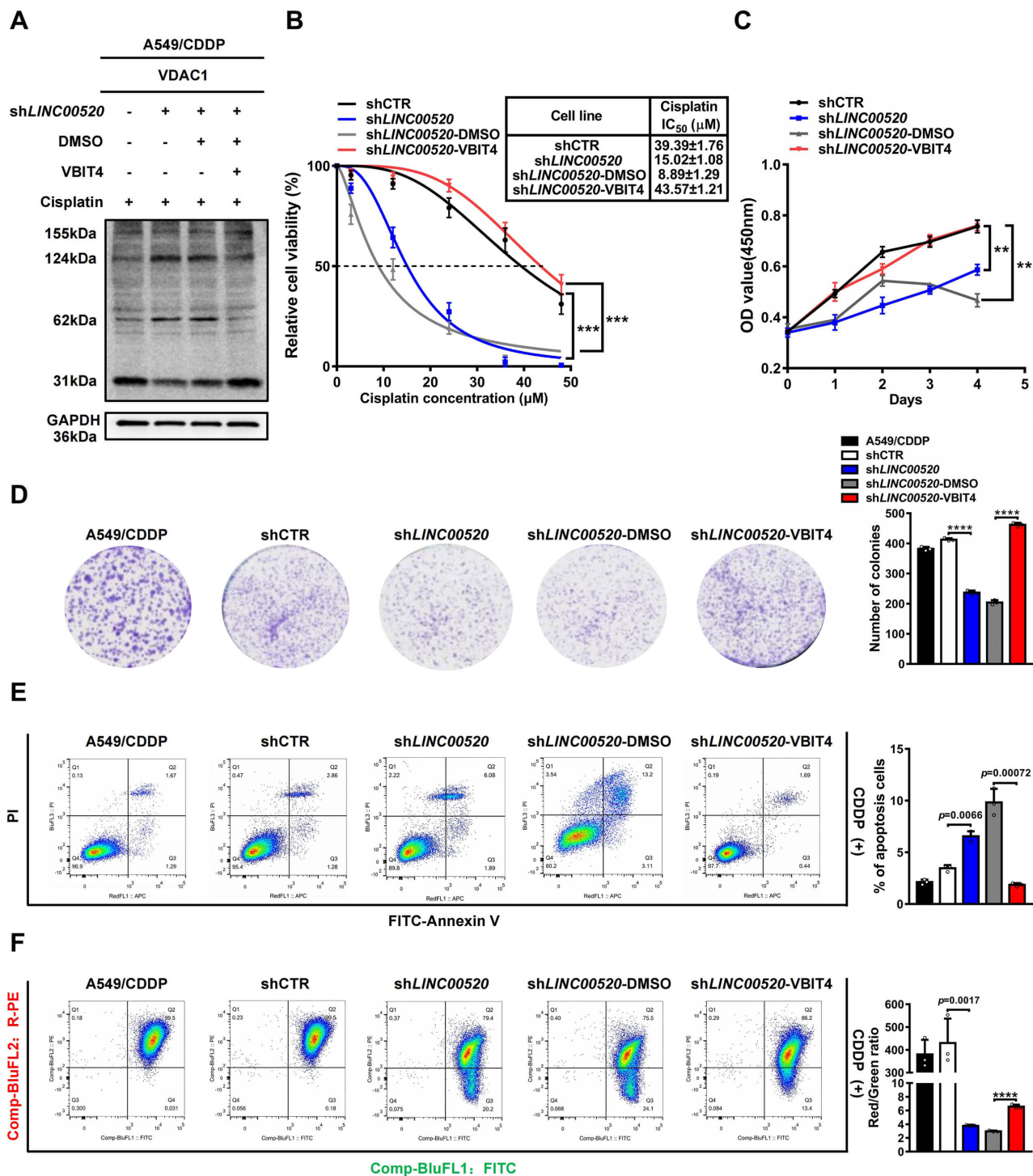


Fig. 6. LINC00520 confers cisplatin resistance in LUAD cells by suppressing VDAC1 oligomerization. (A) Western blot analysis of VDAC1 oligomerization in A549/CDDP-shCTR and A549/CDDP-shLINC00520 cells following cisplatin treatment, with the latter additionally treated with either DMSO or VBIT4 as indicated. GAPDH served as the loading control. (B) Cisplatin IC₅₀ values for the indicated cell lines. Data are presented as mean ± SD. ***, $p = 0.00052$ (shLINC00520 vs. shCTR); ***, $p = 0.00024$ (shLINC00520+VBIT4 vs. shLINC00520+DMSO). (C) Cell proliferation assessed in the indicated cell lines. Data are presented as mean ± SD. **, $p = 0.0025$ (shLINC00520 vs. shCTR); **, $p = 0.0061$ (shLINC00520+VBIT4 vs. shLINC00520+DMSO). (D) Colony formation in the indicated cell lines. (E) Apoptosis rate in the indicated cell lines. (F) MMP in the above cell lines was evaluated. Data are presented as mean ± SD. ****, $p < 0.0001$; exact p -values for other comparisons are indicated in the figure.

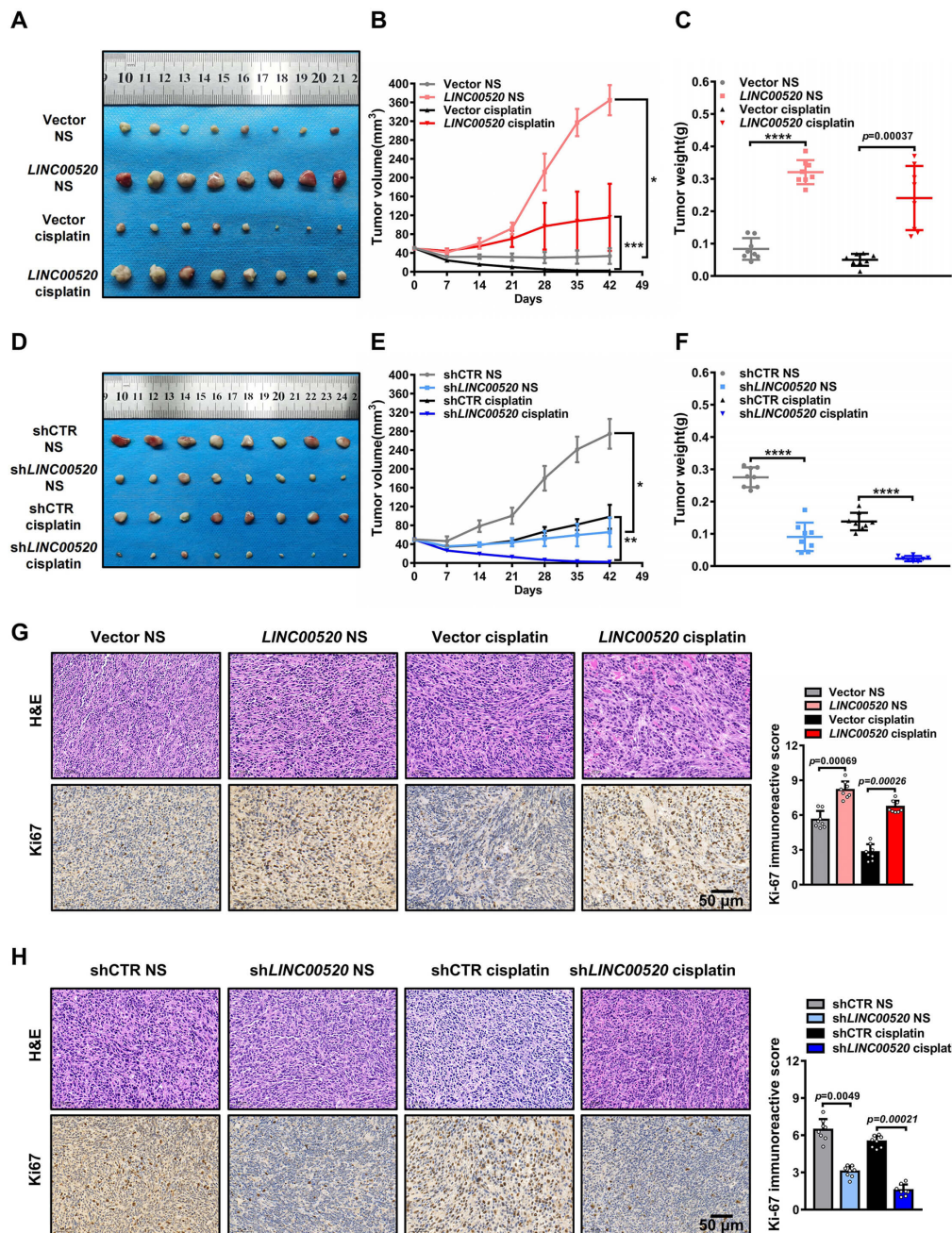


Fig. 7. LINC00520 promotes tumor growth and confers cisplatin resistance in LUAD *in vivo*. (A–C) Nude mice bearing A549-Vector or A549-LINC00520 xenografts were treated with cisplatin or NS for 6 weeks (total $n = 32$ mice, 8 per group). (A) Representative tumor images. (B) Tumor volume curves of the indicated groups. Data are presented as mean $\pm$ SD. *, $p = 0.021$ (LINC00520 NS vs. Vector NS); ***, $p = 0.00077$ (LINC00520 cisplatin vs. Vector cisplatin). (C) Final tumor weights of the resected xenografts. Data are presented as mean $\pm$ SD. ****, $p < 0.0001$ (LINC00520 NS vs. Vector NS); $p = 0.00037$ (LINC00520 cisplatin vs. Vector cisplatin). (D–F) Nude mice bearing A549/CDDP-shCTR or A549/CDDP-shLINC00520 xenografts were treated with cisplatin or NS for 6 weeks (total $n = 32$, 8 per group). (D) Representative tumor images. (E) Tumor volume curves comparing shCTR versus shLINC00520 groups. Data are presented as mean $\pm$ SD. *, $p = 0.039$ (shLINC00520 NS vs. shCTR NS); **, $p = 0.0015$ (shLINC00520 cisplatin vs. shCTR cisplatin). (F) Final tumor weights of the resected xenografts. Data are presented as mean $\pm$ SD. ****, $p < 0.0001$. (G,H) H&E staining and IHC analysis for Ki-67 expression in subcutaneous tumor tissues. Scale bars: 50 μ m. Quantification of Ki-67 IHC staining was performed using a semi-quantitative immunoreactive score system (range 0–12), and the quantitative scoring data are presented as mean $\pm$ SD ($n = 8$ per group). Statistical significance between groups was determined using the Mann–Whitney U test, and exact p-values for other comparisons are indicated in the figure. NS, normal saline; H&E, hematoxylin and eosin; IHC, immunohistochemistry.

cise molecular mechanism by which LINC00520 physically prevents VDAC1 oligomerization remains unclear. VDAC1 oligomerization is known to involve its N-terminal domain and specific transmembrane segments. We speculate that LINC00520 binding may sterically hinder the interaction between VDAC1 monomers or stabilize VDAC1 in a conformation unfavorable for oligomerization. Nevertheless, mapping the precise binding interface requires future structural or mutagenesis studies.

Previous studies have implicated several lncRNAs in cisplatin resistance in LUAD, primarily through transcriptional regulation or microRNA sponging. Beyond these classical mechanisms, emerging evidence suggests that lncRNAs can directly modulate programmed cell death pathways—including apoptosis, autophagy, and ferroptosis—to influence chemosensitivity [41]. LncRNAs have also been recognized as innovative multifunctional players in drug resistance across various cancer types, with established roles in regulating apoptosis and cancer stem cell properties [42,43]. Our study extends this paradigm by demonstrating that an lncRNA can directly interact with a mitochondrial protein to regulate the apoptosis threshold, thereby expanding the functional repertoire of LINC00520 in chemotherapy resistance beyond its previously reported roles.

This study has several limitations that should be noted. First, the MS2-TRAP screen identified 72 potential LINC00520-interacting proteins, suggesting that VDAC1 may not be the only functional target. In addition, whether LINC00520 affects VDAC2 oligomerization or the interaction between VDAC1 and pro-apoptotic proteins such as B-cell lymphoma 2 antagonist/killer or Bcl-2-associated X protein remains unknown. Second, while we observed increased mitochondrial cytochrome c retention upon LINC00520 overexpression, we did not measure cytosolic cytochrome c release or caspase-3/9 activation, which would more directly confirm blockade of the mitochondrial apoptosis pathway. Third, the rescue experiments relied on VBIT4, a pharmacological inhibitor of VDAC1 oligomerization; genetic manipulation of VDAC1 (knockdown or overexpression) would provide stronger evidence that the effects of LINC00520 are specifically mediated via VDAC1. Fourth, clinical validation was limited to 30 LUAD patients without detailed cisplatin treatment outcome data. Addressing these limitations in future studies will help solidify the mechanistic understanding and translational potential of LINC00520 in LUAD cisplatin resistance.

5. Conclusions

This study identifies LINC00520 as a critical mediator of cisplatin resistance in LUAD, acting through VDAC1-dependent suppression of mitochondrial apoptosis. Clinically, its association with poor prognosis, together with its functional role in chemoresistance, highlights LINC00520

as a promising biomarker and a potential therapeutic target for enhancing cisplatin efficacy in LUAD.

Availability of Data and Materials

All data generated or analyzed during this study are available from the corresponding authors upon reasonable request. Public datasets used in this work were obtained from publicly accessible databases as described in the Methods section. All reagents and materials used are commercially available.

Author Contributions

YC and ZL conceived and supervised the study. ZW and BC designed and conducted the majority of the experiments, prepared all figures and tables, and wrote the original draft of the manuscript. WX, JL, CC, and AW collected patient tissue samples and contributed to selected experiments. DY and YC optimized experimental protocols, performed statistical analysis, and contributed to data visualization. LF and PX performed validation experiments, and provided technical assistance. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study was carried out in accordance with the guidelines of the Declaration of Helsinki. The present study involving human participants was approved by the Ethics Committee of Hunan Cancer Hospital (Ethical lot number: KYJJ-2023-004). Informed consent was obtained from all participants. All animal experiments were performed in compliance with the Regulations for the Administration of Affairs Concerning Experimental Animals of the People's Republic of China and were approved by the Ethics Committee of Hunan Cancer Hospital (GZ2023-012). The study was carried out in accordance with the ARRIVE guidelines (<https://arriveguidelines.org>). In accordance with the institutional guidelines, the maximal tumor size permitted was 1.5 cm in diameter, and we confirm that the maximal tumor burden was not exceeded in this study. The ARRIVE 2.0 Essential 10 checklist has been completed and is provided as **Supplementary Material**.

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Conflicts of Interest

The authors declare no conflicts of interest.

Declaration of AI and AI-Assisted Technologies in the Writing Process

During the preparation of this work, the authors used DeepSeek in order to check spelling and grammar. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/FBL53197>.

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