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CNOT3 contributes to cisplatin resistance in lung cancer through inhibiting RIPK3 expression

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Abstract

Chemotherapeutic resistance always results in poor clinical outcomes of cancer patients and its intricate mechanisms are large obstacles in overcoming drug resistance. CCR4-NOT transcription complex subunit 3 (CNOT3), a post-translational regulator, is suggested to be involved in cancer development and progression. However, its role in chemotherapeutic resistance is not well understood. In this study, after screening the *CNOT3* mRNA in a cancer microarray database called Oncomine and examining the expression levels of *CNOT3* mRNA in normal tissues and lung cancer tissues, we found that *CNOT3* was up-regulated in lung cancer tissues. Besides, its high-expression was associated with poor prognosis of lung cancer patients. We also found higher expression level of CNOT3 and lower expression level of receptor-interacting protein kinase 3 (RIPK3) in cisplatin-resistant A549 (A549/DDP) cells, and knocking down CNOT3 expression could sensitize A549/DDP cells to cisplatin-induced apoptosis. We demonstrated that CNOT3 depletion up-regulated the expression level of RIPK3 and the enhanced apoptosis was mediated by the elevated RIPK3 to further trigger Caspase 8 activation. Taken together, our results reveal a role of CNOT3 in cisplatin resistance of lung cancer and provide a potential target for lung cancer therapy.

Keywords Cisplatin resistance · CNOT3 · Lung cancer · Apoptosis · RIPK3

Introduction

According to the latest cancer statistics, lung cancer is still the most commonly diagnosed cancer and the leading cause of cancer death in China [1]. Although many chemotherapeutics kill lung cancer cells effectively, drug resistance always occurs and their effects diminish soon. Mechanisms of drug resistance can be summarized as mutations and adaptive changes in cancer cells. Alterations at molecular levels often lead to behavioral changes in which cell death resistance is included. Hitherto, apoptosis resistance is widely acknowledged to contribute to cisplatin-induced chemoresistance [2]. Hence, solutions are being studied, such as to trigger alternative cell death pathway or to regain sensitivity to apoptosis [3–5]. Though great progress has been made in this field, it is far from enough, and more works need to be done in order to identify new predictive markers of cisplatin resistance or to overcome death resistance.

CNOT3 belongs to a multimeric complex called carbon catabolite repression 4 (CCR4) –negative on TATA-less (NOT) which was originally identified and characterized in yeast. In mammals, various physiological functions including transcriptional regulation, protein modification and deadenylation of mRNA are regulated by CCR4-NOT complex. CNOT3 works as a positive modulator of the catalytic activities of the CCR4-NOT deadenylase, thus to promote mRNA decay [6–8]. Hence, alterations in CNOT3 expression level will lead to multiple changes in cell functions. CNOT3 deficiency results in embryo lethality in mice [9]. Reduction in CNOT3 causes up-regulation of receptor

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activator of nuclear factor-κB (RANK) mRNA expression in bone marrow-derived macrophages through regulating mRNA decay [10]. A microarray analysis suggests that the expression of cytoplasm organization-, autophagy-, cell death- and cell proliferation-related genes are up-regulated in CNOT3-depleted mouse embryonic fibroblasts (MEFs) [11]. Dysregulation of CNOT3 expression is found to be relevant to human diseases such as ankylosing spondylitis [12], retinitis pigmentosa [13] and even cancers [6]. CNOT3 is identified as a tumor suppressor and its mutation is found in T cell acute lymphoblastic leukemia [14]. Besides, the expression level of CNOT3 is correlated with the prognosis of early-stage colorectal cancer [15]. However, less is known about the relationship between CNOT3 and lung cancer and whether its dysregulation is involved in cisplatin resistance of lung cancer cells.

Here, we report that CNOT3 is overexpressed in lung cancer tissues and its high expression predicts poor prognosis of lung cancer patients. In A549/DDP cells, CNOT3 is up-regulated, which reduces RIPK3 expression level and ultimately leads to cisplatin resistance. Besides, depletion of CNOT3 sensitizes lung cancer cells to cisplatin-triggered apoptosis through a mechanism of RIPK3 up-regulation and the increased RIPK3 drives more assembly of (Fas-associated death domain) FADD and Caspase 8 to transduce apoptotic signal.

Materials and methods

Reagents and antibodies

Cisplatin was obtained from Qilu Pharmaceutical. z-VAD-(OME)-FMK and z-IETD-fmk were purchased from MedChemExpress (HY-16658 and HY-101297). Actinomycin D was obtained from Amresco (J608).

The antibodies against human MLKL and phosphorylated MLKL (S358) were from Abcam (ab184718 and Ab187091). The antibody against human RIPK1 was purchased from BD Biosciences (610458). The antibodies against human RIPK3 and Caspase 3 were from Abcam (ab56164 and ab13847). The antibodies against GFP and human FADD were purchased from Santa Cruz Biotechnology (sc-9996 and sc-271520). The anti-CNOT3 antibody was purchased from Proteintech (11135-1-AP). The anti-PARP-1 and the anti-Caspase 8 antibodies were from Cell Signaling Technology (9532 and 9746).

Cell culture, cell death induction and inhibition

A549 and 95-D cells were purchased from American Type Culture Collection and were cultured in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin, and maintained at 37 °C in a 5% CO_2 atmosphere. A549/DDP cells were established in our lab and A549 cells were used as the parent cells to obtain A549/DDP cells by continuously exposure to increasing concentrations of cisplatin for 16 months. A549/DDP cells were cultured under the above conditions additionally with 1 μg/ml cisplatin in the medium. To induce death, A549 and 95-D cells were treated with 4 μg/ml cisplatin for 48 h and A549/DDP cells were treated with 24 μg/ml cisplatin for 48 h. In some experiments, the cells were pre-incubated with zVAD (80 μM) or z-IETD-fmk (80 μM) for 1 h before cisplatin treatment.

Cell survival, cytotoxicity and cell proliferation assay

Cell viability was measured by Cell Counting Kit 8 (CCK-8) purchased from Engreen (EC008). After treatment, a 10 μl CCK-8 solution was added to the culture medium of cells growing in 96-well plates (5000–10,000 cells/well) and incubated in a humidified CO_2 incubator at 37 °C for 3 h. Then, samples were measured at 450 nm and the data was normalized to background readings of media only.

Cell death was estimated by determining LDH released into the culture medium. LDH released into the medium was detected using LDH cytotoxicity assay kit purchased from Beyotime Institute of Biotechnology (C0017) according to the manufacturer's instructions.

To perform proliferation assay, A549 and A549/DDP cells were seeded in 96-well plates after transfection and allowed to adhere. A 10 μl CCK-8 solution was added to each well at the indicated times and then measured absorbance at 450 nm.

Flow cytometry analysis

A FITC Annexin V Apoptosis Detection Kit with Propidium iodide was purchased from Keygen Biotech (KGA107). Cell death was recorded on a FACSCalibur flow cytometer (Becton, Dickinson and Company) in the total population (10,000 cells), and the data were analyzed using FlowJo software (Version 7.6.1).

Construction of recombinant plasmid and transfection

The sequence encoding full-length human CNOT3 was cloned from cDNAs and amplified via PCR and then cloned into pEGFP-N1 (Clontech). For transfection, cells were seeded in a 6-well plate at a density of 800,000–900,000 cells per well on day 0 and transfected with DNA on day 1. A549 cells were transfected with 4 μg plasmids and 10 μl Lipofectamine 2000 reagent (Invitrogen, Life Technologies) according to the manufacturer's instructions. The

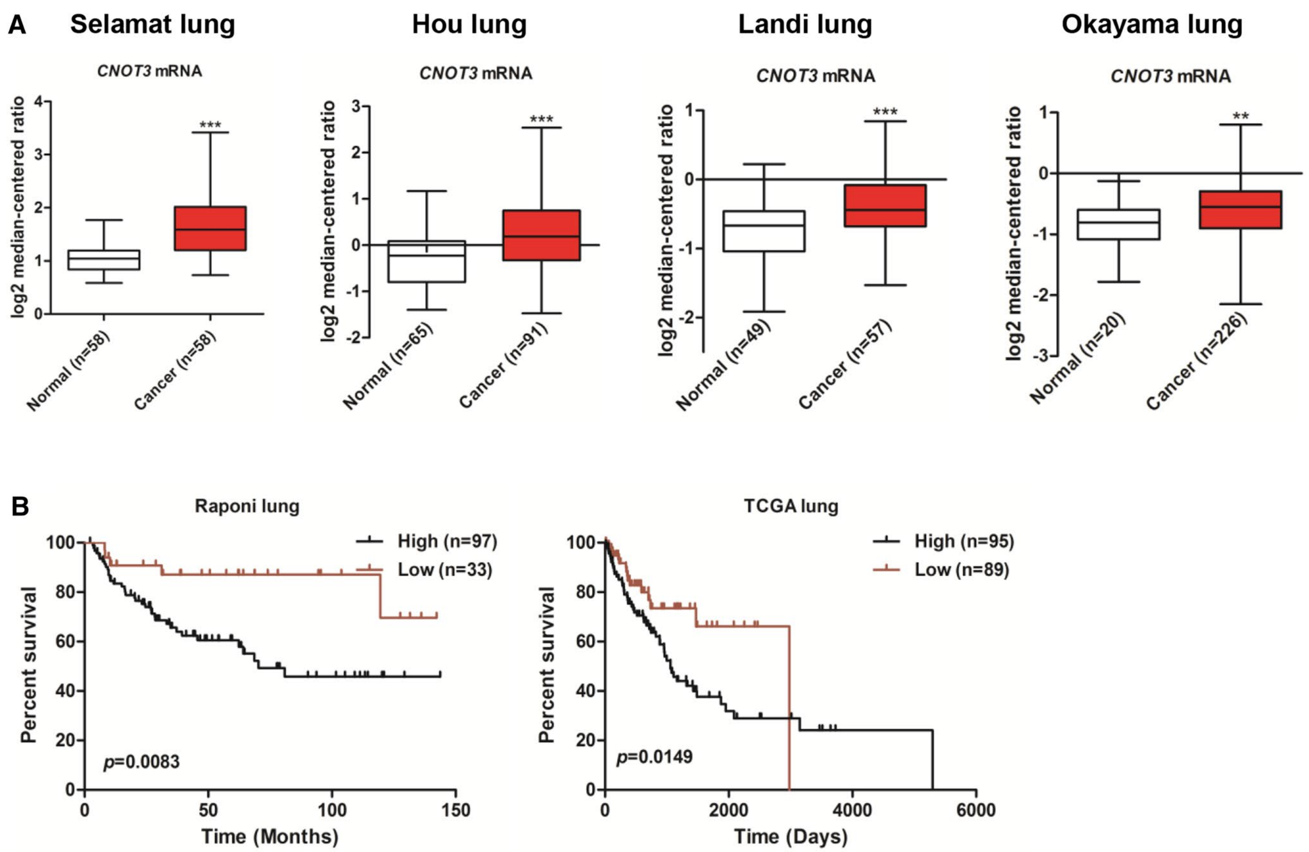


Fig. 1 CNOT3 is up-regulated in lung cancer and its high mRNA level predicts poor prognosis of lung cancer patients. **a** Comparison of *CNOT3* mRNA level between lung cancer tissues and normal tissues downloaded from four different datasets. **b** Kaplan–Meier overall survival curves for *CNOT3* expression levels ($P=0.0083$ and $P=0.0149$). **a** Mann–Whitney *U* test. $^{***}P<0.001$ or $^{**}P<0.01$ for comparisons between the indicated groups

culture medium was changed 6 h after transfection. A549 cell lines with stable transfection of the vector or the recombinant plasmid were screened by G418 and flow cytometry. Lentiviral productions were generated by iGeneBio. A549/DDP cells were infected according to the manufacturer's instructions.

RNA interference

The siRNAs for human CNOT3, RIPK3, RIPK1, FADD and the non-target siRNA were designed by and obtained from GenePharma. Sense strand and antisense strand of CNOT3 siRNA: 5′-GGACAAGCGCAAACUCCAATT-3′ and 5′-UUGGAGUUUGCGCUUGUCCTT-3′. Sense strand and antisense strand of RIPK3 siRNA: 5′-CCAGAGACCUCAACUUUCA-3′ and 5′-UGAAAGUUGAGGUCUCUGG-3′. Sense strand and antisense strand of RIPK1 siRNA: 5′-GCAAAGACCUUACGAGAAUTT-3′ and 5′-AUUCUCGUAAGGUCUUUGCTT-3′. Sense strand and antisense strand of FADD siRNA: 5′-CACAGAGAAGGAGAACGCA-3′ and 5′- UGCGUUCUCCUUCUCUGUG-3′. Sense strand and antisense strand of non-target siRNA: 5′–UUCUCCGAACGUGUCACGUTT-3′ and 5′-ACGUGACACGUUCGGAGAATT-3′.

For transfection, the cells were seeded in a 6-well plate at a density of 300,000 cells per well on the day before transfection. Cells were transfected with the siRNAs (100 pmol) and the Lipofectamine 2000 reagent (5 µl) according to the manufacturer's instructions. The medium was changed 6 h after transfection.

Quantitative PCR (q–PCR) analysis

To perform the analysis, about 1,000,000 cells were collected. Total RNA was isolated with the E.Z.N.A.® Total RNA Kit II (Omega Bio-tek) according to the manufacturer's protocol. cDNAs were synthesized using PrimeScript™ RT reagent Kit (RR037A, Takara) and the reaction system comprised 4 µl of 5 × PrimeScript Buffer, 1 µl of PrimeScript RT Enzyme Mix I, 1 µl of Oligo dT Primer (50 µM),

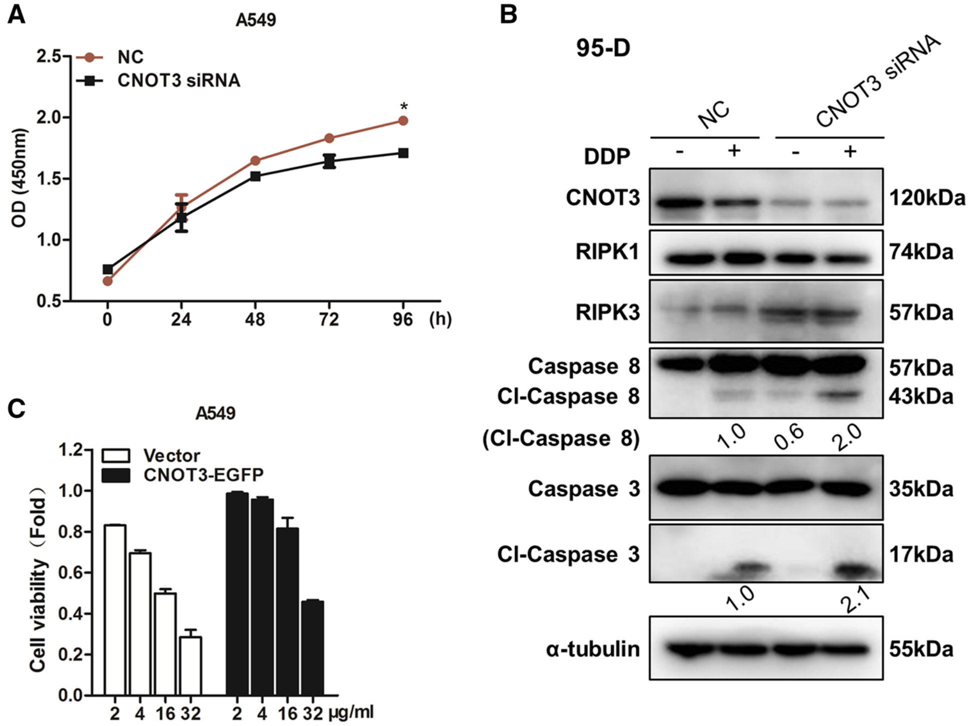


Fig. 2 CNOT3 regulates cell proliferation and apoptosis of lung cancer cells. **a** A549 cells were transfected with a control or CNOT3 siRNA and cell proliferation was measured via the CCK-8 assay. **b** 95-D cells were transfected with a control or CNOT3 siRNA. Twenty-four hours after transfection, cells were treated with DDP and then subjected to western blotting to assess protein expression levels. **c** A549 cells were transiently transfected with the empty vector or the recombinant CNOT3 plasmid. Twenty-four hours after transfection, cells were treated with indicated doses of DDP. Cytotoxicity was determined via the CCK-8 assay. Data are shown as the mean ± S.E.M. n = 3. **a** Two-way ANOVA and Bonferroni post hoc test. $^{*}P < 0.05$ for comparisons between the indicated groups. Numbers below the blots correspond to relative expression levels compared with the reference point set to 1

1 µl of Random 6 mers (100 µM), 1 µg of RNA and RNase Free dH_2O up to 20 µl. The reaction protocol was 37 °C for 30 min, 85 °C for 5 s.

Quantitative PCR analysis was performed using the SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (RR820A, Takara). To measure mRNA stability, cells were treated with Actinomycin D (5 µg/ml), and total RNA was extracted at the indicated times and subjected to q-PCR analysis. Human CNOT3 forward: 5′-GGACGTTCCACAGACAGTGA-3′; reverse: 5′- GAGGGTGCTGGTTGCTGT-3′. Human RIPK3 forward: 5′-AATTCGTGCTGCGCCTAGAAG-3′; reverse: 5′- TCGTGCAGGTAAAACATCCCA-3′. Human GAPDH forward: 5′-GCACCGTCAAGGCTGAGAAC-3′; reverse: 5′-TGGTGAAGACGCCAGTGGA-3′. The reaction system comprised 0.5 µl of forward primer, 0.5 µl of reverse primer, 1 µl of cDNA, 10 µl of SYBR and 8 µl of RNase Free dH_2O. And the reaction protocol was carried out for 40 cycles, comprising 95 °C for 30 s, 60 °C 30 s and 72 °C 1 min.

Western blotting and immunoprecipitation

For western blotting, about 1,000,000 cells were dissociated and collected. RIPA lysis buffer obtained from Beyotime Institute of Biotechnology (P0013B) was added to the cells to generate cell lysates. The protein concentrations of the samples were determined with a Pierce™ BCA Protein Assay Kit (23225, Thermo Fisher Scientific) and then equalized. An equal volume of each cell lysate was resolved via 12% SDS-PAGE, transferred onto Immobilon-P membranes (Millipore), and then incubated 4 °C overnight with primary antibodies diluted 1:500 with PBS-T buffer (136 mM NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.05% Tween-20) followed by incubation with an HRP-conjugated secondary antibody (1:3000) for 1 h. To perform immunoprecipitation, about 3,000,000 cells were dissociated and collected. The Pierce™ Co-Immunoprecipitation Kit (26149, Thermo Fisher Scientific) was used, and all of the experimental procedures were performed according to the manufacturer's instructions. Carestream Molecular Imaging software was used to measure the intensity of each band and then relative protein expression level was quantified by densitometry analysis.

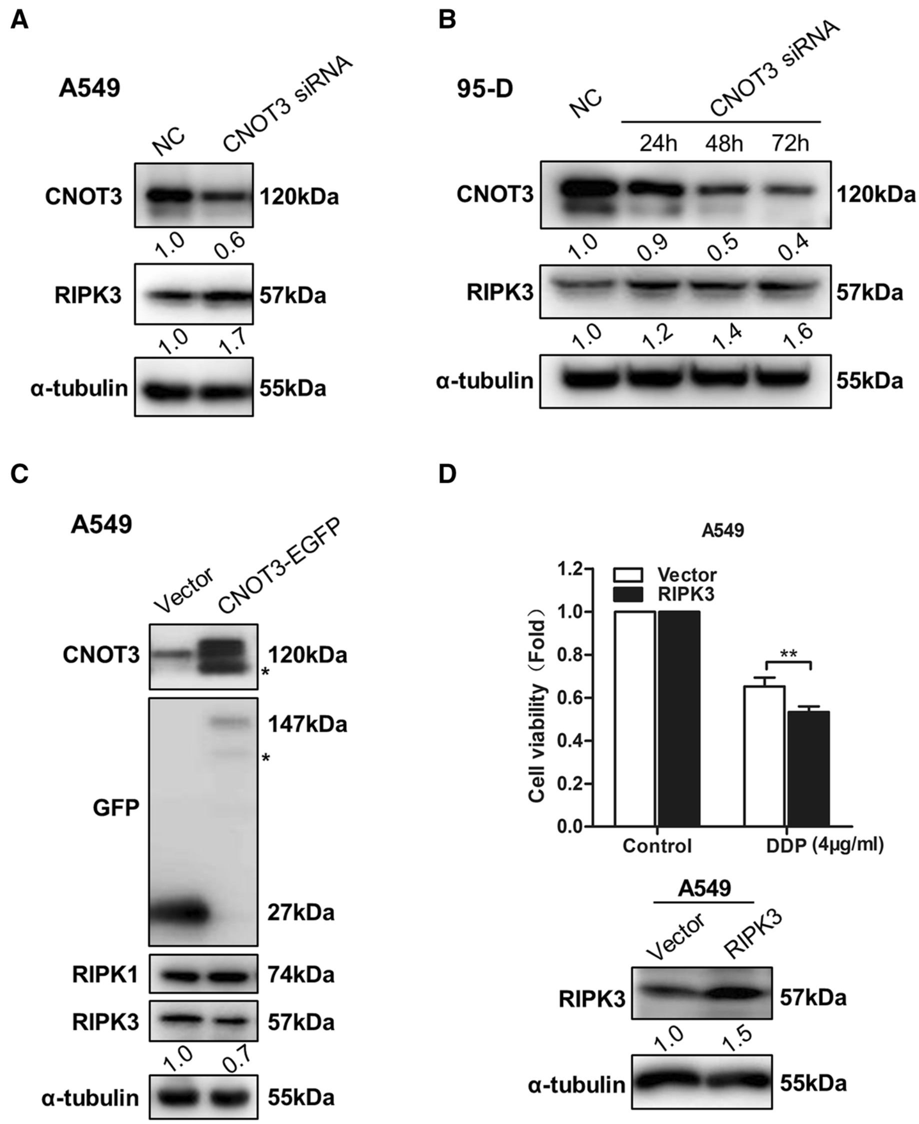


Fig. 3 RIPK3 expression level which influences cisplatin sensitivity of lung cancer cells is regulated by CNOT3. Cells were transfected with a control or CNOT3 siRNA. **a** A549 cells were subjected to western blotting at 48 h post transfection to assess CNOT3, RIPK3 and α-tubulin expression levels. **b** 95-D cells were subjected to western blotting at indicated time to assess CNOT3, RIPK3 and α-tubulin expression levels. **c** A549 cells were transiently transfected with the empty vector or the recombinant CNOT3 plasmid. Twenty-four hours after transfection, cells were subjected to western blotting for assessing CNOT3, RIPK1, RIPK3 and α-tubulin expression levels. **d** Forty-eight hours after infection, RIPK3-overexpressed A549 cells and A549 cells were treated with indicated doses of DDP for 48 h. Cytotoxicity was analyzed via the CCK-8 assay (up panel). Seventy-two hours after infection, RIPK3-overexpressed A549 cells and A549 cells were subjected to western blotting to assess RIPK3 and α-tubulin expression levels (down panel). Data are shown as the mean ± S.E.M. n = 3. **d** Two-way ANOVA and Bonferroni post hoc test. $^{**}P < 0.01$ for comparisons between the indicated groups. Numbers below the blots correspond to relative expression levels compared with the reference point set to 1

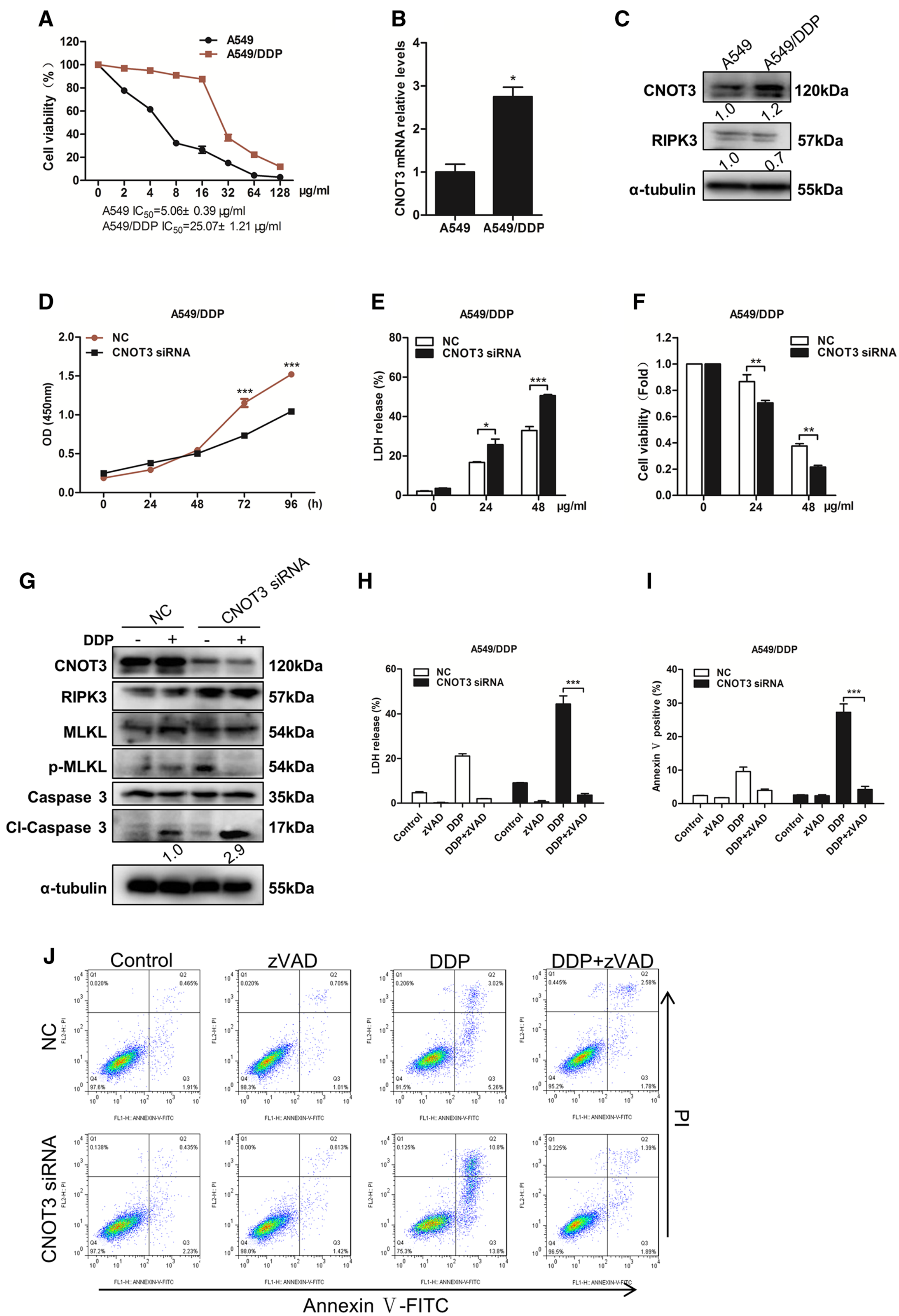
Statistical analysis

Data are expressed as mean ± S.E.M. of three independent experiments. Statistical analysis was performed using Student's *t* test, Mann–whitney *U* test or ANOVA. Survival curves were analyzed using the Kaplan–Meier method with significance determined by the Logrank test. The GraphPad Prism 5.0 program was used for the statistical analysis.

Results

CNOT3 is up-regulated in lung cancer and its high mRNA level predicts poor prognosis of lung cancer patients

We explored the *CNOT3* mRNA expression in human lung cancer tissues and normal tissues using a cancer microarray


A
Cell viability (%)
A549
A549/DDP
μg/ml
A549 IC50=5.06± 0.39 μg/ml
A549/DDP IC50=25.07± 1.21 μg/ml
B
CNOT3 mRNA relative levels
A549
A549/DDP
C
A549
A549/DDP
CNOT3
120kDa
RIPK3
57kDa
α-tubulin
55kDa
D
A549/DDP
NC
CNOT3 siRNA
OD (450nm)
(h)
E
A549/DDP
LDH release (%)
μg/ml
F
A549/DDP
Cell viability (Fold)
μg/ml
G
NC
CNOT3 siRNA
DDP
CNOT3
RIPK3
MLKL
54kDa
p-MLKL
Caspase 3
35kDa
Cl-Caspase 3
17kDa
α-tubulin
H
A549/DDP
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Control
zVAD
DDP
DDP+zVAD
I
A549/DDP
Annexin V positive (%)
J
Control
zVAD
DDP
DDP+zVAD
NC
CNOT3 siRNA
PI
Annexin V-FITC

Fig. 4 The increased CNOT3 in A549/DDP cells contributes to cisplatin resistance. **a** A549 and A549/DDP cells were treated with indicated doses of DDP for 48 h, and then cell viability and IC_{50} of cisplatin were determined using the CCK-8 assay. **b** *CNOT3* mRNA levels in A549 and A549/DDP cells were measured by q-PCR. **c** A549 and A549/DDP cells were subjected to western blotting for assessing CNOT3, RIPK3 and α-tubulin expression levels. **d** A549/DDP cells were transfected with a control or CNOT3 siRNA and cell proliferation was measured via the CCK-8 assay. **e, f** A549/DDP cells were transfected with a control or CNOT3 siRNA. Twenty-four hours after transfection, cells were treated with the indicated doses of DDP. Cytotoxicity was analyzed via the LDH release (**e**) and CCK-8 assay (**f**). **g** A549/DDP cells were treated as in (**e**, **f**) and then cells were subjected to western blotting to assess protein expression levels. **h–j** A549/DDP cells were transfected with a control or CNOT3 siRNA. Twenty-four hours after transfection, cells were pretreated with or without zVAD then exposed to DDP or left untreated. Cell death was analyzed via the LDH release assay (**h**) and flow cytometry (**i**, **j**). Data are shown as the mean ± S.E.M. n = 3. **b** Student's *t* test. (**d**–**f**) Two-way ANOVA and Bonferroni post hoc test. (**h** and **i**) One-way ANOVA with Tukey post hoc test. $^{***}P<0.001$, $^{**}P<0.01$ or $^{*}P<0.05$ for comparisons between the indicated groups. Numbers below the blots correspond to relative expression levels compared with the reference point set to 1

database called Oncomine (http://www.oncomine.org). Several lung cancer datasets showed increased expression levels of *CNOT3* in lung cancer (Fig. 1a). Then, we investigated the correlation between CNOT3 expression and lung cancer patient prognosis also using Oncomine. According to the receiver operating characteristic (ROC) curve, we defined the cut-off point to distinguish high and low *CNOT3* mRNA expression levels. Patients in each dataset were divided into low-expression and high-expression groups according to the cut-off point. Kaplan–Meier analysis of overall survival was performed and the results suggested that high expression of CNOT3 mRNA was significantly associated with poor prognosis of the lung cancer patients (Fig. 1b). To sum up, CNOT3 is correlated with lung cancer malignancy.

CNOT3 regulates cell proliferation and apoptosis of lung cancer cells

Sustained cell proliferation and death resistance which are hallmarks of cancer contribute to cancer malignancy [16]. Though it has been suggested in some studies that CNOT3 regulates cell proliferation [11, 17, 18], we used A549 and 95-D cells in our study to confirm this result. CNOT3 was knocked down in these two cell lines and then CCK-8 assay was performed to measure its effect on lung cancer cell proliferation. Our results showed that CNOT3 knockdown inhibited cell proliferation ability in A549 cells (Fig. 2a and Supplemental Fig. 1). However, in CNOT3-depleted 95-D cells, the cell number began to decrease at day 3 (data not shown). Besides, a slight increase of cleaved Caspase 8 could be detected by western blolting, which suggested that CNOT3 depletion triggers apoptosis in 95-D cells (Fig. 2b). In addition, we found that cisplatin-induced apoptosis was enhanced in CNOT3-knockdown 95-D cells, indicating that CNOT3 may influence the sensitivity of lung cancer cells to cisplatin (Fig. 2b). To confirm this, we overexpressed CNOT3 in lung cancer A549 cells and the results of CCK-8 assay showed that A549 cells with CNOT3 overexpression were less sensitive to cisplatin treatment (Fig. 2c).

RIPK3 expression level which influences cisplatin sensitivity of lung cancer cells is regulated by CNOT3

A previous study has suggested that CNOT3 regulates mRNA stabilization of RIPK3, a protein that participates in cell death signaling [11, 19, 20]. Thus, we tried to figure out in lung cancer cells whether RIPK3 expression is regulated by CNOT3. We found that in CNOT3-knockdown A549 cells, RIPK3 expression level was markedly up-regulated at 48 h post transfection and similar results were observed in CNOT3-knockdown 95-D cells (Figs. 2b and 3a, b). CNOT3 with EGFP tag was then transiently transfected into A549 cells. We found that compared with the vector group, RIPK3 expression level significantly decreased in cells with CNOT3 overexpression (Fig. 3c). The same result was obtained in A549 cell line stably overexpressing CNOT3 (Supplemental Fig. 2). The above data showed that CNOT3 negatively regulates RIPK3 expression in lung cancer cells.

As RIPK3 was up-regulated in CNOT3-knockdown cells, we investigated next whether up-regulating RIPK3 expression could sensitize lung cancer cells to cisplatin. We infected A549 cells with lentivirus vector expressing RIPK3 and then subjected cells to cisplatin treatment. Our results showed that RIPK3-overexpressed lung cancer cells were more sensitive to cisplatin-triggered cell death (Fig. 3d). To sum up, these results indicated that CNOT3 may influence cisplatin sensitivity of lung cancer cells via regulating RIPK3 expression.

The increased CNOT3 in A549/DDP cells contributes to cisplatin resistance

To further investigate if CNOT3 is involved in the mechanism of cisplatin resistance, A549/DDP cells and their parent cells (A549 cells) were used in our study. The result of CCK-8 assay showed that A549/DDP cells were less sensitive to cisplatin treatment (Fig. 4a). CNOT3 expression at both mRNA level and protein level were then determined in these two cell lines and the results showed increased CNOT3 expression levels in A549/DDP cells (Fig. 4b and c). At the same time, we noticed that RIPK3 was down-regulated in A549/DDP cells (Fig. 4c).

Then, we knocked down the expression of CNOT3 in A549/DDP cells and found that CNOT3 depletion also inhibited cell proliferation ability in A549/DDP cells (Fig. 4d and

Supplemental Fig. 3). Besides, cytotoxicity was measured via CCK-8 and LDH release assay and the data showed that CNOT3 depletion sensitized A549/DDP cells to cisplatin-induced cell death (Fig. 4e and f). As shown by western blotting, the cleaved Caspase 3 was increased in CNOT3-knockdown A549/DDP cells following cisplatin treatment (Fig. 4g). Our previous study has suggested that cisplatin could induce necroptosis in A549 cells [21], whereas it was not the case in A549/DDP cells (Fig. 4g). Thus, to figure out whether CNOT3 depletion is able to sensitize A549/DDP cells to necroptosis, MLKL phosphorylation was detected in CNOT3-depleted cells with or without cisplatin treatment. Surprisingly, we noticed that CNOT3 depletion induced MLKL phosphorylation only when cells were not exposed to cisplatin (Fig. 4g). The above data suggested that apoptosis but not necroptosis was enhanced in cisplatin treated CNOT3-knockdown A549/DDP cells.

To confirm the above results, we next introduced z-VAD-(OME)-FMK, a pan-Caspase inhibitor, to inhibit apoptosis. As shown by LDH release assay, zVAD reversed the increased cell death in cisplatin-treated CNOT3-knockdown A549/DDP cells (Fig. 4h). Annexin V-FITC was used to detect apoptotic cells and the result of flow cytometry also showed that the increased apoptosis was markedly reduced by zVAD (Fig. 4i and j). Taken together, the up-regulated CNOT3 contributes to cisplatin resistance of A549/DDP cells.

CNOT3 down-regulates RIPK3 to facilitate cisplatin resistance

To better illustrate how CNOT3 contributes to cisplatin resistance of A549/DDP cells, we first investigated whether the RIPK3 down-regulation was a result of CNOT3 up-regulation. Thus, we knocked down its expression in A549/DDP cells and noticed that RIPK3 increased when CNOT3 was depleted (Fig. 5a). We also analyzed the time course of its mRNA expression level following Actinomycin D (Act. D) treatment and found that the half-life of *RIPK3* mRNA was prolonged in CNOT3-knockdown A549/DDP cells, suggesting the increased RIPK3 expression due in part to stabilization of *RIPK3* mRNA (Fig. 5b).

We then infected A549/DDP cells with lentivirus vector expressing RIPK3 to address whether its overexpression could help to overcome cisplatin insensitivity. Overexpression of RIPK3 was confirmed via western blotting (Fig. 5c). Cytotoxicity measured via the CCK-8 and LDH release assay showed that overexpression of RIPK3 sensitized A549/DDP cells to cisplatin-induced cell death (Fig. 5d and e). Western blotting showed increased cleaved PARP-1 and phosphorylated MLKL levels, which suggested that RIPK3 overexpression not only facilitated apoptosis, but also triggered necroptosis in cisplatin-treated A549/DDP cells (Fig. 5f). As is indicated above, CNOT3 contributes to cisplatin resistance through down-regulating RIPK3 in A549/DDP cells.

RIPK3 up-regulation enhances extrinsic apoptosis in CNOT3-knockdown lung cancer cells

We further verified in this study whether the increased apoptosis in cisplatin-treated CNOT3-knockdown cells relies on RIPK3 up-regulation. As shown by western blotting, when RIPK3 was simultaneously reduced, cisplatin could not enhance apoptosis in CNOT3-knockdown A549/DDP cells, which indicates that the up-regulated RIPK3 contributes to the enhanced apoptosis (Fig. 6a). This result was confirmed via the LDH release assay (Fig. 6b). Furthermore, we found that MLKL phosphorylation could also be ascribed to the increased RIPK3 caused by CNOT3 depletion (Fig. 6c), which is consistent with the report that the increased RIPK3 phosphorylates MLKL to mediate necroptotic signaling [22].

A previous study has suggested that RIPK3 can drive assembly of FADD, RIPK1 and Caspase 8 to trigger extrinsic apoptosis [23]. In our study, the activation of Caspase 8 was determined to address whether the increased RIPK3 facilitates extrinsic apoptotic signaling in CNOT3-knockdown A549/DDP cells. We noticed that Caspase 8 activation was enhanced in CNOT3-knockdown A549/DDP cells following cisplatin treatment (Fig. 6a and d). Besides, as shown by western blotting, CCK-8 assay and flow cytometry, the Caspase 8 inhibitor, z-IETD-fmk, reversed the enhanced apoptosis (Fig. 6d–g). The above data suggested that in A549/DDP cells with CNOT3 depletion, cisplatin enhanced cell death through intensifying extrinsic apoptotic signaling. We also observed that when RIPK3 was simultaneously knocked down, the enhanced activation of Caspase 8 could be attenuated (Fig. 6a), which indicated that Caspase 8 functions downstream of RIPK3.

FADD is an essential adaptor in Caspase 8 activation [24]. Thus, we additionally knocked down FADD in CNOT3-knockdown A549/DDP cells to verify its involvement in Caspase 8 activation. The results of western blotting and CCK-8 assay showed that reduction in FADD reversed the enhanced Caspase 8 activation and cell death (Fig. 7a and b). To our surprise, when we additionally knocked down RIPK1 in CNOT3-knockdown A549/DDP cells, the results of western blotting showed that the enhanced apoptosis could not be effectively reversed (Supplemental Fig. 4). Then, we performed immunoprecipitation and found that in CNOT3-knockdown A549/DDP cells, more RIPK3 and Caspase 8 were recruited to form assembly with FADD, while RIPK1 was not involved in the assembly (Fig. 7c).

To sum up, our data suggested the elevated RIPK3 can recruit more FADD and Caspase 8 to facilitate apoptosis when CNOT3 is depleted.

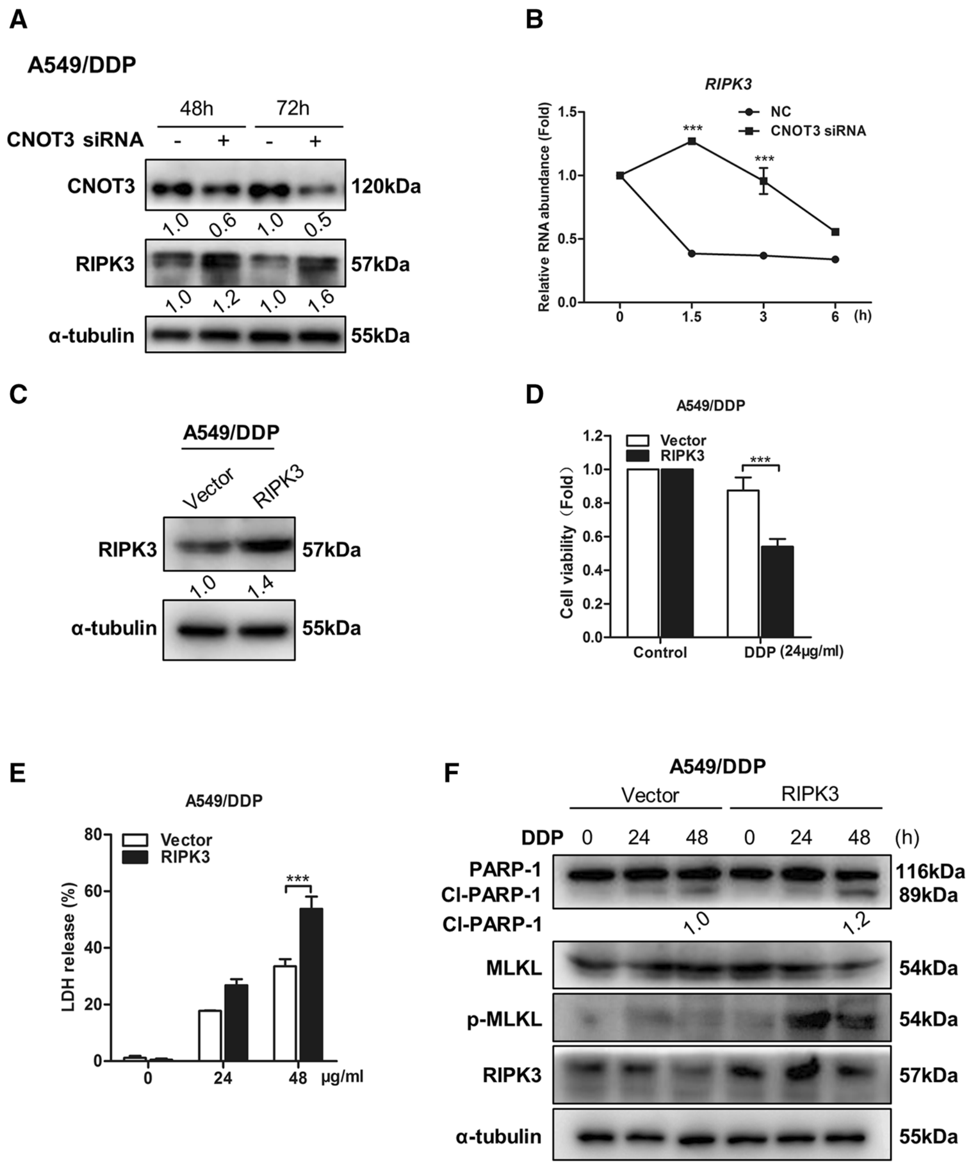

Fig. 5 CNOT3 down-regulates RIPK3 to facilitate cisplatin resistance. **a** A549/DDP cells were transfected with a control or CNOT3 siRNA. Cells were subjected to western blotting at indicated time to assess CNOT3, RIPK3 and α-tubulin expression levels. **b** A549/DDP cells were transfected with a control or CNOT3 siRNA followed by Act. D treatment. Relative mRNA levels were determined by q-PCR at the indicated time after Act. D treatment and normalized to the *GAPDH* mRNA level. mRNA level prior to Act. D treatment (0 h) was set to 1. **c** Seventy-two hours after infection, RIPK3-overexpressed A549/DDP cells and A549/DDP cells were subjected to western blotting to assess RIPK3 and α-tubulin expression levels. **d**–**f** Forty-eight hours after infection, RIPK3-overexpressed A549/DDP cells and A549/DDP cells were treated with indicated doses of DDP for 48 h or for the indicated period of time. Cytotoxicity was analyzed via the CCK-8 (**d**) and LDH release assay (**e**). Cells were subjected to western blotting to assess PARP-1, p-MLKL, MLKL, RIPK3 and α-tubulin expression levels (**f**). Data are shown as the mean ± S.E.M. n = 3. (**b**, **d** and **e**) Two-way ANOVA and Bonferroni post hoc test. $^{***}P < 0.001$ for comparisons between the indicated groups. Numbers below the blots correspond to relative expression levels compared with the reference point set to 1

Discussion

CNOT3, a subunit of CCR4-NOT complex, is a posttranscriptional regulator which promotes degradation of the target mRNA [6]. It participates in many biological processes such as cardiomyocyte autophagy [25], energy metabolism [9, 26], and bone formation [10]. Though some studies have reported that CNOT3 is involved in tumor formation and progression, its role in cancer remains elusive [15, 27–29]. It is suggested in our study that CNOT3 is up-regulated in lung

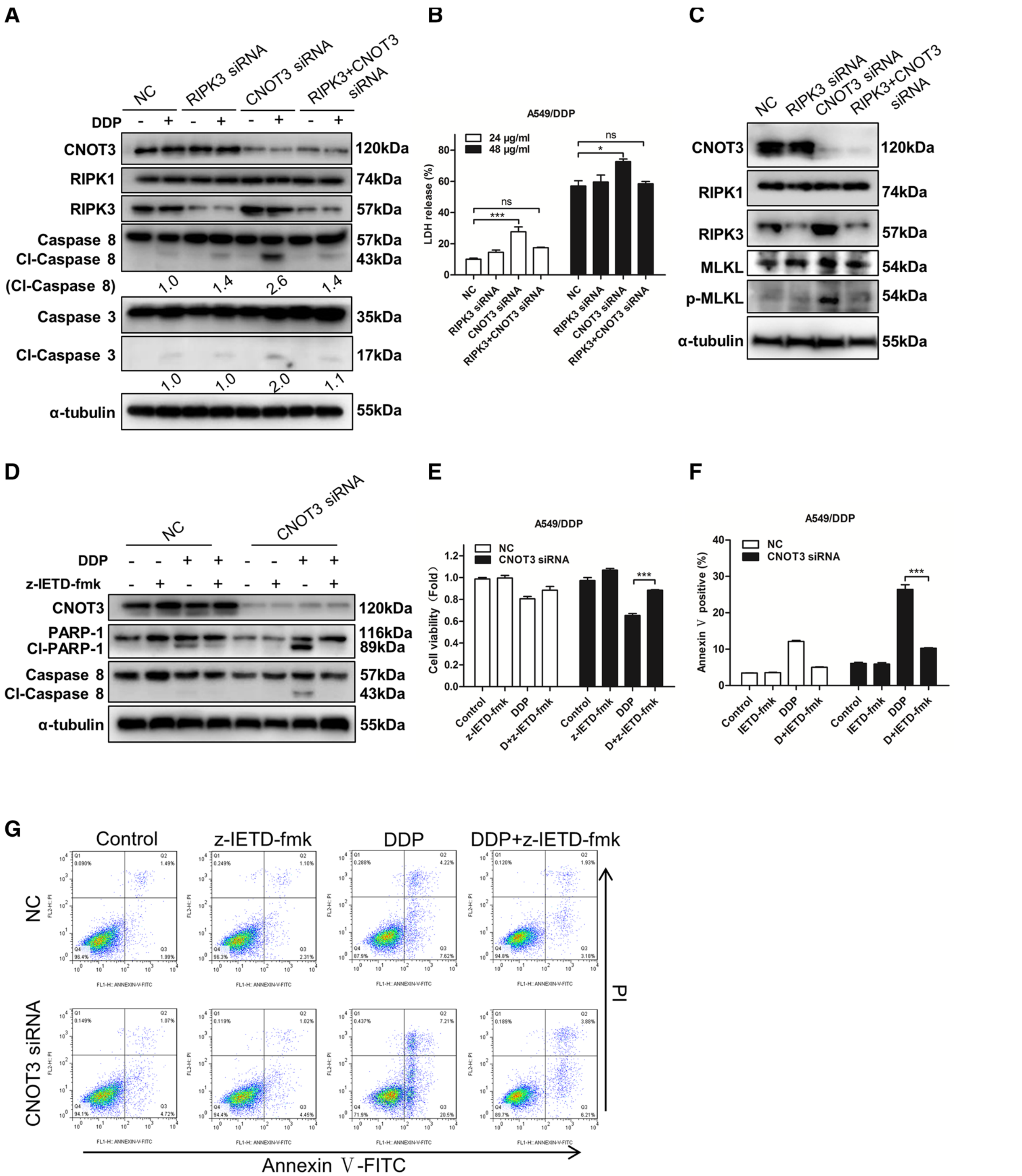

Fig. 6 RIPK3 up-regulation enhances extrinsic apoptosis in CNOT3-knockdown lung cancer cells. **a, b** A549/DDP cells were simultaneously transfected with CNOT3 siRNA and RIPK3 siRNA followed by DDP treatment. Cells were subjected to western blotting to assess protein expression levels (**a**). Cell death was analyzed via the LDH release assay (**b**). **c** A549/DDP cells were simultaneously transfected with CNOT3 siRNA and RIPK3 siRNA, and then were subjected to western blotting to assess protein expression levels. **d**–**g** A549/DDP cells were transfected with a control or CNOT3 siRNA. Twenty-four hours after transfection, cells were pretreated with or without z-IETD-fmk then exposed to DDP or left untreated. Cells were subjected to western blotting to assess protein expression levels (**d**). Cytotoxicity and cell death were measured respectively via the CCK-8 assay (**e**) and flow cytometry (**f**, **g**). Data are shown as the mean ± S.E.M. n = 3. (**b**, **e** and **f**) One-way ANOVA with Tukey post hoc test. $^{***}P < 0.001$ or $^{*}P < 0.05$ for comparisons between the indicated groups. Numbers below the blots correspond to relative expression levels compared with the reference point set to 1

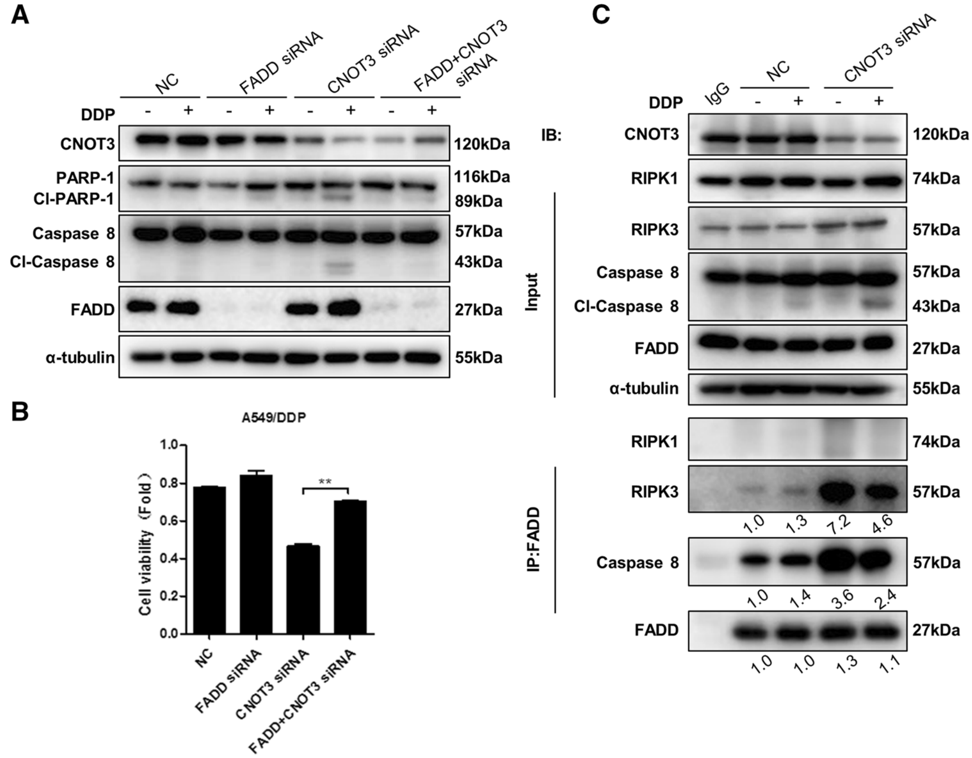

Fig. 7 Elevated RIPK3 recruits FADD to activate Caspase 8 and mediate apoptosis. **a, b** A549/DDP cells were simultaneously transfected with CNOT3 siRNA and FADD siRNA followed by DDP treatment. Cell lysates were subjected to western blotting with the indicated antibodies (**a**). Cytotoxicity was determined via the CCK-8 assay (**b**). **c** A549/DDP cells were transfected with a control or CNOT3 siRNA. FADD immunoprecipitates were analyzed by western blotting with the indicated antibodies. Data are shown as the mean ± S.E.M. n = 3. (**b**) One-way ANOVA with Tukey post hoc test. $^{**}P < 0.01$ for comparisons between the indicated groups. Numbers below the blots correspond to relative expression levels compared with the reference point set to 1

cancer tissues and regulates lung cancer cell proliferation, which is consistent with a previous report [18]. Additionally, we discussed in this study about how CNOT3 regulates lung cancer cell death and especially about its role in cisplatin resistance.

It is broadly acknowledged that RIPK1 is involved in TNF-α-stimulated apoptotic and necroptotic signaling. At the same time, RIPK3 is more considered to participate in necroptotic signaling [30]. Nevertheless, reports that RIPK3 contributes to apoptosis also exist [31, 32]. For example, in conditions of cIAP1/2 depletion or TAK1 kinase inhibition, RIPK3 is able to contribute to TNF-α-mediated RIPK1-dependent apoptosis [33]. And our results happen to be consistent with the idea.

Some studies have suggested that in TNF-α-triggered apoptotic signaling, the processing of Caspase 8 relies on RIPK1 and even though RIPK3 can drive assembly of FADD and Caspase 8, RIPK1 is necessary for the recruitment [23, 34]. However, it is also reported that when its expression level is elevated, RIPK3 is capable of binding and activating Caspase 8 in the absence of RIPK1, though the processing may be less efficient [31, 35, 36]. Similarly, in our study, we found that when RIPK3 expression level was elevated by CNOT3 depletion, Caspase 8 activation could be enhanced and it was independent of RIPK1.

It is described in other studies that RIPK3 overexpression is able to promote necroptosis [36–38]. Owing to the increased RIPK3, MLKL phosphorylation could be detected in CNOT3-knockdown A549/DDP cells. However, interestingly and surprisingly, though MLKL phosphorylation was detectable in CNOT3-knockdown A549/DDP cells, cell death increase could not be measured (Fig. 4e, h and i). Given the previous reports [39, 40], we speculate that it was probably because the MLKL activation was limited and/or the phosphorylated MLKL was extruded from cells in exosomes so that the cells were able to sustain survival.

Previous studies have demonstrated that depletion of CNOT1 or CNOT2, other two subunits of CCR4-NOT complex, leads to apoptosis by destroying the CCR4-NOT-associated deadenylase activity [41, 42]. However, the relationship between CNOT3 depletion and apoptosis is not well understood. Though a study has shown that knocking down CNOT3 in A549 cells does not trigger apoptosis [18], we noticed in our study that CNOT3 depletion triggered apoptosis in 95-D cells. In addition, it's intriguing that unlike RIPK3-overexpressed A549/DDP cells, only apoptosis was enhanced in CNOT3-depleted A549/DDP cells after they were treated with cisplatin. Based on our results and other studies, we analyze here how CNOT3 depletion facilitates apoptosis. On the one hand, the elevated RIPK3 recruited FADD and Caspase 8 to form larger complex in CNOT3-knockdown cells regardless of cisplatin treatment, thus it was easier to activate apoptotic signaling upon stimulation. On the other hand, a microarray data has suggested that mRNAs encoding proteins which positively regulate apoptosis (e.g. DAPK1, GNA13) are up-regulated in CNOT3-depleted cells, which may also help to trigger apoptosis [11, 43–45].

In conclusion, our study briefly delineates the relationship between CNOT3 and lung cancer and verifies CNOT3 high-expression contributes to cisplatin resistance of lung cancer cells. We show that CNOT3 depletion can sensitize lung cancer cells to cisplatin-induced apoptosis via up-regulating RIPK3 expression levels, which indicates that CNOT3 could be a possible target to enhance chemosensitivity of lung cancer cells and even to overcome cisplatin resistance.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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