The arginine methyltransferase PRMT5 and PRMT1 distinctly regulate the degradation of anti-apoptotic protein CFLAR_L in human lung cancer cells

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Abstract

Background: CFLAR_L, also known as c-FLIP_L, is a critical anti-apoptotic protein that inhibits activation of caspase 8 in mammalian cells. Previous studies have shown that arginine 122 of CFLAR_L can be mono-methylated. However, the precise role of arginine methyltransferase of CFLAR_L remains unknown. PRMT5 and PRMT1, which are important members of the PRMT family, catalyze the transfer of methyl groups to the arginine of substrate proteins. PRMT5 can monomethylate or symmetrically dimethylate arginine residues, while PRMT1 can monomethylate or asymmetrically dimethylate arginine residues.

Methods: Lung cancer cells were cultured following the standard protocol and the cell lysates were prepared to detect the given proteins by Western Blot analysis, and the protein interaction was assayed by co-immunoprecipitation (Co-IP) or GST pull-down assay. CFLAR_L ubiquitination level was evaluated by proteasomal inhibitor treatment combined with HA-Ub transfection and WB assay. PRMT1 and PRMT5 genes were knocked down by siRNA technique.

Results: We show that PRMT5 up-regulated the protein levels of CFLAR_L by decreasing the ubiquitination and increasing its protein level. Additionally, PRMT1 down-regulated the protein level of CFLAR_L by increasing the ubiquitination and degradation. The overexpression of PRMT5 can inhibit the interaction between CFLAR_L and ITCH, which has been identified as an E3 ubiquitin ligase of CFLAR_L, while overexpressed PRMT1 enhances the interaction between CFLAR_L and ITCH. Furthermore, we verified that dead mutations of PRMT5 or PRMT1 have the same effects on CFLAR_L as the wild-type ones have, suggesting it is the physical interaction between CFLAR_L and PRMT1/5 that regulates CFLAR_L degradation other than its enzymatic activity. Finally, we showed that PRMT5 and PRMT1 could suppress or facilitate apoptosis induced by doxorubicin or pemetrexed by affecting CFLAR_L in NSCLC cells.

Conclusions: PRMT5 and PRMT1 mediate the distinct effects on CFLAR_L degradation by regulating the binding of E3 ligase ITCH in NSCLC cells. This study identifies a cell death mechanism that is fine-tuned by PRMT1/5 that modulate CFLAR_L degradation in human NSCLC cells.

Keywords: CFLAR, PRMT1, PRMT5, ITCH, Apoptosis
Introduction

CFLAR, which is a CASP8 and FADD-like apoptosis regulator, also known as c-FLIP, is an important regulatory protein in the extrinsic apoptotic pathway in mammalian cells. Several transcript variants encoding different isoforms have been reported. The short form, i.e., CFLAR \(_s\) (c-FLIP\(_s\)), contains two N-terminal death effector domains (DED), whereas the long form, i.e., CFLAR \(_l\) (c-FLIP\(_l\)), contains an additional pseudo-caspase domain in which the active center cysteine residue that confers the proteolytic activity of caspases is substituted by a tyrosine residue [1]. CFLAR\(_l\) can inhibit and prevent apoptosis by interfering with procaspase 8/10 for binding to the FADD domain to decrease caspase 8 activation. This binding prevents further death-inducing signaling complex (DISC) formation and subsequent activation of the caspase cascade [2].

High levels of CFLAR\(_l\) have been found in many different types of human cancers, and the excessive expression of the protein indicates a high degree of tumor malignancy [3]. In the clinic, CFLAR\(_l\) can be used as an independent adverse prognostic biomarker of colorectal cancer (CRC) [4]. Many chemotherapeutic agents have been shown to down-regulate CFLAR\(_l\) at the protein and mRNA level. Silencing its expression has been shown to facilitate apoptosis in chemotherapeutic agent-induced apoptosis. Therefore, CFLAR\(_l\) is a promising therapeutic target in some cancer treatments.

CFLAR\(_l\) can be regulated at both the transcriptional and post-translational level. NF-κB can induce the up-regulation of CFLAR\(_l\) at the mRNA and protein level and inhibit Fas, TNFR1 and TRAIL receptor-induced apoptosis [5]. c-MYC, FOXO3a, and c-Fos inhibit the transcription of CFLAR\(_l\) [6, 7]. Additionally, CFLAR\(_l\) has been shown to be down-regulated following treatment with compounds such as cycloheximide (CHX) and anisomycin [8, 9]. As the half-life of CFLAR\(_l\) is short, the ubiquitin-proteasome system plays an important role in regulating CFLAR\(_l\) degradation and stability. CFLAR\(_s\) is highly prone to ubiquitination and degradation likely due to its unique C-terminal tail [10]. The E3 ubiquitin ligase ITCH is thought to be responsible for CFLAR\(_l\) ubiquitination and degradation [11]. ITCH has also been shown to be an important regulator of CFLAR\(_s\) ubiquitination and stability [12, 13]. Furthermore, phosphorylation events play a vital role in the regulation of CFLAR\(_l\) protein levels; for example, the serine residue 273 of CFLAR\(_l\) is phosphorylated by AKT, which is important for the reduction of CFLAR\(_l\) via an ITCH-dependent mechanism [14]. The proteins that interact with CFLAR\(_l\) can also affect its stability. Recently, XRCC6 was shown to interact with, stabilize, and protect CFLAR\(_l\) from ubiquitin-proteasomal degradation [15]. XRCC6 usually forms a stable heterodimer consisting of two subunits (XRCC6 and XRCC5) [16].

Evidence suggests that XRCC proteins modulate ATM activity following DNA damage [17]. XRCC6 acts as an ATP-dependent single strand DNA helicase and has been found to play an important role in immune system disorders, aging and carcinogenesis [18].

Post-translational modifications, including phosphorylation, methylation, acetylation, ubiquitination, ADP-methylation, and SUMOylation, are highly important for the regulation of protein functioning in eukaryotic cells. Among these post-translational modifications, protein arginine methylation governs many cellular processes, such as cell growth, proliferation, differentiation and development [19]. The PRMT family members play a pivotal role in the regulation of the arginine methylation of both histones and other cellular proteins [20]. Three distinct types of methylated arginine residues, namely, omega-N\(^{G}\)-mono-methylarginine (MMA), symmetric omega-N\(^{G}\),N\(^{G}\)-dimethylarginine (sDMA), and asymmetric omega-N\(^{G}\),N\(^{G}\)-dimethylarginine (aDMA), have been identified in mammalian cells [21]. The PRMT enzymes are classified into two groups depending on the type of modification they catalyze. Type I PRMT enzymes (PRMT1–4, PRMT6 and PRMT8) generate MMA and aDMA, whereas type II PRMT enzymes (PRMT5, PRMT7 and PRMT9) catalyze MMA and sDMA [22]. PRMT5 is a type II methyltransferase that modulates cell growth and transformation. PRMT5 hypermethylates histones H3R8 and H4R3 in promoters and restrains the cell cycle and tumor suppressor genes [23–25]. PRMT5 interacts with and methylates P53 at R333, R335, and R337 when DNA is damaged, inhibiting oligomerization between MDM2 and P53 [26, 27]. In particular, PRMT5 can co-localize with EGFR and regulate its monomethylation. R1175 methylation modulates EGFR-induced EGFR trans-autophosphorylation at Y1173 [28].

PRMT1 was the first mammalian protein arginine methyltransferase identified [29]. Most protein arginine methylation is catalyzed by PRMT1 [30]. PRMT1 preferentially methylates arginine residues flanked by one or more glycine residues [31]. According to its three-dimensional structure, PRMT1 is active as a homodimer [32]. Moreover, PRMT1 has been reported to be a negative protein in Wnt/beta-catenin signaling by the methylation of Axin [33]. However, the mechanism by which the members of the protein arginine methyltransferase family, including PRMT5 and PRMT1, modulate apoptosis remains to be elucidated. Our work suggests that PRMT5 and PRMT1 regulate apoptosis by affecting CFLAR\(_l\) turnover.

Methods

Cell lines and cell culture

The lung cancer cell lines A549, H157, H460 and H1299 were cultured in RPMI 1640 supplemented with 5% (v/v) NBCS. The HEK293FT cell line was cultured in DMEM...
medium supplemented with 5% (v/v) newborn calf serum. All cell lines were originally obtained from the American Type Culture Collection (Manassas, VA) and maintained at 37 °C in a humidified atmosphere consisting of 5% CO₂ and 95% air. The cells were used are routinely authenticated and tested for mycoplasma contamination.

Antibodies and reagents
Doxorubicin and pemetrexed powder was purchased from Sigma Aldrich (Merck, Darmstadt, Germany) and diluted in dimethyl sulfoxide (DMSO). Stock solutions were stored at −20 °C and diluted to the desired concentrations with growth medium before use. The PRMT5 (P4847), PRMT1 (G1544) and FLAG (F7425) antibodies were purchased from Sigma Aldrich (America). The CFLARL (ALX-804-961-0100) antibody was purchased from Enzo Biochem. The monomethyl arginine (8015S), CASP8 (9746 L) and PARP-1 (#9542) antibodies were purchased from Cell Signaling Technology (Boston, Massachusetts, US). The symmetric dimethyl arginine and asymmetric dimethyl arginine antibodies were purchased from Millipore.

Western blot analysis
The preparation of whole-cell protein lysates and procedures used for the western blot analysis have been previously described [34]. The cells were harvested and rinsed with pre-chilled PBS. Then, the cells were lysed and centrifuged at 4 °C for 15 min. Samples of the whole-cell protein lysates (35 μg) were electrophoresed on a 12% denaturing polyacrylamide slab gel and then transferred to a polyvinylidene fluoride (PVDF) membrane by electroblotting. The proteins were probed with the appropriate primary antibodies and subsequently the secondary antibodies. Antibody binding was detected by an HRP system according to the manufacturer’s protocol.

siRNA transfection
The siRNAs were synthesized by Boshang. The small interfering RNA (siRNA) duplexes used for the controls have been previously described [34]. The PRMT5 siRNA duplexes target the sequences 5'-GCCAGGUUGGAGAU GCCUU-3' (#1) and 5'-GGCCUAUGGACCUUGGAA-3' (#2). The PRMT1 siRNA duplexes target the sequences 5'-CCACAGGCCCCAGUCCC-3' (#1) and 5'-ACCAGCAAUUGUUUCGA-3' (#2). The negative control was 5'-UUUCUCCAGGACGGGGCU-3'. The siRNA transfections were carried out with the jet PRIME® siRNA Transfection Reagent (Polyplus) following the manufacturer’s instructions.

Construction of the plasmids
The PRMT5 gene was amplified by PCR from H1299 cell genomic DNA using the following primers: MYC-PRMT5 sense: 5'-CGGATCCGGCGCCACCATGGAACAAAAAC TCATCTCAAGAAGGATCTGGCCGGATGGCGGT CG-3', PRMT5 sense: CCGATCCGGCGCCACCATG GCGCGATGGCGTGTCG antisense: 5'-CAAACTCTT AGAGGCAATGTTATGAG-3'; PRMT1 sense: 5'-CGGATCCGGCGCCACCATG GCGCGAGGGCGGCGG antisense: 5'-CGAATTCGTCGTA TGTAGTCGTT-3'; CFLAR sense: 5'-GGTACCGCGCCACCATGTCCGTAATCCACAT-3' CFLAR antisense: 5'-CGGTCGAGTTTGTTGTGAAGTTA AGATAAG-3'; HA-ITCH sense: 5'-CGGATCCGGCGCCCA CCATGTACCCCTACGACGTGCCCGACTACGCCTCT GACAGTGGATCACACT-3'; and ITCH sense: 5'-CGGA TCGGGCCACCATGTCCGTAATCCACAT-3' antisense: 5'-CGGCCCCTTACTCTTTGTCCAAATCTT CTTG-3'. The plasmid construction procedures have been previously described [35, 36].

Immunoprecipitation
Cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM Na₂EDTA; 1 mM EGTA; 2.5 mM sodium pyrophosphate; 1 mM β-glycerophosphate; 1 mM Na₃VO₄; 0.5% Triton) on ice for 30 min then purified via centrifugation for 15 min at 4 °C. The supernatants were incubated with antibody at 4 °C for 1 h. Then the mixture was incubated with protein A beads (ThermoFisher) at 4 °C for 2 h. The beads were washed twice with 1 ml of lysis buffer. 20 μl 2 × SDS buffer were added for elution (100 °C, 10 min). Samples were centrifuged for western blot analysis.

GST pull-down assay
The HEK293FT cells were collected, lysed in immunoprecipitation lysis buffer and incubated on ice for 30 min. The lysates were purified via centrifugation for 15 min at 4 °C. The supernatants were incubated with glutathione sepharose at 4 °C overnight. The beads were washed twice with 900 μl of IP lysis buffer (1% PIC) buffer, followed by incubation with 2X SDS at 100 °C for 10 min. Then, the beads were centrifuged for 5 min at room temperature. Finally, the supernatants were thoroughly collected for SDS-PAGE and western blot analysis.

Flow cytometry analysis
Annexin V-FITC Apoptosis Detection Kit (Biobox Bio tech, Nanjing, China) was used for cell apoptosis analysis according to the manufacturer’s protocol.
Statistical analysis
GraphPad Prism version 5.00 was used for statistical analysis. All data are presented as the mean ± SD. Differences between groups were identified using Student’s t-test. \( P < 0.05 \) was considered statistically significant.

Results
PRMT5 and PRMT1 regulate protein level of CFLARL and its sensitivity to chemotherapeutic agents
Given that mass spectrometry analyses have revealed that the arginine 122 residue of CFLARL can be methylated [37], we sought to explore the role of the methylation of CFLARL in human lung cancer cells. Since arginine methyltransferases (PRMTs) can catalyze the monomethylation of histones and non-histones [38, 39], we examined whether PRMT5 and PRMT1 could alter the level of CFLARL. We transfected NSCLC cells with siRNAs targeting the corresponding candidates and performed an immunoblot analysis to assess the expression status of CFLARL. As shown in Fig. 1A, CFLARL expression was reduced by knocking down PRMT5 in human NSCLC cells. Consistently, the overexpression of PRMT5 led to an increase in CFLARL in the tested cells (Fig. 1B). Similar results were also obtained in NSCLC cells treated with doxorubicin; the protein level of CFLARL was lower in the PRMT5 siRNA-transfected cells than in the control cells (Fig. 1C). The overexpression experimental results are consistent with the results mentioned above (Fig. 1D). Thus, doxorubicin alters CFLARL levels in cells when PRMT5 is silenced in NSCLC cells. Similarly, we blocked PRMT1 expression using PRMT1 siRNA in H157 and H1299 cells, and the western blot analysis showed that the expression of CFLARL was much increased (Fig. 1E). Consistently,

![Image](https://example.com/image.png)
the overexpression of PRMT1 led to a reduction in the CFLAR\textsubscript{L} levels (Fig. 1F). In NSCLC cells treated with doxorubicin, the protein level of CFLAR\textsubscript{L} was considerably higher in the PRMT1 siRNA-transfected cells than in the control siRNA-transfected cells (Fig. 1G). This effect was also observed in the PRMT1-overexpressed cells (Fig. 1H). Thus, the inhibition of PRMT1 could protect CFLAR\textsubscript{L} from doxorubicin-mediated degradation.

Both PRMT5 and PRMT1 can interact with CFLAR\textsubscript{L}

To examine whether PRMT5 and PRMT1 interact with CFLAR\textsubscript{L}, we carried out GST pull-down assays by cotransfecting PRMT5 or PRMT1 plasmids and pEBG-GST-CFLAR\textsubscript{L} in 293FT cells. The results show that CFLAR\textsubscript{L} interacts with both PRMT5 and PRMT1. To further verify the physical interaction between PRMT5 and CFLAR\textsubscript{L}, 293FT cells were transfected with plasmids carrying control plasmid pDNA3.1 or MYC-tagged PRMT5. After immunoprecipitation with the MYC antibody, MYC-PRMT5 was pulled down. The western blot analysis showed that PRMT5 certainly interacts with CFLAR\textsubscript{L} (Fig. 2A, B). We used similar methods to verify the relationship between PRMT1 and CFLAR\textsubscript{L} (Fig. 2C, D). The endogenous interaction between CFLAR\textsubscript{L} and PRMT1/5 was also examined (Fig. 2E). To determine the region in PRMT5 and PRMT1 critical for binding CFLAR\textsubscript{L}, we constructed several deletion mutants of PRMT5 and PRMT1. The full-length CFLAR\textsubscript{L} mainly bound to the TIM barrel domain and oligomerization domain of PRMT5 (Fig. S1A). And, the full-length CFLAR\textsubscript{L} could bind two truncated parts of PRMT1 (Additional file 1: Figure S1B). Further co-immunoprecipitation analyses revealed that the full-length PRMT5 and PRMT1 could bind the caspase-like domain but not the DED domain of CFLAR\textsubscript{L} (Additional file 1: Figure S1C, S1D). Thus, the P20 and P12 domains (caspase-like domain) of CFLAR\textsubscript{L} are important for the interaction between PRMT5/PRMT1 and CFLAR\textsubscript{L}.

PRMT1 and PRMT5 regulates CFLAR\textsubscript{L} degradation independently of their enzymatic activity

Considering that PRMT5 and PRMT1 belong to a major class of enzymes that catalyze the three types of arginine methylation reactions, we examined the types of CFLAR\textsubscript{L} arginine methylation in vivo. Thus, we carried out pull-down assays by overexpressing pEBG-GST-CFLAR\textsubscript{L} in H1299 cells. As shown in Fig. 3A, the level of monomethyl arginine in CFLAR\textsubscript{L} was decreased after the PRMT5 overexpression. However, the level of symmetric dimethyl arginine in CFLAR\textsubscript{L} was increased after PRMT5 was overexpressed (Fig. 3B). Then, we tested the methylation of CFLAR\textsubscript{L} after PRMT1 was overexpressed. By performing the pull-down assay, we found that the level of monomethyl arginine in CFLAR\textsubscript{L} was decreased after PRMT1 was overexpressed (Fig. 3C); however, the level of asymmetric dimethyl arginine of CFLAR\textsubscript{L} was increased (Fig. 3D).

In order to characterize whether the arginine methylation or the physical interaction of CFLAR\textsubscript{L} and PRMT1/5 regulates the degradation of CFLAR\textsubscript{L}, we constructed the plasmids containing PRMT1 or PRMT5 genes with

![Fig. 2](image-url)
dead mutations as described before [40, 41]. The plasmids are designated as pcDNA3.1-PRMT5-T139/144A and pcDNA3.1-PRMT1- G98R, respectively. As shown in Fig. 3E, CFLAR L was upregulated after overexpression of both wild-type PRMT5 and its dead mutation in A549 cells. Similarly, CFLAR L was downregulated after overexpression of both wild-type PRMT1 and its dead mutation in A549 cells (Fig. 3F), suggesting it is the physical interaction of CFLAR L and PRMT1/5 that regulates CFLARL degradation other than its enzymatic activity.

**PRMT1 and PRMT5 regulate poly-ubiquitination of CFLAR L by affecting the interaction between CFLAR L and ITCH**

Recent studies have reported that CFLAR L is degraded predominantly by the ubiquitin-proteasome degradation pathway as well as lysosomal pathway [42, 43]. Since PRMT5 and PRMT1 could modulate the protein levels of CFLAR L, we examined whether they affect CFLAR L ubiquitination and degradation. Therefore, we blocked PRMT5 expression using PRMT5 siRNA in H1299 cells and then treated the cells with 20 μmol/L MG132 for 4 h or 15 μmol/L E64D for 6 h. MG132 is a specific, potent, reversible and cell-permeable proteasome inhibitor that reduces the degradation of ubiquitin-conjugated proteins in mammalian cells via the 26S complex. E64D, which is a synthetic analog of E-64 and an ethyl ester of E-64C, is an irreversible, membrane-permeable inhibitor of lysosomal and cytosolic cysteine proteases. E-64D inhibits calpain and the cysteine protease cathepsins F, K, B, H, and L [44, 45]. Our data indicated that the silencing of PRMT5 by small interfering RNA could down-regulate the protein levels of CFLAR L. The PRMT5 knockdown-mediated CFLAR L degradation was prevented by the addition of the proteasome inhibitor MG132 (Fig. 4A). Similarly, we found that PRMT1 also decreased the protein expression of CFLAR L after PRMT1 was overexpressed (Fig. 4B). Subsequently, we examined the ubiquitination level of CFLAR L, and our results suggest that the PRMT5 knockdown

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**Fig. 3** PRMT1 and PRMT5 regulates CFLAR L independently of their enzymatic activity. 

**a** H1299 cells were transfected with the pEBG-CFLAR L plasmids and co-transfected with pcDNA3.1 or pcDNA3.1-MYC-PRMT5. The cells were harvested and prepared for the GST pull-down assay after 24 h, and the arginine monomethylation of the CFLAR L protein was detected by western blotting. 

**b** The symmetric arginine dimethylation of the CFLAR L protein was detected by western blotting. 

**c** Cells were transfected with the pEBG-CFLAR L plasmids and co-transfected with pcDNA3.1 or pcDNA3.1-MYC-PRMT1. The cells were harvested and prepared for the GST pull-down assay after 24 h, and the arginine monomethylation of the CFLAR L protein was detected by western blotting. 

**d** The asymmetric arginine dimethylation of the CFLAR L protein was detected by western blotting. 

**e** and **f** A549 cells were seeded in 6-well plates. PRMT5-WT and PRMT5-T139/144A plasmids or PRMT1-WT and PRMT1-G98R plasmids were transfected for 24 h, while pcDNA3.1 was transfected as control. Cells were harvested for western blot analysis.
promoted the polyubiquitination level of CFLAR_L (Fig. 4C). In addition, the polyubiquitination level of CFLAR_L decreased after PRMT1 was knocked down (Fig. 4D). ITCH has been reported to be the E3 ubiquitin ligase of CFLAR_L. To determine whether PRMT5 and PRMT1 regulate CFLAR_L by affecting the interaction between CFLAR_L and ITCH, we carried out GST pull-down assays and co-immunoprecipitation assays. Our data indicate that the overexpression of PRMT5 reduced the interaction between CFLAR_L and ITCH (Fig. 4E). After we overexpressed PRMT1, the interaction between CFLAR_L and ITCH was enhanced (Fig. 4F). Taken together, we conclude that PRMT5 and PRMT1 regulate the ubiquitination of CFLAR_L by affecting the interaction between CFLAR_L and ITCH.

PRMT5 and PRMT1 regulate degradation of CFLAR_L

Considering that the polyubiquitination level of CFLAR_L is regulated by PRMT5 and PRMT1, we explored whether they regulated CFLAR_L degradation. We conducted cycloheximide chase assays in H460 cells. We used 10 μg/ml cycloheximide (CHX) to block protein synthesis and examined CFLAR_L protein turnover in control and PRMT5 or PRMT1 plasmid-transfected cells for the indicated time points. As shown in Fig. 5A and B, the half-life of CFLAR_L...
was significantly lengthened in the PRMT5 overexpressed cells compared with that in the control cells, while the half-life of CFLAR_L was shortened in the PRMT1 overexpressed cells compared with that in the control cells. Taken together, we conclude that PRMT5 and PRMT1 contribute to the turnover of CFLAR_L as regulators.

PRMT5 and PRMT1 modulate caspase 8 cleavage and apoptosis induced by the anti-cancer drugs in NSCLC cells

CFLAR_L is known as a crucial anti-apoptotic regulator that suppresses death receptor-induced apoptosis by interfering with the processing of procaspase 8 in DISC [20]. Therefore, we explored the effect of PRMT1/5 on caspase cleavage and activation. We overexpressed PRMT5 in lung cancer cells for 24 h and then treated the cells with 2 μmol/L doxorubicin for 24 h. Several apoptosis-related proteins were examined by performing a western blot analysis. The data showed that CFLAR_L was up-regulated, and weak cleavage of CASP8, CASP3 and PARP was induced by doxorubicin in the PRMT5-overexpressed cells after the exposure to doxorubicin (Fig. 6A), indicating that PRMT5 protects cells from caspase activation and apoptosis induced by doxorubicin. Coincidently, we also knocked down PRMT5 expression using siRNA in lung cancer cells. The data demonstrated that CFLAR_L was down-regulated, and caspase activation induced by doxorubicin was strengthened after PRMT5 was silenced (Fig. 6B). We also overexpressed PRMT1 in lung cancer cells for 24 h and then treated the cells with 2 μmol/L doxorubicin for 24 h. The western blot analysis showed a high cleavage of CASP8, CASP3 and PARP induced by doxorubicin in the PRMT1-overexpressed cells after exposure to doxorubicin, indicating that PRMT1 promotes caspase activation after doxorubicin exposure (Fig. 6C). In addition, we knocked down PRMT1 and found that CFLAR_L was up-regulated and that cellular apoptosis induced by doxorubicin was markedly weakened after PRMT1 was silenced (Fig. 6D). We also examined the percentage of pemetrexed-induced apoptotic cells after PRMT5 and PRMT1 alteration using FACS technique. Cellular apoptosis was reduced after PRMT5 overexpression or PRMT1 knockdown, and cellular apoptosis was increased after PRMT5 knockdown (Fig.S2). Taken together, we conclude that PRMT5 and PRMT1 truly affect apoptosis induced by anti-cancer drugs doxorubicin and pemetrexed in NSCLC cells.
PRMT5 and PRMT1 modulate apoptosis in NSCLC cells by regulating protein levels of CFLAR L

Since PRMT5 and PRMT1 could modulate apoptosis and CFLAR L levels, we considered whether PRMT5 and PRMT1 affect human NSCLC cell apoptosis by regulating CFLAR L expression. Accordingly, we constructed an A549 cell line that could stably overexpress CFLAR L. The cleavage of pro-caspases and PARP-1 in the PRMT5 knockdown A549-CFLAR L cells was weaker than that in the control knockdown cells after treatment with doxorubicin, suggesting that CFLAR L could prevent cancer cells from PRMT5-knockdown-enhanced apoptosis after doxorubicin treatment (Fig. 7A). Moreover, we overexpressed PRMT1 in A549-LacZ cells and A549-CFLAR L cells and simultaneously treated the cells with doxorubicin. We found that the enhanced apoptosis caused by the PRMT1 overexpression was suppressed in the A549-CFLAR L cells compared with that in the A549-LacZ cells (Fig. 7B). Taken together, we conclude that PRMT5 and PRMT1 affect apoptosis by regulating the CFLAR L level in NSCLC cells. In addition, we found that the PRMT5 knockdown did not affect the expression of PRMT1, and vice versa (Fig. 7C, D).

PRMT5 competes with PRMT1 for binding to CFLAR L

After verifying that both PRMT5 and PRMT1 interact with CFLAR L, we contemplated whether PRMT5 competes with PRMT1 for binding to CFLAR L. Thus, we conducted GST pull-down assays. Our data indicate that the overexpression of PRMT5 could attenuate the interaction between CFLAR L and PRMT1 (Fig. 8A). Silencing the expression of PRMT5 by RNAi facilitated the
interaction between CFLARL and PRMT1 (Fig. 8B). Similarly, after PRMT1 was overexpressed, the interaction between CFLARL and PRMT5 was attenuated (Fig. 8C). The PRMT1 knockdown increased the interaction between CFLARL and PRMT5 (Fig. 8D).

Discussion
In the present study, we report a new finding that PRMT5 and PRMT1, which are both arginine methyltransferases, distinctly regulate the turnover of CFLARL in human NSCLS cells independently of its enzymatic activity. CFLARL is a critical regulator of apoptosis induction and drug resistance in multiple cancers, such as colon, rectum and lung cancer, and has been considered a potential anti-cancer molecular target. Thus, investigating its molecular mechanisms is particularly meaningful. Our present work verified that PRMT5 and PRMT1 could affect its ubiquitination, degradation. Although overexpression of PRMT5 catalyzed CFLARL to produce arginine symmetric dimethylation, and PRMT1 catalyzed CFLARL to produce arginine asymmetric demethylation, the dead mutations of PRMT1 and PRMT2 had the same effect on the CFLARL level as the wild-type ones had, suggesting it is the physical interaction between CFLAR and PRMT1/5 that regulates CFLARL degradation other than its enzymatic activity.

Several reports have shown that PRMT5 overexpression is associated with hyper-proliferation and apoptosis resistance in cancer cells, and the inhibition of its activity leads to the repression of cancer genes and slows growth [46, 47]. PRMT5 plays essential roles in the regulation of protein function, quality control and signal transduction [48]. Our results show that PRMT5 enhances CFLARL stability. The link between PRMT5 and CFLARL protein longevity is novel and reasonable.

In our study, we found that PRMT5 and PRMT1 regulate the protein level of CFLARL and the sensitivity to chemotherapy drugs. The experiments showed that PRMT5 knockdown or PRMT1 overexpression promoted cell apoptosis induced by doxorubicin and pemetrexed. The PRMT5 overexpression and PRMT1 knockdown inhibited apoptosis induced by chemotherapeutic agents. Furthermore, cellular apoptosis induced by doxorubicin along with the PRMT5 knockdown or PRMT1 overexpression could be inhibited by the exogenous replenishment of CFLARL, suggesting that
PRMT5 and PRMT1 affect apoptosis by regulating the protein level of CFLAR_L in NSCLC cells. We speculated how these two arginine methyltransferases contributed to apoptosis induced by chemotherapeutic agents. Therefore, we verified that both PRMT5 and PRMT1 bound CFLAR_L, regulating CFLAR_L by affecting its ubiquitin-proteasome degradation. The level of CFLAR_L polyubiquitination is increased after overexpressing PRMT1 or knocking down PRMT5 in HEK293FT cells. Our data showed that PRMT5 and PRMT1 were involved in the interaction between CFLAR_L and the E3 ligase ITCH. The PRMT5 silencing and PRMT1 overexpression enhanced the interaction between CFLAR_L and ITCH, leading to an altered ubiquitination level and, eventually, the degradation of CFLAR_L.

Conclusions

In summary, in the present study, we explored the role of the interaction between CFLAR_L and PRMT5/PRMT1 in apoptosis in NSCLC cells. We also demonstrated that PRMT1 and PRMT5 had opposing effects on chemotherapeutic agent-mediated apoptosis in lung cancer cells. The identification of PRMT5 and PRMT1 as CFLAR_L regulators involved in cellular apoptosis may help in developing new strategies to increase the sensitivity of cancer cells to chemotherapy, which may eventually benefit lung cancer treatments.
ITCH: tchy E3 ubiquitin protein ligase; MMIA: Monomethylation; NSCLC: non-small cell lung cancer; PARP: poly ADP-ribose polymerase; PRMT1: Protein arginine methyltransferase 1; PRMT5: Protein arginine methyltransferase 5; SDMA: Symmetric demethylation

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Availability of data and materials
Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Authors’ contributions
ML, WA, LX and LS conducted experiments; XL, ML, LS and YL designed experiments and analyzed data; ML, XL and LS prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval
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Competing interests
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