doi:10.4149/neo_2012_003

Over-expressing CYLD augments antitumor activity of TRAIL by inhibiting the NF-κB survival signaling in lung cancer cells

L. L. DENG[1,‡], Y. X. SHAO[2,‡], H. F. LV[3], H. B. DENG[4], F. Z. LV[2,*]

[1]*Department of Oncology, The Second Affiliated Hospital of Harbin Medical University, 150086, 246 Xuefu Road, Nangang District, Harbin, Heilongjiang, P.R. China;* [2]*Department of Respiratory Medicine, The Second Affiliated Hospital of Harbin Medical University, 150086, Heilongjiang, P.R. China;* [3]*Department of Internal Medicine-Oncology, Henan Cancer Hospital, Zhengzhou University, Zhengzhou, P.R. China;* [4]*Department of General Surgery, The Second Affiliated Hospital of Harbin Medical University, 150086, Heilongjiang, P.R. China*

[*]*Correspondence: lvfuzhendoctor@126.com*
[‡]*Contributed equally to this work.*

Received April 30, 2011 / Accepted July 5, 2011

The death ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can selectively induce apoptosis in tumor cells. But studies have demonstrated that many tumor cells were resistant to TRAIL-induced apoptosis. CYLD is recognized as a negative regulator of nuclear factor-kappa B (NF-κB) activity. To explore a correlation between CYLD expression and responsiveness to TRAIL in lung cancer cell lines, we established lung cancer cell lines that stably express CYLD. Our data provided the first evidence that increased expression of CYLD directly blocks TRAIL-induced NF-κB activation, and consequently increases TRAIL-induced apoptosis in lung cancer cells. CYLD may act as a therapeutic target of lung cancer. Targeting CYLD, in combination with TRAIL, may be a new strategy to treat lung cancer with high NF-κB activity.

Key words: lung cancer; CYLD; NF-κB; TRAIL

The death ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily that recently emerged as a potential cancer therapeutic agent because it preferentially induces apoptosis in transformed or malignant cells but hardly in normal cells [1-3]. TRAIL triggers apoptosis by binding to its death receptors DR4 and DR5 [4, 5], and activating both death receptor (extrinsic) and mitochondria (intrinsic) apoptosis pathways [6, 7]. Ligation of DR4/DR5 by TRAIL results in the assembly of a death-inducing signaling complex (DISC), which contains adapter components such as Fas associated death domain (FADD) [8]. DISC, in turn, activates initiator caspases, such as caspase-8[5]. Consequently, activated caspase-8 may induce apoptosis of tumor cells by activating mitochondria-dependent and mitochondria-independent apoptosis pathways [7, 9].

Currently, recombinant human TRAIL is being tested in phase I clinical trials to treat advanced non-small-cell lung cancer, either alone or in combination with other therapies [10, 11]. TRAIL-induced, death receptor-mediated apoptosis has been subject to intense research as a cancer therapeutic approach. TRAIL selectively induces apoptosis in many types of cancer cells in vivo and vitro [12], however, certain types of cancer cells, such as lung cancer cells, have primary or acquired resistance to TRAIL-induced apoptosis [13-16]. It is known that the inhibitor of apoptosis proteins (IAPs), such as survivin [17], XIAP, c-IAP1, c-IAP2 [18], play a critical role in resistance to TRAIL-induced apoptosis. Mounting evidence confirms that expression of almost all IAP proteins is driven by a transcription factor, nuclear factor-kappa B (NF-κB) [19]. NF-κB consists of a number of closely-related protein dimers of the Rel family that bind to a conserved cis-element on chromosome DNA [20]. NF-κB is inactivated by IκB proteins. Signaling through the IκB kinase (IKK) complex, containing the IκB kinases IKKα and IKKβ and a structural component NF-κB essential modulator (NEMO)(or IKKγ),

causes phosphorylation and subsequent degradation of IκB, allowing for activation of NF-κB [20]. As a factor controlling cell growth and oncogenesis, NF-κB induces transcription of a panel of genes that promotes proliferation and oncogenic transformation [1, 19], as well as to protect cells from undergoing apoptosis. Persistent NF-κB activation was suggested to contribute to cancer development and progression [19, 21]. Multiple stimuli, including TRAIL treatment, activates NF-κB [21], [1, 23]. TRAIL-induced NF-κB activation attenuates apoptosis, predominantly by up-regulating various anti-apoptotic genes, including IAPs [1, 23]. Therefore, NF-κB functions as an upstream regulator of IAPs and regulates negative feedback mechanism of TRAIL signaling [24]. Since TRAIL-induced NF-κB activation renders tumor cells resistant to TRAIL-induced apoptosis, it is tempting to speculate that inhibiting NF-κB prior to TRAIL treatment may allow overcoming resistance to TRAIL-induced apoptosis.

Cylindromatosis (CYLD) was originally identified as a tumor suppressor gene whose mutation causes familial cylindromatosis (Brook-Spiedler syndrome), an autosomal dominant predisposition to multiple tumors of the skin appendages [25]. CYLD down-regulation was correlated with development of many human malignancies in liver, colon [26], lung [27], cervical [28] and kidney [29]. Recent studies have identified CYLD as a key negative regulator for NF-κB signaling , which functions by de-ubiquitinating tumor necrosis factor (TNF) receptor-associated factor (TRAF)2, TRAF6, and NEMO[22,23].CYLD binds to NEMO in the IKK complex and inhibits IKK activation, IκB phosphorylation and degradation , thereby prevent NF-κB activation [23]. Loss of CYLD function results in abnormal activation of NF-κB signaling pathway, leading to increased resistance to apoptosis and a high chance of cell transformation [30].

Currently, there are no reports on whether CYLD can increase TRAIL-induced apoptosis in lung cancer cells. We reasoned that ectopically-expressed CYLD in lung cancer cells could inhibit the activity of endogenous NF-κB and consequently increase sensitivity to TRAIL-induced apoptosis. In this study, using lentiviral transduction, we established an A549 human lung adenocarcinoma cell line stably expressing CYLD. Our study, for the first time, examined the tumor suppressive roles of CYLD in human lung cancer cells. Our results showed that CYLD over-expression increased anti-tumor activities of TRAIL by inhibiting TRAIL-induced NF-κB activation.

Materials and methods

Cell lines, Antibodies and Reagents. 293T cells were purchased from System Biosciences (Mountain View, CA). Human normal bronchial epithelial cells BEAS-2B were purchased from American Type Culture Collection (USA). Human lung cancer cell lines A549, H1299, NCL-H460, SK-MES-1, SPC-A-1 were brought from the Cell Bank of the Chinese Academic of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 100U/ml penicillin and 100μg/ml streptomycin (Invitrogen) at 37°C in an incubator with a humidified atmosphere containing 5% CO2. CYLD antibody was purchased from Abcam Corporation (Cambridge, MA, USA). GAPDH antibody, goat anti-rabbit IgG were purchased from cell signaling technology (Beverly, MA, USA). Anti-XIAP, anti-survivin, anti-caspase-8, anti-caspase-3, anti-poly (ADP-Ribose) polymerase (PARP) antibodies and donkey anti-goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant human TRAIL proteins were purchased from Abcam Corporation (Cambridge, MA, USA). Lipofectamine™ 2000 was purchased from Invitrogen Corporation; PACKH1 Lentivector Packaging Kit was purchased from system biosciences (Mountain View, CA, USA).

Construction of recombinant lentiviral vector containing theCYLD Open Reading Frame. The pcDNA-HA-CYLD plasmid waskindly provided by Prof. Shao-Cong Sun (University of Texas M.D.Anderson Cancer Center, Houston, USA)[33]. pCDH expression lentivectors were purchased from System Biosciences (Mountain View, CA). The CYLD open reading frame was PCR amplified using HA-tagged CYLD as template with the following primers: 5′-CGGGATCCGCCACCATGAGTTCAGGCTTATGGAGC-3′(sense) and 5′-ATAAGAATGCGGCCGCTTATTTGTACAAACTCATTGTTG-3′ (antisense). The resulting PCR product was digested with BamHI and NotI and subcloned into the pCDH expression lentivector.

Transduction with lentiviral vectors. Lentivirus was produced from 293T cells by transient cotransfection of pPACKH1 HIV Packaging plasmid Mix and the Lenti-CYLD plasmid using Lipofectmaine ™ 2000 reagent (Ivitrogen, CA).

Lentivirus infection and establishment of stable cell lines. Cells were infected with lentivirus at different MOI at 37°C for 24 hours. Efficiency of gene delivery was estimated by expression of GFP visualized by Fluorescence microscopy. Cells infected with the lentivirus were isolated. CYLD expression in these cells was determined by Quantitative Real-time PCR and Western blot.

Quantitative Real-time PCR. Total RNA was extracted from cells using TRIzol reagent (Invitrogen). Reverse transcription reaction with 1 μg of total RNA in 100μl was carried out using M-MLV Reverse Transcriptase (D2640A,Takara, Japan). For quantitative PCR, 10 μl SYBR Premix Ex Taq (Takara, Japan) with 10 μM of each primer and 10 ng cDNA template in 20μl of reaction volume was applied. Primers used for amplification were designed as: CYLD, sense, 5′- GCGTTCCCACAATTCAGCAGT-3′ and antisense, 5′- TCCGGATCGTCGTAGCATTCTC-3′; β-actin (as an internal control), sense, 5′-CCTGTACGCCAACACAGTGC-3′ and antisense, 5′ – ATACTCCTGCTTGCTGATCC-3′. All reactions were performed on the TaKaRa PCR Thermal Cycler Dice. To calculate values of mRNA levels, 2(-Delta Delta C(T)) method was used, where CT is the threshold cycle. Target gene mRNA levels were normalized to that of actin mRNA in the same sample.

Western blotting. Whole cell proteins were isolated by RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.25% Sodium deoxycholate and 1 mM EDTA) with freshly added protease inhibitor cocktail (Roche). Whole cell lysates were clarified by centrifugation at 12,000 × g for 15 minutes at 4°C. Total protein concentrations were determined by BCA Protein Assay (Pierce, Rockford, IL).Equal amounts of proteins were loaded to pre-cast 4-20% SDS-PAGE gels (Invitrogen). Proteins resolved on gels were transferred to PVDF membranes (IPVH00010, Millipore). After electro-transfer, membranes were blocked with 5% nonfat milk in TBS-T buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20), and probed with the desired primary antibodies directed against CYLD, XIAP, survivin and GAPDH. Blots were then incubated with horseradish- conjugated secondary antibodies and detected with the SuperSignal West Pico chemiluminescence substrate (Pierce, Rockford, IL). Optical density of the bands was determined using the LabWork software (UVP Laboratory Products, CA). All experiments were repeated at least three times.

NF-κB Luciferase Reporter Assays. To construct a NF-κB-responsive luciferase reporter plasmid, the cDNA fragment containing NF-kappaB binding site (5′-GGGAATTTCC-3′) was generated by reverse transcription PCR using primers as follows: 5′-CGGGAATTTCCGGGAATTTCCGGGACTTTCCGGGACTTTCCGGGACTTTCCGGGACTTTCCGGGACTTTCCA-3′(sense) and 5′-AGCTTGGAAAGTCCCGGAAAGTCCCGGAAAGTCCCGGAAAGTCCCGGAAAGTCCCGGAAATTCCCGGAAATTCCCGGTAC-3′ (antisense) and inserted into the BamHI and NotI sites of pGL3-basic vector (Promega). For reporter gene assay, cells were transfected with 770ng of the NF-κB-responsive luciferase plasmid and 30ng of a pRL–TK control luciferase expression plasmid encoding Renilla luciferase (5 ng/cm^2), as a control for differences in transfection efficiency, with Lipofectmaine reagent. Twenty four hours after transfection, TRAIL was added into cell culture system. After 24 hours, cells were harvested and firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega Italia, Milano, Italy), according to the manufacturer's instruction. Firefly luciferase activity was normalized to Renilla luciferase activity. The assays were performed in triplicates.

Measurement of cell viability by trypan blue exclusion assay. Stable A549 cell lines or control cells were seeded at a density of 5 x 10^4 cells per 10-cm plate in triplicate and allowed to grow for various periods of time. Next, cells were harvested, and the number of viable cells was determined by trypan blue dye exclusion. Briefly, for trypan blue exclusion assay, trypsinized cells were pelleted and resuspended in 0.2 ml of medium, 0.5 ml of 0.4% trypan blue (Sigma-Aldrich) solution, and 0.3 ml of phosphate-buffered saline solution (PBS). The samples were mixed thoroughly, incubated at room temperature for 15 min and examined under a light microscope. Absolute cell numbers were determined in three separate experiments under identical culture conditions.

Measurement of cell proliferation by MTT assay. Cells were plated in 96-well plates at a final concentration of 1×10^4 cells/well and incubated with different concentration of TRAIL (0,1,5,10ng/ml) for 12,24 and 48hours.The MTT solution (5 mg/ ml) was prepared in DMEM medium, and 15 μl were added to each well after the treatment periods. Absorbance of converted dye was measured at a wavelength of 570 nm. Data were presented as percentage of MTT reduction as compared to that in the control cells. Each experiment was repeated three times.

Cells apoptosis assay. For apoptosis analysis by flow cytometry, the Annexin V assay was performed using the Annexin V: FITC Apoptosis Detection Kit II (BD, CA, USA). Briefly, cells were treated with or without 5 ng/ml of TRAIL. Twenty hours after incubation, cells were harvested and washed twice with ice-cold PBS, then co-stained with 5 μl of Annexin V-FITC and 10 μl of propidium iodide (50 μg/ml) for 15 min at room temperature in the dark. The population of Annexin V-positive cells was evaluated by flow cytometry (FACS Calibur, BD, CA, USA) using 488nm laser excitation.

Statistical analysis. All data were expressed as means with standard deviation (SD) and were analyzed using independent samples t test and One-way ANOVA. p values less than 0.05 were defined as statistically significant.

Results

Down-regulated CYLD expression in lung cancer cells. Initially, we evaluated CYLD mRNA expression in five human lung cancer cell lines (A549, H1299, NCL-H460, SK-MES-1, SPC-A-1) compared with that in a normal non-tumorous bronchial epithelial cells (BEAS-2B) using quantitative real-time PCR. We found strong reduction in the expression of CYLD mRNA in all lung cancer cell lines compared with normal control ($p < 0.01$) (Figure 1A). Among all five lung cancer cell lines, CYLD mRNA expression in A549 cells was the lowest (Figure 1A). We also analyzed CYLD protein levels in five tumor cell lines in comparison with normal non-tumorous control cells using western blotting. We detected remarkably low levels of CYLD protein in all lung cancer cell lines comparing to that in the non-neoplastic cells (Figure1B). CYLD protein expression in A549 cells was the lowest among five cancer cell lines (Figure 1B), which was consistent with that of mRNA expression. These data indicated that CYLD was highly expressed in normal cells, whereas less expressed in malignant cells.

Over-expression of CYLD sensitizes TRAIL-induced growth inhibition Since CYLD has the lowest level of expression in A549 cells, we established an A549-Lv-CYLD cell line that stably expressed CYLD using a lentiviral expression system. The over-expression of CYLD was determined by Real-time PCR and Western blotting as presented in Figure 2. We obtained increased CYLD expression in cells transfected with the CYLD lentivector, and no changes in CYLD expression in cells transfected with empty vectors (A549-Lv-plasmid) (Figure 2).

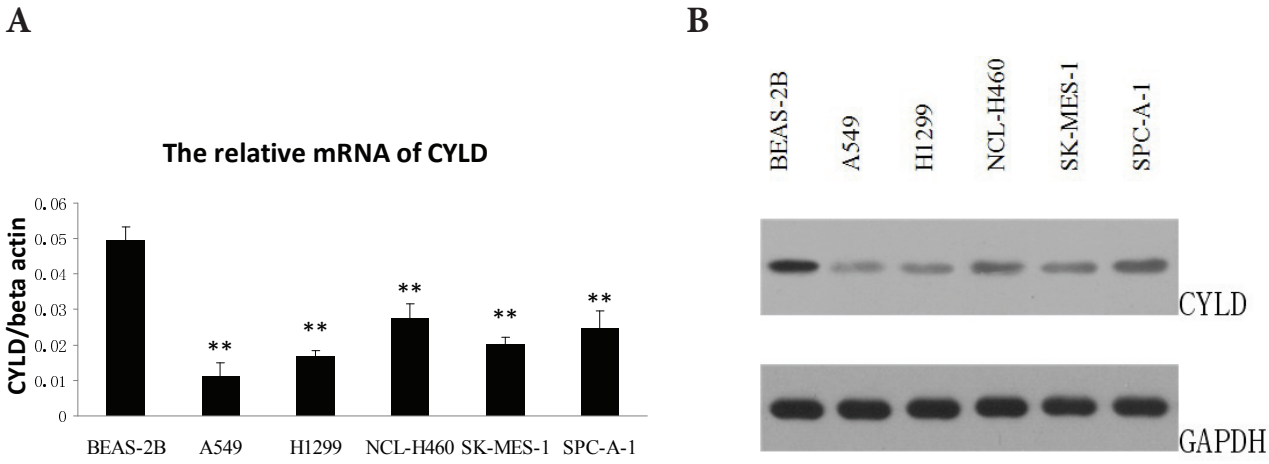


Figure 1. CYLD expression in tumor cell lines compared with corresponding normal cells. A, CYLD mRNA expression was analyzed in five lung cancer cell lines A549, H1299, NCL-H460, SK-MES-1, SPC-A-1 and a human normal bronchial epithelial cells BEAS-2B by quantitative real-time PCR, using primers specific to CYLD and β-actin as a control. Individual data points are the mean ± SD of triplicate determinations. ** p<0.01. B, CYLD protein expression was analyzed in five lung cancer cell lines and corresponding normal cells by western blotting with anti-CYLD antibody and anti-GAPDH antibody as a control.

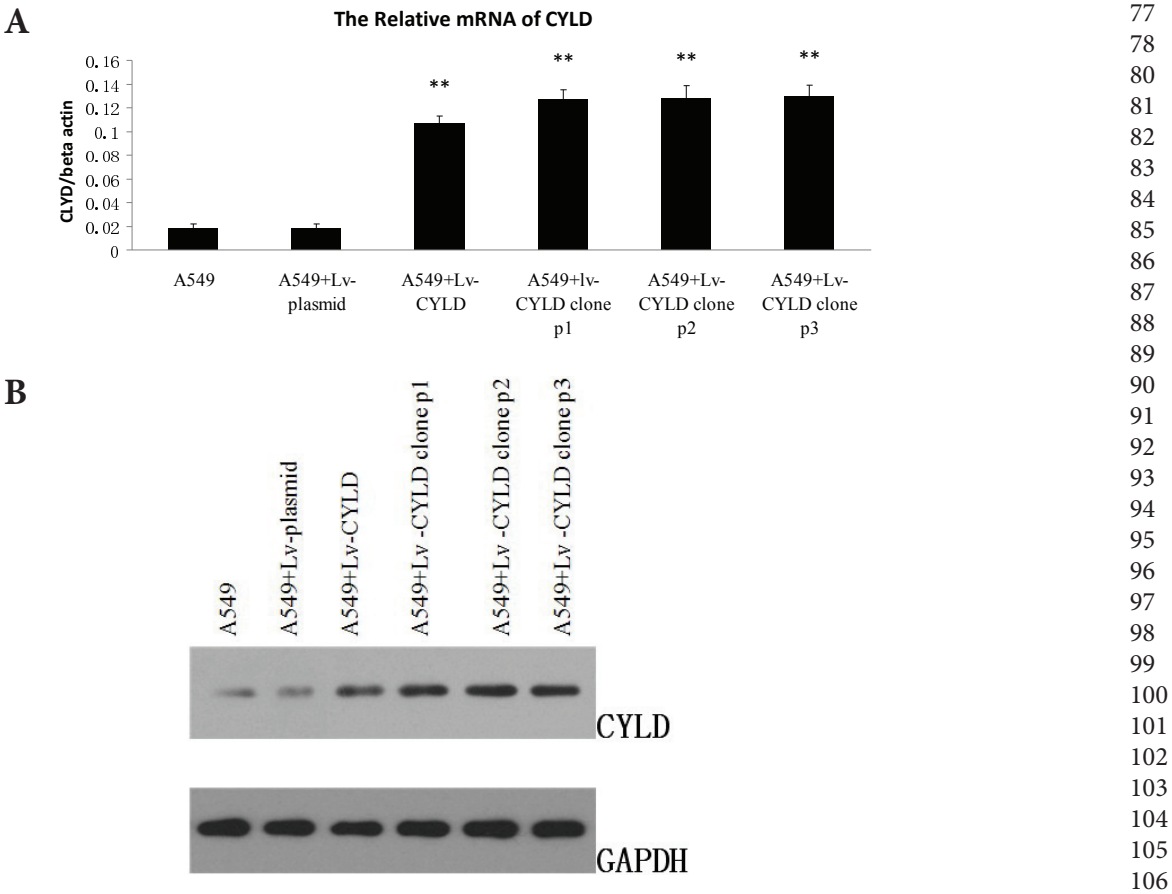


Figure 2. Lentivirus-mediated establishment of an A549-Lv-CYLD cell line that stably expressed CYLD.A549 cells were transduced with letiviral vectors expressed CYLD, transduced empty vectors as a control (A549-Lv-plasmid). A, Real-Time Quantitative PCR analysis was performed, using primers specific to CYLD and β-actin as a control, in RNA samples respectively prepared from A549, A549+Lv-plasmid (cells were infected by Lv-plasmid 72 hours later), A549+Lv-CYLD (cell were infected by Lv-CYLD 72 hours later) and A549+Lv-CYLD clone p1,p2,p3 cells. ** p<0.01. B, The whole cell lysates were analyzed by western blotting with antibody against CYLD. GAPDH was used as an internal control. The level of CYLD expression was quantified and normalized to GAPDH expression, in protein samples respectively prepared from cells as described for A.

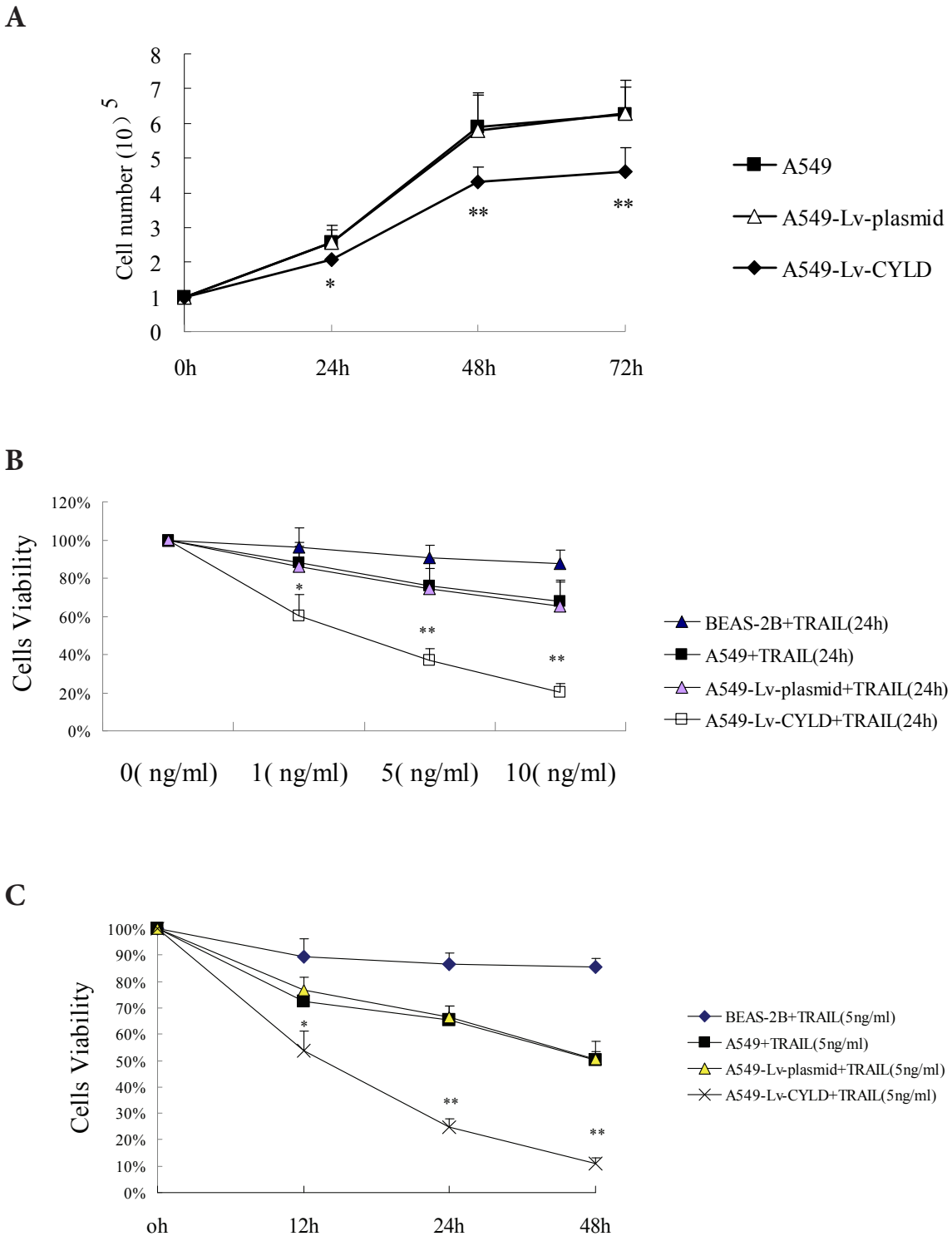


Figure 3. Cytotoxicity induced by CYLD alone and combined with TRAIL. A, Trypan blue exclusion assay was performed to evaluate the effects of CYLD alone on the growth of A549, A549-Lv-plasmid, A549-Lv-CYLD cells. Each point represents the mean ± SD of triplicate determinations. Error bars correspond to standard deviation.*p < 0.05, ** p < 0.01.B, Cells were treated with TRAIL at indicated concentrations (0, 1, 5, 10 ng/ml) for 24 hours. Cell viability was measured by MTT assays. Individual data points are the mean ± SD of triplicate determinations. Error bars correspond to standard deviation. *p < 0.05, ** p < 0.01 versus TRAIL treated cells at the same dose point. C, Cells treated with TRAIL (5ng/ml) for indicated periods (12, 24, 48 hours) were subjected to measurement of viability using MTT assays. Data were presented as mean ± SD (n = 3). Error bars correspond to standard deviation.*p < 0.05, ** p < 0.01 versus TRAIL treated cells at the same time point.

Then we sought to test whether CYLD has growth inhibitory effect, alone or in combination with TRAIL. A549 cells and its variants stably transfected with CYLD or empty vectors were treated with TRAIL at a series of doses, and the percentage of cell survival was determined using the MTT assay 24 hours post-treatment. As shown in Figure 3B, ectopic CYLD expression increased TRAIL-induced lung cancer cell growth inhibition in a dose-dependent manner. At twenty four hours, colorimetric readings of the combined treatment group were statistically significantly lower than those of the control groups ($p < 0.05$) (Figure 3B). Similar results were observed while cells were treated with TRAIL (5ng/ml) for the indicated periods. As presented in Figure 3C, increased expression of CYLD resulted in a time-dependent reduction in TRAIL-regulated cell viability ($p < 0.05$). However, TRAIL caused no remarkable cell viability reduction in the normal cell line BEAS-2B, regardless of dosage or period of treatment.

Notably, in A549 cells, increased expression of CYLD alone achieved significant inhibitory effects on cell viability compared with control group ($p < 0.05$) (Figure 3A). These data demonstrated that increased expression of CYLD may potentiate TRAIL-mediated cytotoxicity in A549 cells.

Over-expression of CYLD enhances TRAIL-induced apoptosis. It is known that TRAIL selectively induces apoptosis in tumor cells [31]. To determine whether apoptosis contributes to TRAIL-induced cell death that we observed, we directly detected apoptosis by measuring annexin V-positive cells and caspase cleavage in cells exposed to TRAIL alone, increased expression of CYLD alone and in combination. A549, A549-Lv-plasmid, A549-Lv-CYLD stable cells and BEAS-2B cells were treated or not with TRAIL (5ng/ml) for 24 hours. In agreement with cell survival data, the combination of the stably over-expressed CYLD and TRAIL treatment was much more potent than each single agent alone to induce cleavage of caspase-8, caspase-3 and poly(ADP-Ribose) polymerase (PARP) (Fig. 4C) and increase Annexin V-positive cells (i.e., apoptotic cells) (Figure 4A).Notably, increased expression of CYLD alone and TRAIL alone induced approximately 21% and 17% of cells to undergo apoptosis, respectively, however, the combination of CYLD and TRAIL caused about 62% apoptosis ($p < 0.01$) (Figure 4A and B), which is a more than additive effect. In contrast, as shown in Figure 4 A and B, BEAS-2B cells did not undergo apoptosis in response to TRAIL. Collectively, these results indicate that increased expression of CYLD sensitizes lung cancer cells to TRAIL-induced apoptosis.

In addition, we found that ectopic CYLD induced approximately 21% of cells to undergo apoptosis (Figure 4 A and B) comparing to wild type A549 and A549-Lv-plasmid cells. This indicates that CYLD directly induces apoptosis in lung cancer cells.

Over-expression of CYLD inhibits TRAIL-induced NF-κB activation. It is well known that CYLD negatively regulates the activity of NF-κB [23, 26]. Since inhibiting NF-κB activation may contribute to TRAIL-induced apoptosis [1, 23, 32], we evaluated the involvement of NF-κB in CYLD mediated sensitization to TRAIL. A549 cells and its variants stably transfected with CYLD or empty vectors were transfected with a NF-κB-responsive luciferase reporter construct and then treated with 5ng/ml of TRAIL. As shown in Figure 5, NF-κB activity almost tripled after stimulation with TRAIL. Ectopic CYLD expression significantly attenuated TRAIL-induced NF-κB activation ($p < 0.01$) (Figure 5). However, treatment of TRAIL did not induce NF-κB activity in normal BEAS-2B cells (Figure 5).These results demonstrated that CYLD negatively regulated NF-κB activation induced by TRAIL.

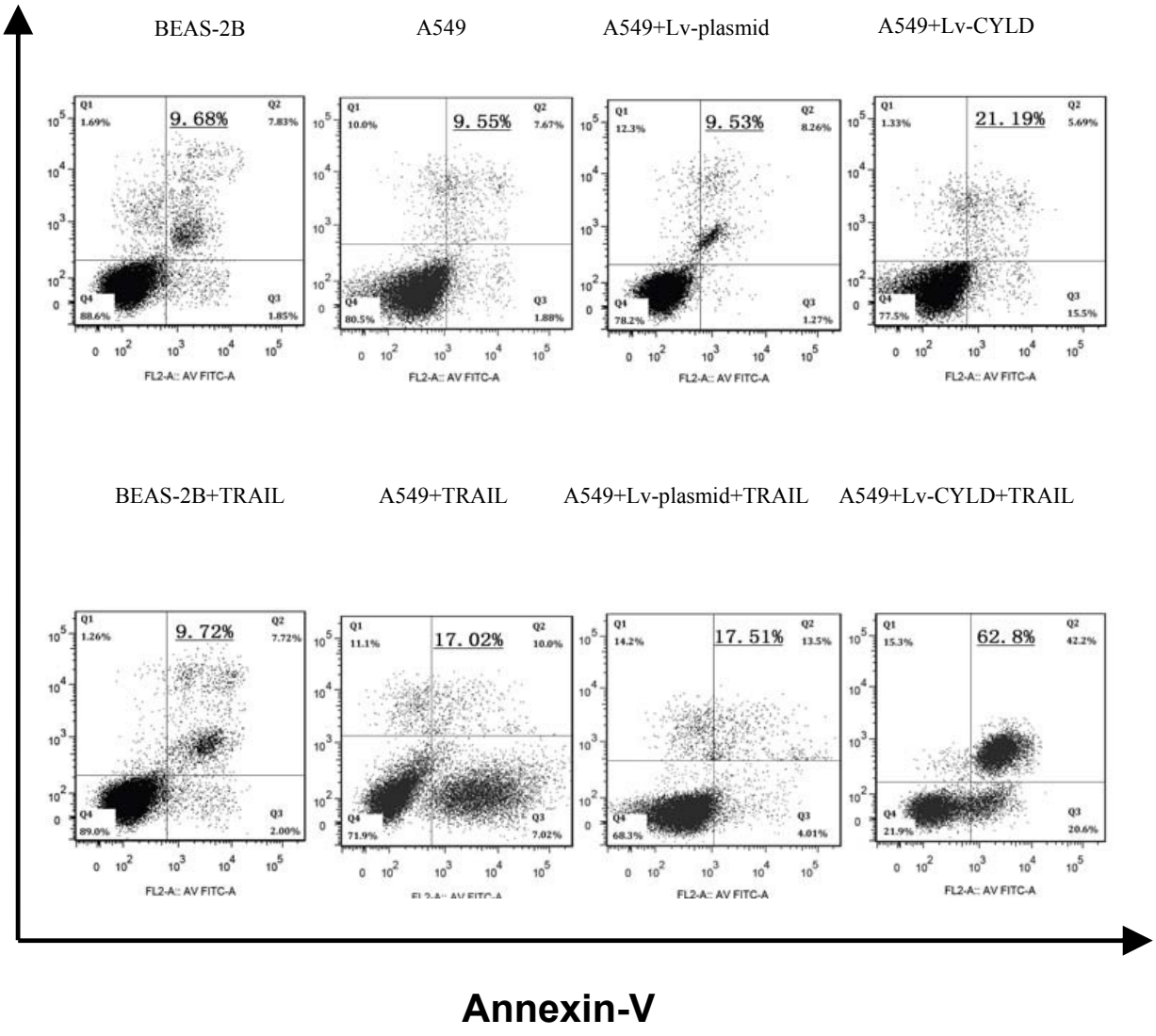
Over-expression of CYLD reverses IAPs expression induced by TRAIL. It is well established that NF-κB functions as an upstream regulator of IAPs and negatively regulates TRAIL signaling [1, 23, 32]. To investigate a potential link between CYLD, IAPs and resistance to TRAIL-induced apoptosis, we adopted immunoblotting to examine the expression of survivin and XIAP, two important members of IAPs in response to TRAIL (5ng/ml). In A549 cells, TRAIL treatment resulted in increased expression of survivin and XIAP (Figure 6). This was antagonized by ectopic CYLD expression ($p < 0.01$) (Figure 6B). These results suggest that CYLD may serve as a negative regulator of NF-κB and block expression of NF-κB-dependent anti-apoptotic genes.

Discussion

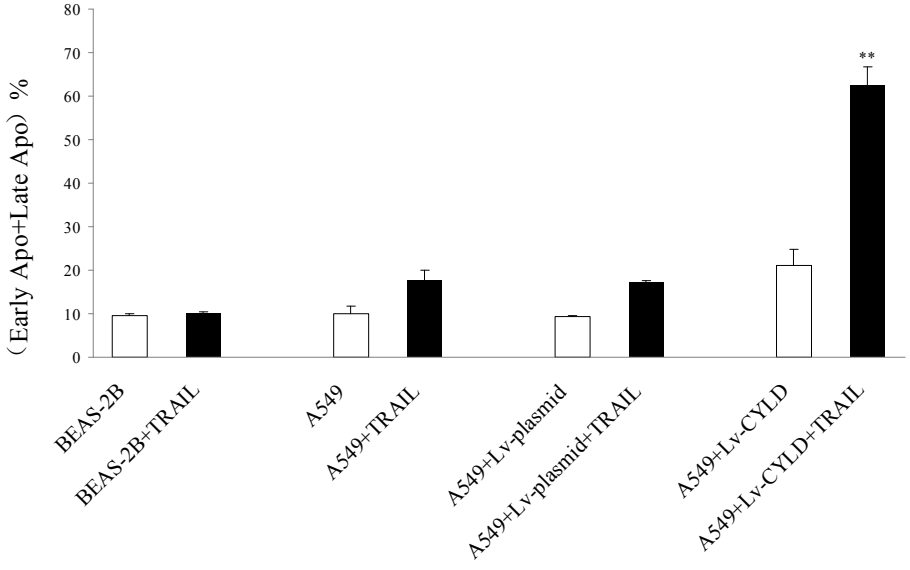
The tumor suppressor CYLD is a member of de-ubiquitinating enzyme family that negatively regulates cytokine-mediated activation of nuclear factor-κB, a transcription factor highly implicated in carcinogenesis [22]. CYLD was previously shown to directly bind to IKK, reverse the ubiquitination of TRAF2 and consequently suppress NF-κB activation induced by TRAIL in hepatocellular carcinoma cells (HCC) [23]. However, whether CYLD exerted similar effects in human lung cancer cells has not been reported. Interestingly, in lung cancer cell lines, no strict correlation has been observed between CYLD expression and TRAIL responsiveness. Here we demonstrated that suppression of NF-κB survival signaling by CYLD augmented the antitumor effect of TRAIL in A549 lung cancer cells.

Resistance to chemo- or radiotherapy, which is often associated with lung cancer recurrence after prior therapy, remains a severe clinical problem. TRAIL is currently being evaluated in Phase I/II clinical trials [10, 11], alone or in combination with other therapies, for the treatment of lung cancer. TRAIL can induce apoptosis in tumor cells. However, a few studies have demonstrated that many types of cancers are resistant to apoptosis induced by TRAIL [13, 14]. Some researchers found chemotherapy or radiotherapy in combination with TRAIL could restore sensitivity of tumor cells towards TRAIL [34, 35]. Previous studies demonstrated that during the process of apoptosis, TRAIL could bind to DR4, DR5 to activate NF-kappa B-Inducing Kinase (NIK) through TNF-receptor-associated death domain(TRADD), TRAF2, and receptor-interaction

A


BEAS-2B
A549
A549+Lv-plasmid
A549+Lv-CYLD
9. 68%
9. 55%
9. 53%
21. 19%
BEAS-2B+TRAIL
A549+TRAIL
A549+Lv-plasmid+TRAIL
A549+Lv-CYLD+TRAIL
9. 72%
17. 02%
17. 51%
62. 8%
PI
Annexin-V
FL2-A:: AV FITC-A

B


（Early Apo+Late Apo）%
80
70
60
50
40
30
20
10
0
**
BEAS-2B
BEAS-2B+TRAIL
A549
A549+TRAIL
A549+Lv-plasmid
A549+Lv-plasmid+TRAIL
A549+Lv-CYLD
A549+Lv-CYLD+TRAIL

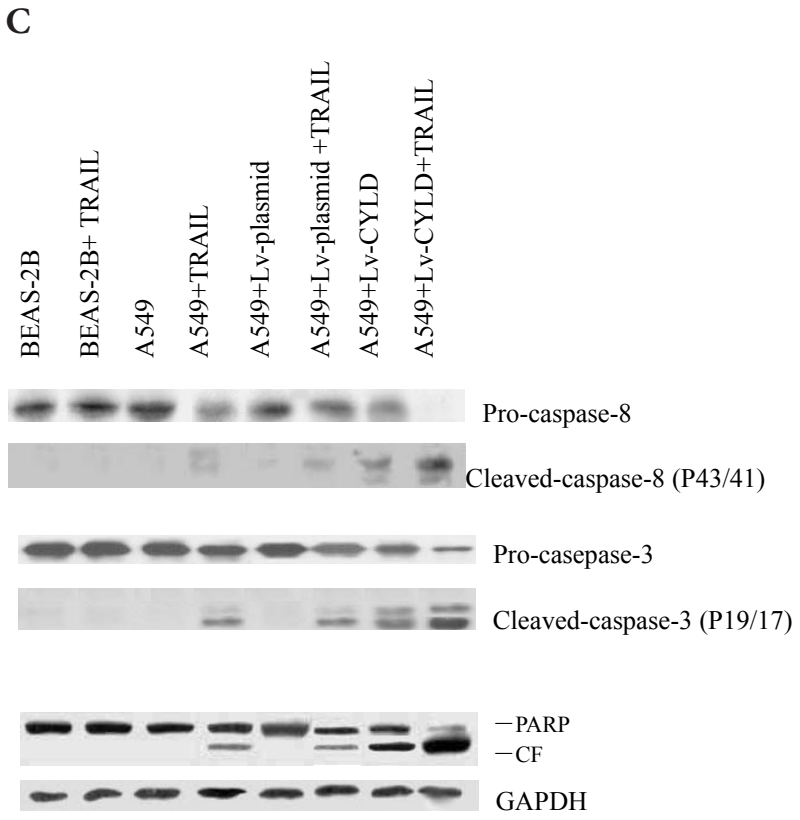


Figure 4. CYLD enhanced TRAIL-induced apoptosis as evaluated by Annexin V staining (A) and caspase activation (C). A, The indicated cell lines (BEAS-2B, A549, A549-Lv-plasmid, A549-Lv-CYLD cell lines) were prepared and treated with or without TRAIL (5ng/ml). 24hours after incubation, cells were harvested and processed for Annexin V-FITC and PI staining by flow cytometry. Numbers represented total apoptosis (Annexin V positive cell population). Data represented one of three independent experiments. B, A summary of studies represented the effects of TRAIL on TRAIL-induced apoptosis. Cells were treated with TRAIL (5ng/ml) for 24 hours or untreated before the percentage of apoptotic cells in each cell population was determined by Annexin V staining and flow cytometry. Each bar represents the mean ± S.D. of triplicate experiments. **p < 0.01 versus TRAIL alone treated cells. C, The indicated cell lines were treated or not with TRAIL (5ng/ml). After 24 hours, the cells were subjected to preparation of whole-cell protein lysates for detecting caspase cleavage using Western blotting. Membranes were probed with antibodies against caspase-8, caspase-3, caspase-9 and PARP. GAPDH was shown as a loading control. P43/41, P19/17 and P89 were cleavage products of caspase-8, caspase-3 and PARP, respectively. CF indicates cleaved fragment.

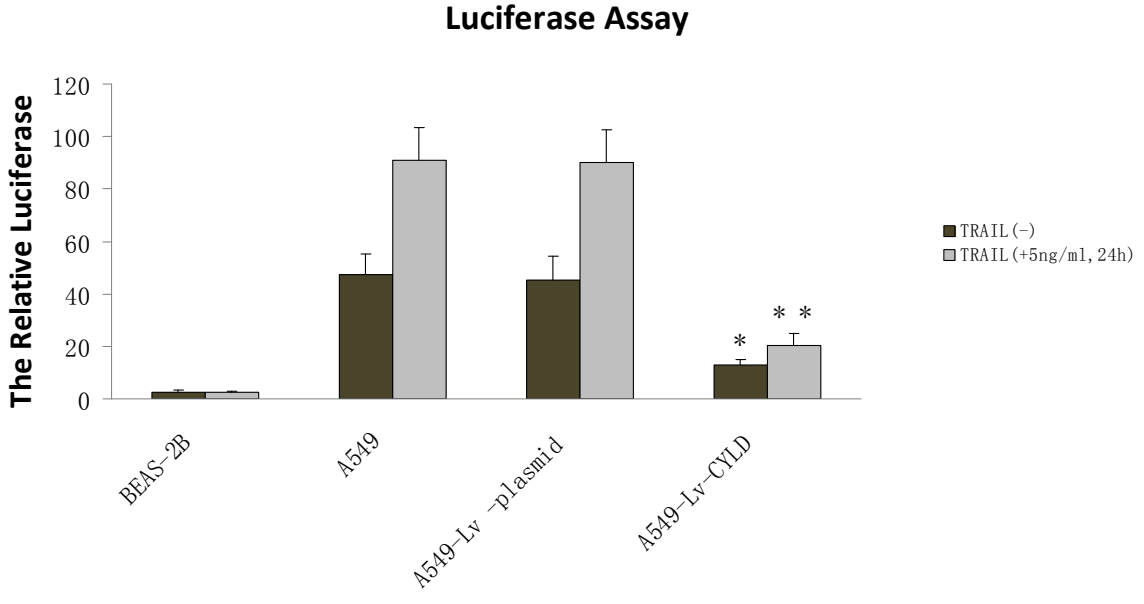


Figure 5. CYLD negatively regulates NF-κB activation induced by TRAIL. Transfection of BEAS-2B, A549, A549-Lv-plasmid, A549-Lv-CYLD cells with a NF-κB-dependent luciferase plasmid and a pRL–TK expression plasmid encoding Renilla luciferase was performed. Twenty hours after transfection, TRAIL was added in accordance with final concentration (5ng/ml). After 24 hours, cells were lysed for NF-κB luciferase assay. Firefly and Renilla luciferase activities were measured. Firefly luciferase activity was normalized to Renilla luciferase activity. Results are the mean ± SD relative luciferase activity from three separate experiments. Error bars correspond to standard deviation. *p < 0.05 versus A549 or A549-Lv-plasmid cells treated without TRAIL, ** p < 0.01 versus TRAIL alone treated cells.

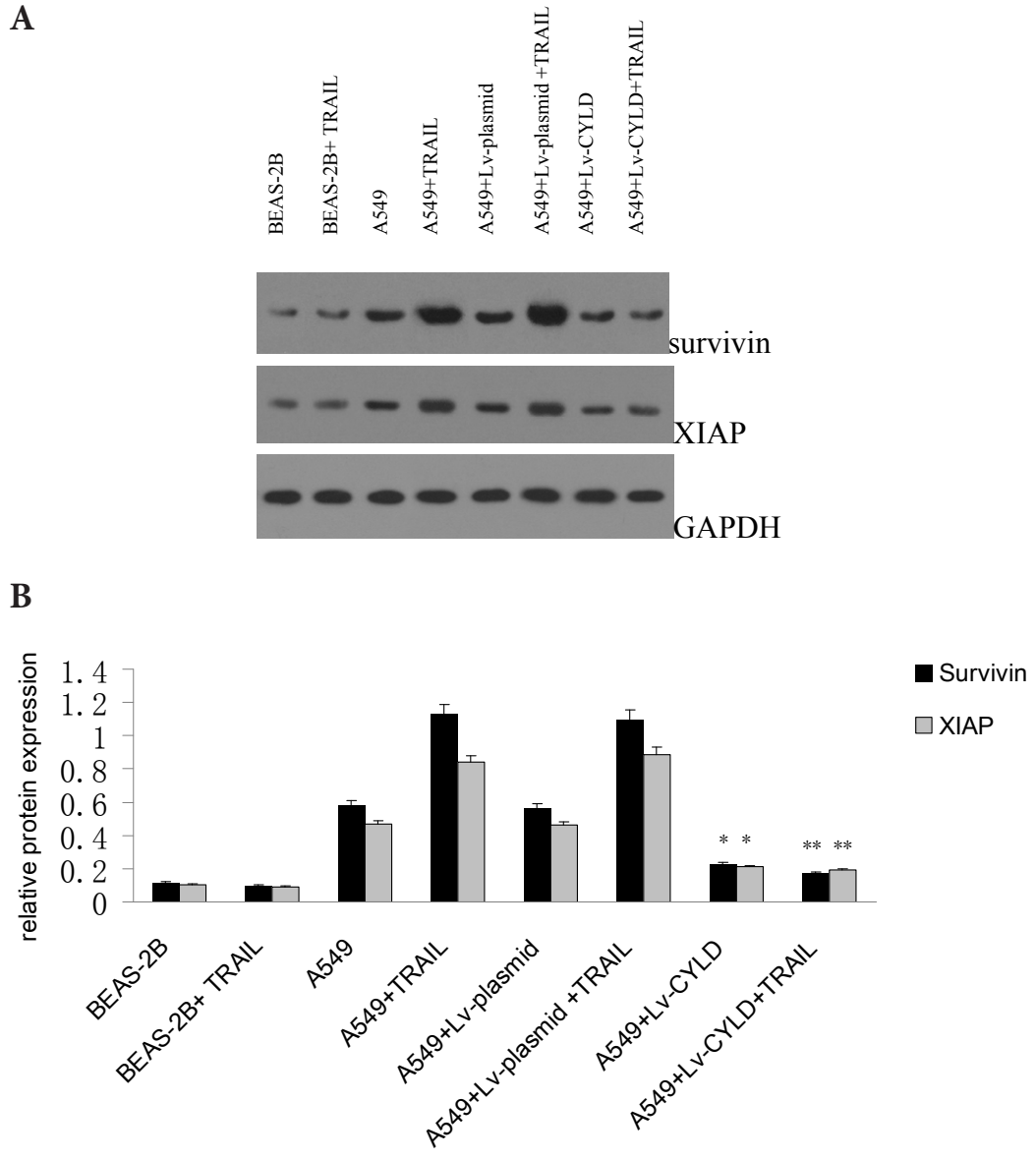


Figure 6. CYLD decreases enhanced IAPs expression induced by TRAIL. A, The indicated cell lines (BEAS-2B, A549, A549-Lv-plasmid, A549-Lv-CYLD cells) were treated with 5ng/ml of TRAIL for 24 hours, untreated groups as control. The whole cell lysates were analyzed by western blotting with antibodies against survivin and XIAP. GAPDH was used as an internal control. Levels of survivin and XIAP expression were quantified and normalized to GAPDH expression respectively. Each band represents one of three independent experiments. B, A summary of studies represented the effects of increased expression of CYLD on expression of survivin and XIAP in a panel of lung cancer cell lines and a normal bronchial epithelial cell line. Cells were treated with TRAIL (5 ng/ml) for 24 hours or untreated before western blotting was performed using antibodies against survivin and XIAP. Relative expression of survivin and XIAP was shown by dividing band intensity by that of GAPDH. The data shown are the mean ± SD of three individual experiments. Error bars correspond to standard deviation. *p < 0.05 versus A549 or A549-Lv-plasmid cells treated without TRAIL, ** p < 0.01 versus TRAIL alone treated cells.

proteins (RIP)[20,37], and thereafter activated NF-κB. Therefore, inhibiting NF-κB activation may enhance the antitumor effect of TRAIL.

Downregulation or loss of CYLD has been implicated in solid tumors of the colon and liver [26], kidney [29], cervix [28] and prostate [40], and vector-driven expression of CYLD in HCC cells decreased NF-kB activity, induced rapid apoptosis [23]. We aim to extend the tumor suppressive role of CYLD to other organs. Particularly, we interested in knowing whether CYLD expression is altered in human lung cancer cells and whether CYLD can affect NF-κB activity in these cells. We found reduced CYLD mRNA and protein levels in all five lung cancer cell lines examined compared to those in a normal bronchial epithelial cell line (Figure1). To understand the

mechanism by which CYLD sensitizes lung cancer cell lines to TRAIL-induced apoptosis, we investigated whether CYLD regulates NF-κB activity. We showed a drastically increased NF-κB activation in response to TRAIL treatment in A549 cells, which associated to attenuated response of these cells to TRAIL-induced apoptosis. However, when CYLD was ectopically over-expressed in A549 cells, NF-κB activation was markedly blocked (Figure 5). Our data provided the first evidence that increased expression of CYLD directly blocks TRAIL-induced NF-κB activation, and consequently increases TRAIL-induced apoptosis in lung cancer cells.

At the tested concentrations and time periods of TRAIL, we verified that over-expression of CYLD may potentiate TRAIL-mediated cytotoxicity in A549 cells (Figure 3). We also found a minor effect on apoptosis induced by TRAIL alone in A549 cells, which is remarkably increased upon ectopic CYLD expression (Figure 4). When CYLD was over-expressed, TRAIL-induced apoptosis almost tripled. TRAIL treatment and ectopic CYLD exerted a more-than-addative pro-apoptotic activity, indicating that TRAIL cooperates with CYLD to augment apoptosis. Interestingly, in this study, over-expression of CYLD alone significantly suppressed the growth of A549 cells in culture. Indeed, CYLD as a single agent moderately induces apoptosis of A549 cells. Our study suggested that CYLD, acting as a tumor suppressor gene, not only initiated apoptosis, but also plays an important role to increase TRAIL mediated apoptosis. Our data are in partial disagreement with a recent study, which showed in hepatocellular carcinoma cells that CYLD failed to initiate apoptosis in the absence of apoptotic stimuli although it can negatively regulate NF-kB activation [23]. Thus we suggest that CYLD may act differently in tumor cell lines from different organs.

NF-κB is a well documented pro-survival factor that is involved in mediating resistance to TRAIL-induced apoptosis in tumor cells [39]. NF-κB regulates the transcription of XIAP and survivin, which function as inhibitors of various different kinds of caspases [18-20], such as caspase-3, 7, 9.Constitutively active NF-κB signaling leads to resistance to TRAIL by up-regulating XIAP and survivin in multiple human cancer cell lines [40]. Through inhibition of caspases activity, these two factors can prevent apoptosis in response to cytotoxic stresses [18, 19], including that induced by TRAIL [38]. In our study, we provided evidences that CYLD enhances TRAIL-induced apoptosis while down-regulating XIAP and survivin, suggesting that XIAP and survivin may be critical factors in modulating TRAIL induced apoptosis in human lung cancer cells. In agreement with our data on NF-κB activity, there were high levels of XIAP and survivin expression in A549 cells under quiescent conditions, which helped to explain why A549 cells were not sensitive to apoptosis induced by TRAIL. Treating A549 cells with TRAIL resulted in markedly increased expression of survivin and XIAP. However, increased expression of CYLD antagonized the up-regulation of survivin and XIAP by TRAIL (Figure 6). This is consistent with changes in NF-κB activation and apoptosis in response to TRAIL treatment combined with ectopic CYLD expression.

In conclusion, the finding that CYLD sensitizes lung cancer cells to TRAIL-induced apoptosis by antagnoizing NF-κB activity and down-regulating IAP expression is of particular importance to explain why certain lung cancer cells are relatively resistant to TRAIL induced apoptosis. Increasing CYLD expression in chemo- or radio-resistant lung cancer may yield a potential therapeutic benefit of TRAIL-based therapy. Our future work aims to specifically express CYLD in other solid tumors cell lines, especially in those with high levels of endogenous NF-κB, to detect the mechanisms by which CYLD regulates tumorigenesis.

Acknowledgements: We thank Yang Yi for assistance with gene sequencing.

References

[1] DAI Y, LIU M, TANG W, LI Y, LIAN J et al. A Smac-mimetic sensitizes prostate cancer cells to TRAIL-induced apoptosis via modulating both IAPs and NF-kappaB. BMC Cancer 2009; 9: 392-399. http://dx.doi.org/10.1186/1471-2407-9-392

[2] HOLOCH PA, GRIFFITH TS.TNF-related apoptosis-inducing ligand (TRAIL): a new path to anti-cancer therapies.Eur J Pharmacol 2009; 625: 63-72. http://dx.doi.org/10.1016/j.ejphar.2009.06.066

[3] KIM YH, LEE DH, JEONG JH, GUO ZS, LEE YJ. Quercetin augments TRAIL-induced apoptotic death: Involvement of the ERK signal transduction pathway. Biochem Pharmacol 2008; 75: 1946-58. http://dx.doi.org/10.1016/j.bcp.2008.02.016

[4] JUNG YH, HEO J, LEE YJ, KWON TK, KIM YH. Quercetin enhances TRAIL-induced apoptosis in prostate cancer cells via increased protein stability of death receptor 5. Life Sci 2010; 86: 351-7. http://dx.doi.org/10.1016/j.lfs.2010.01.008

[5] PENNARUN B, KLEIBEUKER JH, OENEMA T, STEGEHUIS JH, DE VRIES EG et al. Inhibition of IGF-1R-dependent PI3K activation sensitizes colon cancer cells specifically to DR5-mediated apoptosis but not to rhTRAIL.Anal Cell Pathol (Amst) 2010; 33: 229-44

[6] CORDIER SM, PAPENFUSS K, WALCZAK H. From biochemical principles of apoptosis induction by TRAIL to application in tumour therapy. Results Probl Cell Differ 2009; 49: 115-43. http://dx.doi.org/10.1007/400_2008_27

[7] BERGER A, QUAST SA, PLÖTZ M, HEIN M, KUNZ M et al. Sensitization of melanoma cells for death ligand-induced apoptosis by an indirubin derivative - enhancement of both extrinsic and intrinsic apoptosis pathways. Biochem Pharmacol 2011; 81:71-81. http://dx.doi.org/10.1016/j.bcp.2010.09.010

[8] PENNARUN B, MEIJER A, DE VRIES EG, KLEIBEUKER JH, KRUYT F et al. Playing the DISC: turning on TRAIL death receptor-mediated apoptosis in cancer. Biochim Biophys Acta 2010; 1805: 123-40.

[9] CAO X, POBEZINSKAYA YL, MORGAN MJ, LIU ZG. The role of TRADD in TRAIL-induced apoptosis and signaling. FASEB J 2011; 25: 1353-8. http://dx.doi.org/10.1096/fj.10-170480

[10] HERBST RS, ECKHARDT SG, KURZROCK R, EBBINGHAUS S, O'DWAYER PJ et al. Phase I dose-escalation study of recombinant human Apo2L/TRAIL, a dual proapoptotic receptor agonist, in patients with advanced cancer. J Clin Oncol 2010; 28: 2839-46. http://dx.doi.org/10.1200/JCO.2009.25.1991

[11] SORIA JC, SMIT E, KHAYAT D, BESSE B, YANG X et al. Phase 1b study of dulanermin (recombinant human Apo2L/TRAIL) in combination with paclitaxel, carboplatin, and bevacizumab in patients with advanced non-squamous non-small-cell lung cancer. J Clin Oncol 2010; 28: 1527-33. http://dx.doi.org/10.1200/JCO.2009.25.4847

[12] ABDULGHANI J, El-DEIRY WS. TRAIL receptor signaling and therapeutics. Expert Opin Ther Targets 2010; 14: 1091-108. http://dx.doi.org/10.1517/14728222.2010.519701

[13] LI Y, WANG H, WANG Z, MAKHIJA S, BUCHSBAUM D et al. Inducible resistance of tumor cells to tumor necrosis factor-related apoptosis-inducing ligand receptor 2-mediated apoptosis by generation of a blockade at the death domain function. Cancer Res 2006; 66: 8520-8. http://dx.doi.org/10.1158/0008-5472.CAN-05-4364

[14] JACQUEMIN G, SHIRLEY S, MICHEAU O. Combining naturally occurring polyphenols with TNF-related apoptosis-inducing ligand: a promising approach to kill resistant cancer cells? Cell Mol Life Sci 2010; 67: 3115-30. http://dx.doi.org/10.1007/s00018-010-0407-6

[15] SAHU RP, BATRA S, KANDALA PK, BROWN TL, SRIVASTAVA SK. The role of K-Ras gene mutation in TRAIL-induced apoptosis in pancreatic and lung cancer cell lines. Cancer Chemother Pharmacol 2011; 67: 481-7. http://dx.doi.org/10.1007/s00280-010-1463-1

[16] FRESE-SCHAPER M, SCHARDT JA, SAKAI T, CARBONI GL, SCHMID RA, et al. Inhibition of tissue transglutaminase sensitizes TRAIL-resistant lung cancer cells through up-regulation of death receptor 5. FEBS Lett 2010; 584: 2867-71. http://dx.doi.org/10.1016/j.febslet.2010.04.072

[17] ZHANG HY, DU ZX, LIU BQ, GAO YY, MENG X et al. Tunicamycin enhances TRAIL-induced apoptosis by inhibition of cyclin D1 and the subsequent downregulation of survivin. Exp Mol Med 2009; 41: 362-9. http://dx.doi.org/10.3858/emm.2009.41.5.041

[18] GILL C, DOWLING C, O'NEILL AJ, WASTON RW. Effects of cIAP-1, cIAP-2 and XIAP triple knockdown on prostate cancer cell susceptibility to apoptosis, cell survival and proliferation. Mol Cancer 2009; 8: 39. http://dx.doi.org/10.1186/1476-4598-8-39

[19] GYRD-HANSEN M, DARDING M, MIASARI M, SANTORO MM, ZENDER L et al. IAPs contain an evolutionarily conserved ubiquitin-binding domain that regulates NF-kappaB as well as cell survival and oncogenesis. Nat Cell Biol 2008; 10: 1309-17. http://dx.doi.org/10.1038/ncb1789

[20] LI W, LI H, BOCKING AD, CHALLIS JR. Tumor necrosis factor stimulates matrix metalloproteinase 9 secretion from cultured human chorionic trophoblast cells through TNF receptor 1 signaling to IKBKB-NFKB and MAPK1/3 pathway. Biol Reprod 2010; 83: 481-7. http://dx.doi.org/10.1095/biolreprod.109.082578

[21] CECON E, FERNANDES PA, PINATO L, FERREIRA ZS, MARKUS RP. Daily variation of constitutively activated nuclear factor kappa B (NFKB) in rat pineal gland. Chronobiol Int 2010; 27: 52-67. http://dx.doi.org/10.3109/07420521003661615

[22] JONO H, LIM JH, CHEN LF, XU H, TROMPOUKI E et al. NF-kappaB is essential for induction of CYLD, the negative regulator of NF-kappaB: evidence for a novel inducible autoregulatory feedback pathway. J Biol Chem 2004; 279: 36171-4. http://dx.doi.org/10.1074/jbc.M406638200

[23] CHU L, GU J, HE Z, XIAO T, LIU X. Adenoviral vector expressing CYLD augments antitumor activity of TRAIL by suppression of NF-kappa survival signaling in hepatocellular carcinoma. Cancer Biol Ther 2006; 5: 615-22. http://dx.doi.org/10.4161/cbt.5.6.2662

[24] SARKAR FH, LI Y. NF-kappaB: a potential target for cancer chemoprevention and therapy. Front Biosci 2008; 13: 2950-9. http://dx.doi.org/10.2741/2900

[25] SUN L, GAO J, HUO L, SUN X, SHI X et al. Tumour suppressor CYLD is a negative regulator of the mitotic kinase Aurora-B. J Pathol 2010; 221: 425-32.

[26] HELLERBRAND C, BUMES E, BATAILLE F, DIETMAIER W, MASSOUMI R et al. Reduced expression of CYLD in human colon and hepatocellular carcinomas. Carcinogenesis 2007; 28: 21-7. http://dx.doi.org/10.1093/carcin/bgl081

[27] ZHONG S, FIELDS CR, SU N, PAN YX, ROBERTSON KD. Pharmacologic inhibition of epigenetic modifications, coupled with gene expression profiling, reveals novel targets of aberrant DNA methylation and histone deacetylation in lung cancer.Oncogene 2007; 26: 2621-34. http://dx.doi.org/10.1038/sj.onc.1210041

[28] HIRAI Y, KAWAMATA Y, TAKESHIMA N, FURUTA R, KITAGAWA T et al. Conventional and array-based comparative genomic hybridization analyses of novel cell lines harboring HPV18 from glassy cell carcinoma of the uterine cervix. Int J Oncol 2004; 24 :977-86.

[29] STROBEL P, ZETTLE A, REN Z, STAROSTIK P, RIEDMILLER H, STORKEL S, MULLER-HERMELINK HK, MARX A. Spiradenocylindroma of the kidney: clinical and genetic findings suggesting a role of somatic mutation of the CYLD1 gene in the oncogenesis of an unusual renal neoplasm. Am J Surg Pathol 2002; 26: 119-124. http://dx.doi.org/10.1097/00000478-200201000-00016

[30] BRUMMELKAMP TR, NIJMAN SM, DIRAC AM, BERNARDS R. Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kappaB. Nature 2003; 424: 797-801. http://dx.doi.org/10.1038/nature01811

[31] KELLEY SK, ASHKENAZI A. Targeting death receptors in cancer with Apo2L/TRAIL. Curr Opin Pharmacol 2004; 4: 333-339. http://dx.doi.org/10.1016/j.coph.2004.02.006

[32] FALSCHLEHNER C, EMMERICH CH, GERLACH B, WALCZAK H. TRAIL signalling: decisions between life and death. Int J Biochem Cell Biol 2007; 39: 1462-1475. http://dx.doi.org/10.1016/j.biocel.2007.02.007

[33] WRIGHT A, REILEY WW, CHANG M, JIN W, LEE AJ et al. Regulation of early wave of germ cell apoptosis and spermatogenesis by deubiquitinating enzyme CYLD. Dev Cell 2007; 13: 705-16. http://dx.doi.org/10.1016/j.devcel.2007.09.007

[34] WU GS. TRAIL as a target in anti-cancer therapy. Cancer Lett 2009; 285: 1-5. http://dx.doi.org/10.1016/j.canlet.2009.02.029

[35] FINNBERG N, KIM SH, FURTH EE, LIU JJ, RUSSO P et al. Non-invasive fluorescence imaging of cell death in fresh human colon epithelia treated with 5-Fluorouracil, CPT-11 and/or TRAIL. Cancer Biol Ther. 2005; 4: 937-42. http://dx.doi.org/10.4161/cbt.4.9.2182

[36] LIRDPRAPAMONGKOL K, SAKURAI H, SUZUKI S, KOIZUMI K, PRANGSAENGTONG O et al. Vanillin enhances TRAIL-induced apoptosis in cancer cells through inhibition of NF-kappaB activation. In Vivo 2010; 24: 501-6.

[37] FINGAS CD, BLECHACZ BR, SMOOT RL, GUICCIARDI ME, MOTT J et al. A smac mimetic reduces TNF related apoptosis inducing ligand (TRAIL)-induced invasion and metastasis of cholangiocarcinoma cells. Hepatology 2010; 52: 550-61. http://dx.doi.org/10.1002/hep.23729

[38] MORALES JC, RUIZ-MAGANA MJ, RUIZ-RUIZ C. Regulation of the resistance to TRAIL-induced apoptosis in human primary T lymphocytes: role of NF-kappaB inhibition. Mol Immunol 2007; 44: 2587-2597. http://dx.doi.org/10.1016/j.molimm.2006.12.015

[39] BRAEUER SJ, BUNEKER C, MOHR A, ZWACKA RM. Constitutively activated nuclear factor-kappaB, but not induced NF-kappaB, leads to TRAIL resistance by up-regulation of X-linked inhibitor of apoptosis protein in human cancer cells. Mol Cancer Res 2006; 4: 715-728. http://dx.doi.org/10.1158/1541-7786.MCR-05-0231

[40] KIKUNO N, SHIINA H, URAKAMI S, KAWAMOTO K, HERATA H, TANAKA Y, MAJID S, IGAWA M, DAHIYA R. Genistein mediated histone acetylation and demethylation activates tumor suppressor genes in prostate cancer cells. Int J Cancer 2008; 123: 552-560. http://dx.doi.org/10.1002/ijc.23590