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

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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-Spieled 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 Academy 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 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).

The pcDNA-HA-CYLD plasmid was kindly 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’-CGGGATCC-GCGCACACATGAGTTCAGGCTTATGGAGC-3’ (sense) and 5’-ATAAAGATCGGCCGCTATTGTGACCCACACCAT-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 Lipofectamine™ 2000 reagent (Invitrogen, 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’-GGGATCCGCTCAGCTGATCC-3’ and antisense, 5’-CCGGATCCCATGAGTTCAGGCTTATGGAGC-3’; β-actin (as an internal control), sense, 5’-CCCTGCACTGCTGATCCGACATG-3’ and antisense, 5’-ATCCTTCTGCTGCTGAGTCTC-3’. All reactions were performed on the TaKaRa PCR Thermal Cycler Dice. To calculate values of mRNA levels, 2-∆ΔCT 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. Table 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’-GGGAATTTCCGGGAATTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACTTTCCGGGAGATTTCCGGGACTTTCCGGGAGATTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGGACGTTCCGGGACTTTCCGGG
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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 ß-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 ß-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-kB activation.** It is well known that CYLD negatively regulates the activity of NF-kB [23, 26]. Since inhibiting NF-kB activation may contribute to TRAIL-induced apoptosis [1, 23, 32], we evaluated the involvement of NF-kB in CYLD mediated sensitization to TRAIL. A549 cells and its variants stably transfected with CYLD or empty vectors were transfected with a NF-kB-responsive luciferase reporter construct and then treated with 5ng/ml of TRAIL. As shown in Figure 5, NF-kB activity almost tripled after stimulation with TRAIL. Ectopic CYLD expression significantly attenuated TRAIL-induced NF-kB activation (p < 0.01) (Figure 5). However, treatment of TRAIL did not induce NF-kB activity in normal BEAS-2B cells (Figure 5). These results demonstrated that CYLD negatively regulated NF-kB activation induced by TRAIL.

**Over-expression of CYLD reverses IAPs expression induced by TRAIL.** It is well established that NF-kB 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-kB and block expression of NF-kB-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-kB, 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-kB 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-kB 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 that 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
Annexin-V

BEAS-2B  A549  A549+Lv-plasmid  A549+Lv-CYLD

BEAS-2B+TRAIL  A549+TRAIL  A549+Lv-plasmid+TRAIL  A549+Lv-CYLD+TRAIL

B

Early Apo+Late Apo %
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). 24 hours 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.


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-κB 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 (Figure 1). To understand the effect of TRAIL.

Inhibiting NF-κB activation may enhance the antitumor effect of TRAIL. [28] and prostate [40], and vector-driven expression of CYLD proteins (RIP) [20, 37], and thereby activated NF-κB. Therefore, inhibiting NF-κB activation may enhance the antitumor effect of TRAIL.

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 5 ng/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.
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 4). 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-additive 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, which is remarkably increased upon ectopic CYLD expression (Figure 4). When CYLD was over-expressed, treating A549 cells with TRAIL helped to explain why A549 cells were not sensitive to apoptosis. However, increased expression of CYLD antagonized TRAIL-induced apoptosis in the absence of apoptotic stimuli although it can negatively regulate NF-κB 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, and -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 antagonizing 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 chemoresistant 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 tumorogenesis.

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References


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