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c-Cbl-mediated degradation of TRAIL receptors is responsible for the development of the early phase of TRAIL resistance

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Abstract

We previously reported two modes of development of acquired TRAIL resistance: early phase and late phase [1]. In these studies, we observed that greater Akt activity and the expression of Bcl-xL were related mainly to the late phase of acquired TRAIL resistance.

Recently we became aware of a possible mechanism of early phase TRAIL resistance development through internalization and degradation of TRAIL receptors (DR4 and DR5). Our current studies demonstrate that TRAIL receptors rapidly diminish at the membrane as well as the cytoplasm within four hours after TRAIL exposure, but recover completely after one or two days. Our studies also reveal that Cbl, a ubiquitously expressed cytoplasmic adaptor protein, is responsible for the rapid degradation of TRAIL receptors; Cbl binds to them and induces mono-ubiquitination of these receptors concurrent with their degeneration soon after TRAIL exposure, creating the early phase of acquired TRAIL resistance.

Keywords

c-Cbl; TRAIL; TRAIL receptors; TRAIL resistance

1. Introduction

TRAIL has been shown to induce apoptosis in a number of cancer cell lines while displaying minimal or no toxicity to normal cells, suggesting that this protein may hold potential for development as a new cancer therapeutic agent. However, although TRAIL is regarded as a potential anticancer agent, considerable numbers of cancer cells, especially some highly malignant tumors, are resistant to apoptosis induction by TRAIL, and some cancer cells that

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were originally sensitive to TRAIL-induced apoptosis can become resistant after repeated exposures (acquired resistance) [2]. We observed that, for DU-145 prostate cancer cells with acquired TRAIL resistance, there was greater Akt activity and expression of Bcl-xL [1].

TRAIL induces an apoptotic response in tumor cells by binding to TRAIL receptors. Four distinct TRAIL receptors have been identified: TRAIL-R1/DR4, TRAIL-R2/DR5, TRAIL-R3/DcR1, and TRAIL-R4/DcR2. These receptors have been classified into two groups: death-inducing receptors (TRAIL-R1 and -R2) and death-inhibitory receptors (TRAIL-R3 and -R4) [3,4]. Both TRAIL-R1 and TRAIL-R2 contain a C-terminal death domain that signals downstream caspase activation to mediate TRAIL-induced apoptotic cell death in a variety of tumor cells. In contrast to these death-inducing receptors, TRAIL-R3, and TRAIL-R4 are death-inhibitory receptors, which share homology with the death-inducing TRAIL-R1 and TRAIL-R2 but lack transmembrane and death domains or have a truncated, nonfunctional death domain, respectively. The mechanism of protection by these death-inhibitory receptors was postulated to involve competition for TRAIL or activation of antiapoptotic signals perhaps via activation of NF-κB [5]; TRAIL-R4 cannot transmit a death signal but can weakly activate NF-κB, which may protect cells from TRAIL-mediated apoptosis [6]. It is not fully clear how widespread the decoy receptors' surface expression in tumor or normal cells is, or how these receptors modulate TRAIL signaling since the death-inhibitory receptors may undergo relocation from the nucleus to the cytoplasm and cell membranes [7,8]. A fifth receptor, osteoprotegerin, exists in a secreted form and appears to inhibit TRAIL-induced apoptosis by competitive inhibition of the binding of TRAIL to the death receptors TRAIL-R1 and -R2 [9].

Recently, Kohlhaas et al. [10] reported that endocytosis of TRAIL and its receptors occurred rapidly in a time- and concentration-dependent manner; this suggests a means of TRAIL activity reduction since down regulation of activated receptors reduces constitutive signaling [11]. Many studies about the endocytosis and subsequent degradation of the receptors have been done with receptor tyrosine kinases (RTKs) [12,13]. RTK ubiquitination has been known to be important for both receptor internalization and degradation in the lysosome [12,14]. In the yeast *Saccaromyces cerevisiae*, ubiquitin molecules conjugated to lysine residues of membrane proteins function as endocytosis motifs [15], and mono-Ub (ubiquitin) signals appear to play an evolutionally conserved role in the endosomal sorting of internalized activated receptors for degradation in the lysosome [14] However, under certain physiological conditions, it has been reported that Ub-independent mechanisms can promote epidermal growth factor (EGF) receptor internalization [16,17]. In this case, endocytosis of membrane proteins is typically mediated by signals present in their cytoplasmic domains [18]. Once internalized, the ligand-receptor complex is targeted to early endosomes and the receptor is then either recycled to the cell surface or directed to lysosomes for degradation [19]. In the case of cytosolic and nuclear proteins, polyubiquitin chains are usually added to them for degradation via the proteosome [20].

Cbl is a well known multi-adaptor protein involved in ligand-induced downregulation of receptor tyrosine kinases [21]. The Cbl family of adaptor proteins comprises three mammalian members: c-Cbl, Cbl-b, and Cbl-3. These proteins serve as E3 ubiquitin ligases targeting signaling proteins, such as activated protein tyrosine kinases, to the proteosome or lysosome for degradation [22]. Cbl ubiquitin-protein ligases are modular proteins that contain a conserved N-terminal tyrosine kinase binding (TKB) domain and RING finger domain in addition to other protein interaction motifs [23]. The TKB domain interacts with specific phosphotyrosine residues on RTKs directly, or through phosphotyrosine residues on adaptors indirectly [24,25]; the consensus sequence for recognition by the Cbl TKB domain is NXpY(S/T)XXP. In this study, we observed that c-Cbl interacts with TRAIL receptors and regulates the level of TRAIL receptors through the proteosomal and lysosomal pathways. The

differential role of c-Cbl in these two different degradation pathways is probably determined by the phosphorylation status of c-Cbl at the Tyr-731 residue. We also observed that TRAIL-stimulated c-Cbl-mediated-ubiquitination of TRAIL receptors is responsible for the early phase of acquired TRAIL resistance.

2. Materials and Methods

2.1. Cell culture and survival assay

Human prostate adenocarcinoma DU-145 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and 26 mM sodium bicarbonate for monolayer cell culture. The cells were maintained in a humidified atmosphere containing 5% CO_2 and air at 37° C.

2.2. Reagents and antibodies

MG-132, lactacystin and biotin-X-NHS were purchased from Calbiochem (San Diego, CA, USA). Amantadine was purchased from Sigma-Aldrich (St. Louis, MO, USA). Monoclonal anti-DR4 and anti-DR5 were purchased from Alexis Biochemicals. Polyclonal anti-DR4, anti-DR5, monoclonal/polyclonal anti-Ub and protein G-agarose were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Monoclonal anti-Ub and polyclonal anti-c-Cbl were purchased from Cell Signaling Technology, Inc. (Beverly, CA, USA). Monoclonal anti-PARP was purchased from Biomol International, L.P. (Plymouth Meeting, PA, USA). Monoclonal anti-HA (clone 3F10) was purchased from Roche Applied Science (Indianapolis, IN, USA). Monoclonal anti-actin was purchased from ICN (Costa Mesa, CA, USA).

2.3. Immunofluorescence

After TRAIL treatment for 4 h, DU-145 cells were fixed with 1 % formaldehyde in PBS for 20 min in ice, and permeabilized for 10 min at room temperature with PBS containing 1 % BSA and 0.1 % Triton-X-100. After washing twice with cold PBS, the cells were blocked in 1 % BSA, 10 % rabbit serum for 1 h at room temperature. They were then incubated with goat DR4 antibody having an epitope mapping at the C-terminus (C-20, Santa Cruz, 1:50 dilution) for 1 h at room temperature, followed by three washes with cold PBS. Samples were then incubated with secondary antibody (rabbit Alexa Fluor 555 anti-goat antibody 1:100) for 1 h at room temperature. After washing three times with cold PBS and after mounting with mounting solution, the slides were observed under the fluorescence microscope.

2.4. RNA interference by siRNA c-Cbl

To construct siRNA of c-Cbl, pSilencer 2.1-U6 hygro vector (Ambion, Inc., Austin, TX, USA) was used for expressing siRNA for c-Cbl. The insert for hairpin siRNA into pSilencer was prepared by annealing two oligonucleotides. For human c-Cbl siRNA, the top strand sequence was 5'-GATCCGATGGAGACACTTGGAGAATTCAAGAGATTCTCCAAGTGTCTCCATCTTTTTTG GAAA-3', and the bottom strand sequence was 5'-AGCTTTTCCAAAAAAGATGGAGACACTTGGAGAATCTCTTGAATTCTCCAAGTGTCTCC ATCG-3'. The annealed insert was cloned into pSilencer 2.1-U6 hygro digested with BamH I and Hind III. The correct structure of pSilencer 2.1-U6 hygro-c-Cbl was confirmed by nucleotide sequencing. The resultant plasmid, pSilencer-c-Cbl, was transfected into DU-145 cells. The interference of c-Cbl protein expression was confirmed by immunoblot using anti-c-Cbl antibody (Cell Signaling).

2.5. Protein extracts and polyacrylamide gel electrophoresis

Cells were lysed with 1 × Laemmli lysis buffer (2 % sodium dodecyl sulfate, 10 % glycerol, 0.002 % bromophenol blue, 62.5 mM Tris, pH 6.8) and boiled for 10 min. Protein content was measured with BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). The samples were diluted with 1 × lysis buffer and added β-mercaptoethanol to be 350 mM, then equal amounts of protein were loaded on 10 % or 15 % sodium dodecyl sulfate (SDS)-polyacrylamide gels. SDS-PAGE analysis was performed according to Laemmli using a Hoefer gel apparatus.

2.6. Immunoblot analysis

Proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked with 5 % nonfat dry milk in PBS-Tween-20 (0.1 %, v/v) at 4° C overnight. The membrane was incubated with primary antibody (diluted according to the manufacturer's instructions) for 2 h. Horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody. Immunoreactive protein was visualized by the chemiluminescence protocol (ECL, Amersham, Arlington Heights, IL, USA).

2.7. Biotin labeling to plasma membrane

After TRAIL treatment for the indicated time, DU-145 cells were washed with ice-cold PBS and incubated with 0.5 mg/ml biotin-X-NHS dissolved in a borate buffer (10 mM boric acid, 150 mM NaCl, pH 8.0) for 45 min at 4°C. Biotin coupling was terminated by washing the plates with ice-cold PBS containing 15 mM glycine. After washing with room temperature PBS, proteins were lysed for the immunoprecipitation with mouse anti-DR4 or DR5 antibody (Alexis). Protein biotinylation was detected by peroxidase conjugated streptavidin after western blotting.

2.8. Site-directed mutagenesis

The QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) was used to make point mutations in human Cbl protein. One tyrosine residue in Cbl (Tyr-371 Cbl) was replaced with phenylalanine (Phe-371 Cbl). Sense primer (5'-CAGGAACAATATGAATTATTCTGTGAGATGGGCTCCAC-3') and antisense primer (5'-GTGGAGCCCATCTCACAGAATAATTCATATTGTTCCTG-3') were used for site-directed mutagenesis. PCR reaction was prepared by adding 5 μl of 10X reaction buffer, 20 ng of dsDNA template (pSRα neo-HA-Cbl was kindly provided by Dr. Langdon (University of Western Australia, Australia)), 125 ng of each sense primer, 125 ng of each antisense primer, 1 μl of deoxyribonucleotide triphosphate mix, 3 μl QuickSolution, double-distilled water to a final volume of 50 μl, and 1 μl of *Pfu* Turbo DNA polymerase (2.5 U/μl). PCR was performed with 18 cycles (95° C for 1 min; 57° C for 1 min; 68° C for 12 min) with initial incubation at 95° C for 2 min. Following temperature cycling, the reaction was placed on ice for 2 min to cool the reaction. After PCR, 1 μl of *Dpn* I restriction enzyme (10 U/μl) was added directly to each amplification reaction and incubated at 37° C for 1 h to digest the parental supercoiled dsDNA. The *Dpn* I-treated dsDNA was transformed into *Epicurian coli* XL1-Blue supercompetent cells. Colonies were selected and the resultant plasmid was sequenced using primer (5'-GGCTGAGCTGTACTCGTCTG-3') to confirm mutation.

2.9. Confocal microscope studies

To detect the localization of DR4 and c-Cbl, 1×10^5 tumor cells were seeded overnight on glass slides in 37° C and 5% of CO_2. Next, the culture media was replaced by a fresh one supplemented with TRAIL (200 ng/ml). After 2 h of co-incubation with TRAIL, cells were washed in 0.5% BSA in PBS, fixed in 2% paraformaldehyde for 15 min, permeabilized with 0.05% Triton-X, washed and blocked with 2% BSA in PBS for 45 min to eliminate non-specific

binding of secondary Abs. The following Abs were used for staining: polyclonal goat anti-human DR4 (C-20, Santa Cruz Biotechnology) and polyclonal rabbit anti-human c-Cbl (Cell Signaling). Primary Abs were diluted 1:100-1:200 in 0.5% BSA and incubated 1 h in moist chamber, then washed and incubated with secondary Abs. As a secondary Abs, donkey anti-goat TexasRed-labeled (Santa Cruz Biotechnology) and donkey anti-rabbit FITC-labeled (Santa Cruz Biotechnology) were used at dilution 1:500 and incubated 45 min in moist chamber and in the dark. Control reactions included replacement of primary antibody by 0.5% BSA. Slides were mounted in a medium with 4', 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) in order to trace cell nuclei. Cells were visualized in 0.4-μm sections using an inverted Olympus Fluoview 1000 laser scanning confocal microscope under a × 60 oil immersion objective. For digital image analysis, the software Adobe Photoshop 7.0 version was used.

2.10. In vitro ubiquitination assay

DU-145 cells were transfected with HA-tagged Cbl plasmid (pSRα neo-HA-Cbl). The cells were lysed with lysis buffer, and the lysate was immunoprecipitated with anti-HA antibody and collected with protein G plus agarose. Ubiquitination was carried out with HA-tagged Cbl as a potential E3 ligase in a 20 μl reaction solution (Biomol, 10 × buffer, IPP (20 U/ml), DTT 1 mM, Mg-ATP 5 mM, 20X E1 0.1 μM, Flag-Ub (2.5 μM), GST-DR4, UbcH7 as E2 conjugating enzyme 0.2 mg/ml) at 37 ° C for 1 h. Samples were analyzed by electrophoresis on 15 % SDS-PAGE followed by immunoblotting with anti-ubiquitin antibody (Cell Signaling). For the purification of DR4 as a target protein of ubiquitination, cytoplasmic domain of DR4 was subcloned into BamHI/XhoI site of pGEX4T-1 after PCR of cytoplasmic domain of DR4 by using pCMV1FlagDR4 as a template. pCMV1FlagDR4 was kindly provided by Dr. Vincenz at the University of Michigan. Sense primer was 5'-CATAGGATCCGGCTCAGGTTGTGGAGGGGAC-3', and antisense primer was 5'-CAGTCTCGAGTCACTCCAAGGACACGGCAGAGCC-3'. pGEX-4T-1/DR4 was transformed into JM109, and expressed DR4 corresponding to cytoplasmic domain was purified by using glutathione-Sepharose 4B (Amersham).

3. Results

3.1. Correlation between development and decay of acquired TRAIL resistance and down regulation of TRAIL receptors (DR4, DR5) at the early phase of acquired TRAIL resistance

Previous studies have shown that apoptotic signals of TRAIL are transduced by binding to the TRAIL receptors DR4 and DR5 [8,26,27]. In this study, we hypothesized that TRAIL receptors also play a role in the development of acquired TRAIL resistance, in particular early phase, through their internalization and degradation after ligand-receptor complex formation. To test the hypothesis, we first examined whether the level of surface TRAIL receptors is reduced after TRAIL treatment. For this study, we labeled accessible lysines of surface receptors by using the water-soluble, membrane-impermeant biotin-X-NHS. Data from measurement of TRAIL-induced cytotoxicity show that acquired TRAIL resistance developed immediately and was sustained for 2 days and then gradually decayed (upper panel in Fig. 1A). These observations were consistent with poly(ADP-ribose) polymerase (PARP) cleavage, which is the hallmark feature of apoptosis (lower panel in Fig. 1A). In the next step, we examined the level of total TRAIL receptors or membrane-bound TRAIL receptors immediately or at various times (1–6 days) after treatment with 200 ng/ml TRAIL for 4 h (Figs. 1B and 1C). The level of total and membrane-bound TRAIL receptors decreased rapidly and recovered within 1 or 2 days for DR5 or DR4, respectively (Figs. 1B and 1C). Similar results were observed with human pancreatic cancer MiaPaCa-2 cells (data not shown). We further examined the time course of acquired TRAIL resistance development within 1 day (early phase) and changes in the level of TRAIL receptors during treatment with 200 ng/ml TRAIL. Figure 2A shows that

acquired TRAIL resistance developed immediately after TRAIL treatment and was sustained for 24 h. Data from western blot analysis demonstrate that the levels of total and membrane-bound TRAIL receptors were rapidly decreased within 2 h during treatment with TRAIL (Figs. 2B and 2C). The reduction of membrane-bound DR4 and DR5 during TRAIL treatment was confirmed by using the fluorescence microscope (DR4 only) (Fig. 2D). These results suggest that resistance to secondary TRAIL treatment immediately after TRAIL treatment is related to the reduction of TRAIL receptors at the membrane in what is called desensitization of receptors.

3.2. Monoubiquitination of TRAIL receptors (DR4, DR5)

As shown in Figures 2B and 2C, downregulation of TRAIL receptors (DR4 and DR5) was observed during TRAIL treatment. Several researchers previously reported that membrane receptor kinases are usually monoubiquitinated for endocytic trafficking both at the stage of receptor internalization at the plasma membrane and also in the endosomal compartment, where ubiquitinated receptors are sorted to a lysosomal compartment [16, 17]. In our current study, we investigated whether ubiquitination occurs during downregulation of TRAIL receptors. Figures 3A and 3B clearly demonstrate that DR4 and DR5 were monoubiquitinated during TRAIL treatment. Chloropromazine, an internalization inhibitor, inhibits TRAIL-induced ubiquitination of DR4 (data not shown). These results suggest that ubiquitination of TRAIL receptors occurs after internalization.

3.3 Interaction of TRAIL receptors with c-Cbl during TRAIL treatment

A fundamental question that still remains unanswered is how TRAIL receptors are ubiquitinated during treatment with TRAIL. We hypothesized that c-Cbl, an E3 ubiquitin ligase, is involved in the ubiquitination of TRAIL receptors. To examine this possibility, first of all, we investigated whether c-Cbl interacts with TRAIL receptors during TRAIL treatment. Data from immunoprecipitation analysis in Figures 4A and 4B clearly show that interactions between c-Cbl and TRAIL receptors (DR4/DR5) occurred during TRAIL treatment. To confirm our observations, we employed an immunofluorescent staining analysis with confocal microscope. The immunofluorescent staining studies also demonstrate the interaction between c-Cbl and DR4 during treatment with 200 ng/ml TRAIL for 2 h (Fig. 4C). For the confirmation of biological function of c-Cbl as an E3 ligase, an *in vitro* ubiquitination assay was performed by using HA-tagged c-Cbl as a potential E3 ligase for DR4 during TRAIL treatment. Results from Figure 4D clearly demonstrate direct E3 ligase activity of c-Cbl towards TRAIL receptors.

3.4. Involvement of c-Cbl in TRAIL receptor degradation but not in internalization

After observation of c-Cbl involvement in the reduction of TRAIL receptors during TRAIL treatment, we further investigated the biological role of c-Cbl in the reduction of TRAIL receptors by using the siRNA technique for c-Cbl knockdown. To make a stable cell line of siRNA of c-Cbl, the sequences for siRNA of c-Cbl were inserted into pSilencer 2.1-U6 hygro vector and then DU-145 cells were stably transfected with this expression vector. After hygromycin B-resistant cell clones were isolated, the interference to c-Cbl protein expression was verified by immunoblotting assay using anti-c-Cbl antibody. We obtained several stable transfectants and chose 3 clones (si c-Cbl #1, #3, #4) in which c-Cbl expression was almost completely repressed (Fig. 5A). In the first step of our siRNA analysis, we examined whether c-Cbl knockdown alters TRAIL sensitivity. Figure 5B shows that a pool of si c-Cbl clones in comparison to pSilencer control plasmid transfectant was more sensitive to TRAIL cytotoxicity, but not to another apoptotic agent, cisplatin, cytotoxicity. These results suggest that the increase in sensitivity seen in c-Cbl-depleted cells is specific to TRAIL-induced apoptosis. TRAIL-induced morphological alterations and cell death were increased in the pool of si c-Cbl clones (Fig. 5B). This is probably due to an increase in the level of TRAIL receptors (DR4 and DR5) in c-Cbl knockdown cells (Figs. 5C and 5D). Interestingly, the reduction of

membrane-bound TRAIL receptors during TRAIL treatment was almost the same in the pSilencer control plasmid transfectant and a pool of si c-Cbl clones (Figs. 5C and 5D). However, the reduction of total TRAIL receptors was inhibited in c-Cbl knockdown cells (Figs. 5C and 5D). These data suggest that c-Cbl is involved in the degradation of TRAIL receptors, but not in the internalization. To understand the increased level of DR4 and DR5 at the membrane and total lysates in c-Cbl knockdown cells, we further investigated the possibility that, without TRAIL treatment, c-Cbl is involved in the steady-state turnover of total DR4 and DR5 by the proteosomal degradation pathway. To test the hypothesis, cells were treated with proteosome inhibitors, MG-132 and/or lactacystin. The levels of DR4 and DR5 were significantly increased by treatment with MG-132 and/or lactacystin (lanes 3, 5, and 7 in Fig. 5E). However, the levels of DR4 and DR5 were decreased by treatment with TRAIL even in the presence of MG-132 and/or lactacystin (lanes 4, 6, and 8 in Fig. 5E). These data suggest that the reduction of DR4 and DR5 levels during treatment with TRAIL is not mediated through the proteosomal degradation pathway. Figure 5E also shows that proteosome inhibitors enhanced PARP cleavage. This increase in sensitivity to TRAIL is probably due to an increase in DR4 and DR5 levels. To examine whether the lysosomal pathway is involved in TRAIL receptor regulation during TRAIL treatment, cells were pretreated with amantadine, a lysosome inhibitor prior to TRAIL treatment. Figure 5F shows that inhibition of the lysosomal pathway prevented the reduction of DR5 during TRAIL treatment. To examine the role of proteosomal and lysosomal pathways in the development of acquired TRAIL resistance, the effect of proteosome inhibitor and lysosome inhibitor on the development of acquired TRAIL resistance was assessed. Cells were pretreated with MG-132 and/or amantadine and treated with 20 ng/ml TRAIL and/or 50 ng/ml TRAIL, respectively. These concentrations of TRAIL are the isosurvival doses to 200 ng/ml TRAIL in the control cells. Acquired TRAIL resistance was evaluated by challenging TRAIL treatment. Figure 5G shows that MG-132 and amantadine effectively inhibited the development of acquired TRAIL resistance. To investigate the role of c-Cbl in the development of acquired TRAIL resistance, c-Cbl knockdown cells were treated with 20 ng/ml TRAIL, which is the isosurvival dose to 200 ng/ml TRAIL in the pSilencer control plasmid transfectant. As shown in Fig. 5H, development of acquired TRAIL resistance was partially suppressed in c-Cbl knockdown cells. Moreover, acquired TRAIL resistance rapidly decayed in c-Cbl knockdown cells. To examine whether the rapid decay of acquired TRAIL resistance in c-Cbl knockdown cells is due to the recovery of TRAIL receptors, membrane-bound and total level of TRAIL receptors were determined at various times after TRAIL treatment. Figures 5I and 5J clearly demonstrate that the levels of membrane DR4 and DR5 were recovered to their original levels faster in c-Cbl knockdown cells compared to pSilencer control plasmid transfected cells.

3.5. Monoubiquitination of TRAIL receptors is dependent on c-Cbl

We observed that c-Cbl plays an important role in the reduction of TRAIL receptors and development of acquired TRAIL resistance during treatment with TRAIL. We also observed that ubiquitination of TRAIL receptors occurs during TRAIL treatment. Based on our observations, we hypothesized that c-Cbl which is known as an E3 ubiquitin ligase is involved in monoubiquitination of TRAIL receptors. As shown in Figures 6A and 6B, monoubiquitination of TRAIL receptors (DR4, DR5) decreased in c-Cbl knockdown cells, suggesting that c-Cbl is involved in ubiquitination of TRAIL receptors during TRAIL treatment.

3.6. Phosphorylation of tyrosine 371 residue of c-Cbl is important for ubiquitination of TRAIL receptors

Several researchers have reported that various membrane receptors are internalized by ubiquitin ligase c-Cbl to achieve efficient receptor down-regulation [12]. It is well known that c-Cbl is inducibly phosphorylated by receptor and non-receptor protein tyrosine kinases, but

constitutively phosphorylated by oncogenic tyrosine kinases in various cancer cell lines [28]. The bulk of phosphorylation occurs at the carboxy terminus of c-Cbl which contains multiple tyrosine residues [29–31]. Phosphorylation of Y700, Y731, and Y774 is necessary for the c-Cbl-CIN85 interaction, which mediates endocytosis and downregulation of EGFR [32]; especially, phosphorylation of Y731 of c-Cbl provides a docking site for downstream signaling components such as p85 and Fyn [33,34]. Phosphorylation of Y371 regulates the transfer of ubiquitin from UbcH7 to the substrate [22,35,36]. In this study, we hypothesized that Y371 of c-Cbl plays an important role in the downregulation of TRAIL receptors. To test the hypothesis, tyrosine of 371 was replaced with phenylalanine by site-directed mutagenesis technique. We examined whether ubiquitination of TRAIL was affected by overexpression of mutant-type c-Cbl (Y371F Cbl). Overexpression of Y371F Cbl inhibited reduction of total (not membrane) DR4 and DR5 (Figs. 6C and 6E) and ubiquitination of DR4 and DR5 (Figs. 6D and 6F) during TRAIL treatment. Overexpression of Y371F Cbl also facilitated the decay of acquired TRAIL resistance (Fig. 6G). These results suggest that phosphorylation of Y371 of c-Cbl is important for ubiquitination of TRAIL receptors and the early phase of acquired TRAIL resistance.

Discussion

We have previously shown that TRAIL treatment induces acquired TRAIL resistance in two modes; one is the restoration of biological function of Bcl-xL, and the other is the elevation of the Bcl-xL level [1]. The former mode has been proven to have an important role in the development of acquired TRAIL resistance in the early phase; the latter mode is responsible for the late phase of acquired TRAIL resistance. In the present study, we observed that the downregulation of TRAIL receptors after TRAIL treatment is also involved in the development of acquired TRAIL resistance, particularly in the early phase. Kohlhaas et al. [10] has reported that, unlike CD95 and TNF-R1-induced apoptosis, rapid endocytosis of TRAIL and its receptors is not required for TRAIL-induced apoptosis [37,38]. Thus, a question that remains unanswered is the biological meaning of internalization and degradation of TRAIL receptors during TRAIL treatment. In this study, we mainly focused on TRAIL death receptors (DR4, DR5) because decoy receptors were not well detected in DU-145 cells (data not shown). These observations are consistent with previous report that little DcR expression was frequently observed in cancer cells [39]. Also, DcR expression does not appear to be correlated with resistance to TRAIL [39].

As shown in Figure 2, the level of TRAIL receptors rapidly decreased at the membrane as well as in the whole cell within 4 h during TRAIL treatment, and the reduction of TRAIL receptors coincided with the acquisition of early phase TRAIL resistance. However, acquired TRAIL resistance is still sustained 24 h after TRAIL treatment although the total level of DR5 was recovering during that time (Fig. 1C). These results suggest that the early phase (within 24 h after TRAIL exposure) of acquired TRAIL resistance was contributed by other factors in addition to the reduction of TRAIL receptors, such as the restoration of biological activity of Bcl-xL [1]. Nevertheless, the reduction of TRAIL receptors is a major factor in the development of acquired TRAIL resistance (Fig. 5).

Our studies reveal that rapid degradation following internalization of TRAIL receptors occurs during treatment with TRAIL. Our studies also reveal that ubiquitination of TRAIL receptors is responsible for degradation, but not internalization. These observations somewhat contradict previous reports. In the case of RTKs, recent studies illustrate that RTKs are monoubiquitinated at multiple sites following ligand stimulation and that ubiquitin acts as a signal controlling receptor internalization and routing to the lysosome for degradation [16,17,40], and c-Cbl, an E3 ubiquitin ligase, is involved in RTK ubiquitination [41–43]. We observed that c-Cbl was also involved in the monoubiquitination of TRAIL receptors (DR4, DR5) during TRAIL exposure (Figs. 6A, 6B). However, unlike its role in RTKs, monoubiquitination by c-Cbl is

found to be necessary for degradation of TRAIL receptors rather than internalization (Figs. 5C, 5D). These results are similar to previous observations that Cbl mutants impaired in their ubiquitin ligase activity block receptor degradation by shunting endocytosed receptors from the endosome to the recycling pathway and not by blocking receptor internalization [43], and Cbl-mediated ubiquitination of EGFRs in mouse embryonic fibroblasts is required for endosomal receptor sorting and degradation but is dispensable for receptor internalization [41]. Observations from Levkowitz et al. [43] are consistent with our observations that downregulation of c-Cbl leads to an increase in the level of DR4 and DR5 at the membrane (Figs 5C and 5D) and facilitation of restoration of TRAIL receptors (Figs. 5I and 5J).

As pointed out by Haglund et al. [16,17], c-Cbl mediated ubiquitination of TRAIL receptors at the cellular membrane is not essential for internalization because of the existence of multiple internalization pathways. Nonetheless, c-Cbl-mediated ubiquitination of TRAIL receptors has a main role in the endosomal sorting leading to the degradative pathway (Figs. 6A, 6B, 6D, 6F). The inhibition of c-Cbl-induced ubiquitination would impair endosomal sorting and lysosomal degradation, but not the internalization step [20]. Höller and Dikic [14] suggested that the mechanism underlying ligand-dependent receptor endocytosis seems to be divergent and more complex in mammalian cells, which means endocytosis is not regulated by one exclusive pathway but is, instead, covered by several redundant mechanisms.

Results from our studies strongly suggest that phosphorylation of the tyrosine 371 residue of c-Cbl plays an important role in the activation of E3 ubiquitin ligase activity of c-Cbl. A fundamental question that remains is how c-Cbl is phosphorylated during TRAIL treatment. Interestingly, TRAIL receptors do not contain phosphotyrosine residue consensus recognition sites, suggesting that the presence of non-receptor tyrosine kinase as an adaptor to phosphorylate TRAIL receptors and Cbl is plausible. TKB domain of c-Cbl could be recruited to non-receptor tyrosine kinase instead of TRAIL receptors. This possibility is being investigated, calling for the detection of responsible non-receptor tyrosine kinase to phosphorylate TRAIL receptors and c-Cbl. In addition to this possibility, the fact that internalization of TRAIL receptors is not interrupted in c-Cbl knockdown cells raises a possibility that other endocytosis motifs might be present in TRAIL receptors and that these motifs might function in an ubiquitin-independent manner. We believe that this model will provide a framework for future studies.

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Abbreviations

TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
DR4	5, death receptor4, 5
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
SDS	sodium dodecyl sulfate
Ub	ubiquitin

biotin-X-NHS biotin-X-N-hydroxysuccinimide

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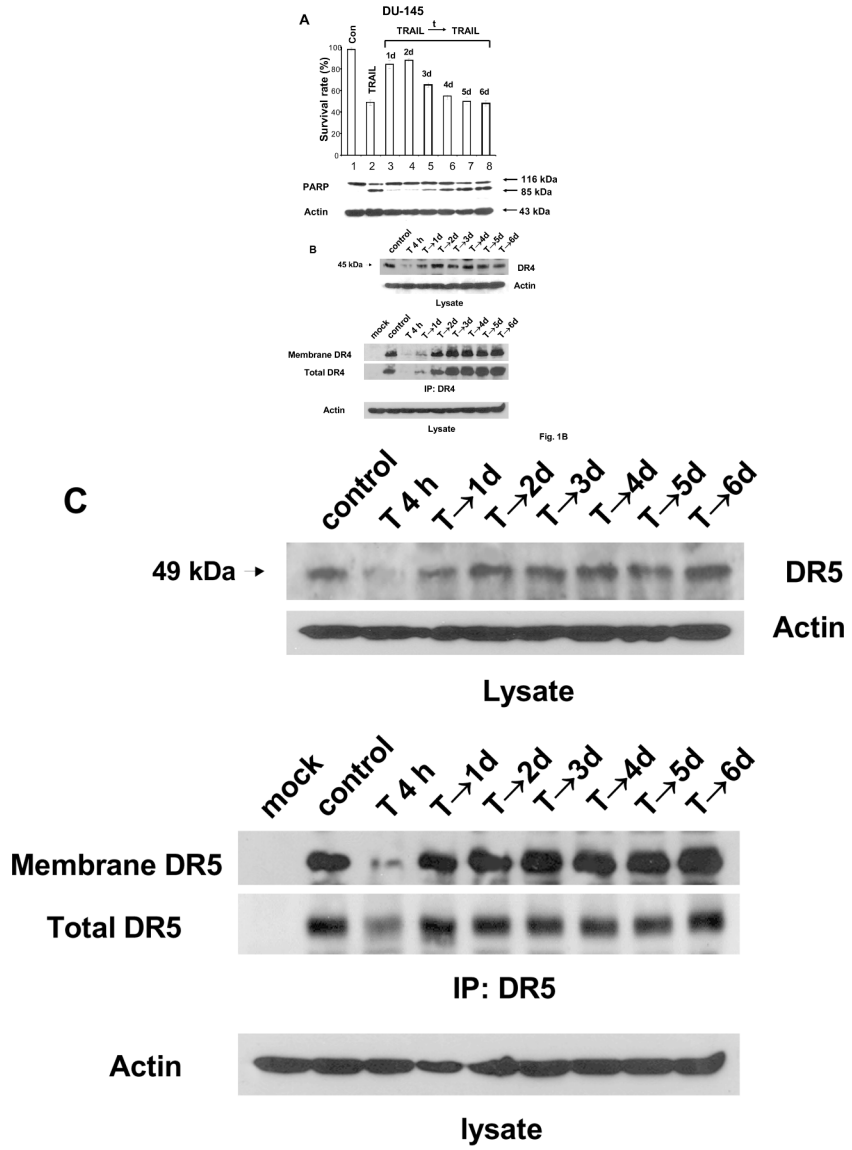


Figure 1. TRAIL-induced development of acquired TRAIL resistance and alteration of levels of TRAIL receptors (DR4, DR5) in DU-145 cells (long term)

(**A**) Cells were first treated with TRAIL (200 ng/ml) for 4 h and then detached cells were removed by washing out with PBS. After removal of detached cells, fresh media was added onto the remaining attached cells and incubated for the time indicated (1–6 d), replenishing with fresh media every day, and then cells were treated a second time with TRAIL (200 ng/ml) for 4 h. Cell survival was determined by trypan blue exclusion assay (upper panel) and cell lysates were subjected to immunoblotting for PARP or actin (lower panels). Con, untreated control cells. Error bars represent the S.E. from three separate experiments. (**B** and **C**) Cells were treated with TRAIL (200 ng/ml) for 4 h and then detached cells were removed by washing out with PBS. After removal of detached cells, fresh media was added onto the remaining attached cells and incubated for the time indicated (0 h, or 1–6 d), and then cells were treated with biotin-X-NHS (0.5 mg/ml) following washing with PBS. After labeling with biotin, cell lysates were immunoprecipitated with anti-DR4 or anti-DR5 antibody. Membrane-bound DR4 or DR5 was detected with peroxidase-conjugated streptavidin. Total DR4 or DR5 was detected with anti-DR4 or anti-DR5 antibody, respectively (B, C).

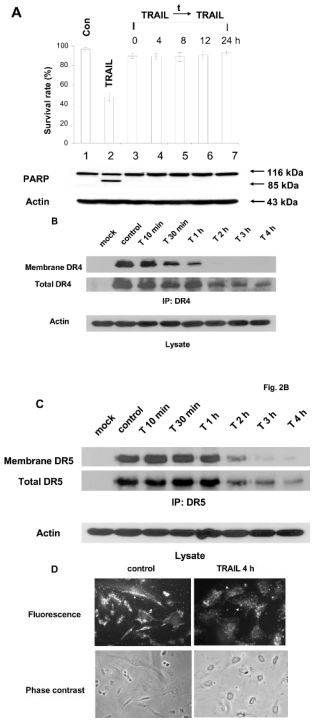


Figure 2. TRAIL-induced development of acquired TRAIL resistance, alteration of levels of TRAIL receptors (DR4, DR5), and internalization of DR4 in DU-145 cells (short term)

(**A**) Cells were first treated with TRAIL (200 ng/ml) for 4 h and then detached cells were removed by washing out with PBS. After removal of detached cells, fresh media was added onto the remaining attached cells and cells were incubated for the time indicated (0 h, 4 h, 8 h, 12 h, 24 h), and then treated a second time with TRAIL (200 ng/ml) for 4 h. Cell survival was determined by trypan blue exclusion assay (upper panel) and cell lysates were subjected to immunoblotting for PARP or actin (lower panels). Con, untreated control cells. Error bars represent the S.E. from three separate experiments. (**B** and **C**) Cells were treated with TRAIL (200 ng/ml) for various times (10 min, 30 min, 1 h, 2 h, 3 h, 4 h). After detached cells were removed, cells were treated with biotin-X-NHS (0.5 mg/ml). After labeling with biotin, cell lysates were immunoprecipitated with anti-DR4 or anti-DR5 antibody of Alexis, respectively. Membrane-bound DR4 or DR5 was detected with peroxidase-conjugated streptavidin. Total DR4 or DR5 was detected with anti-DR4 (H-130) or anti-DR5 (N-19) antibody of Santa Cruz, respectively. (**D**) Internalization of DR4 was determined by immunofluorescent staining as described in “Materials and Methods”. Arrows indicate the localization of DR4.

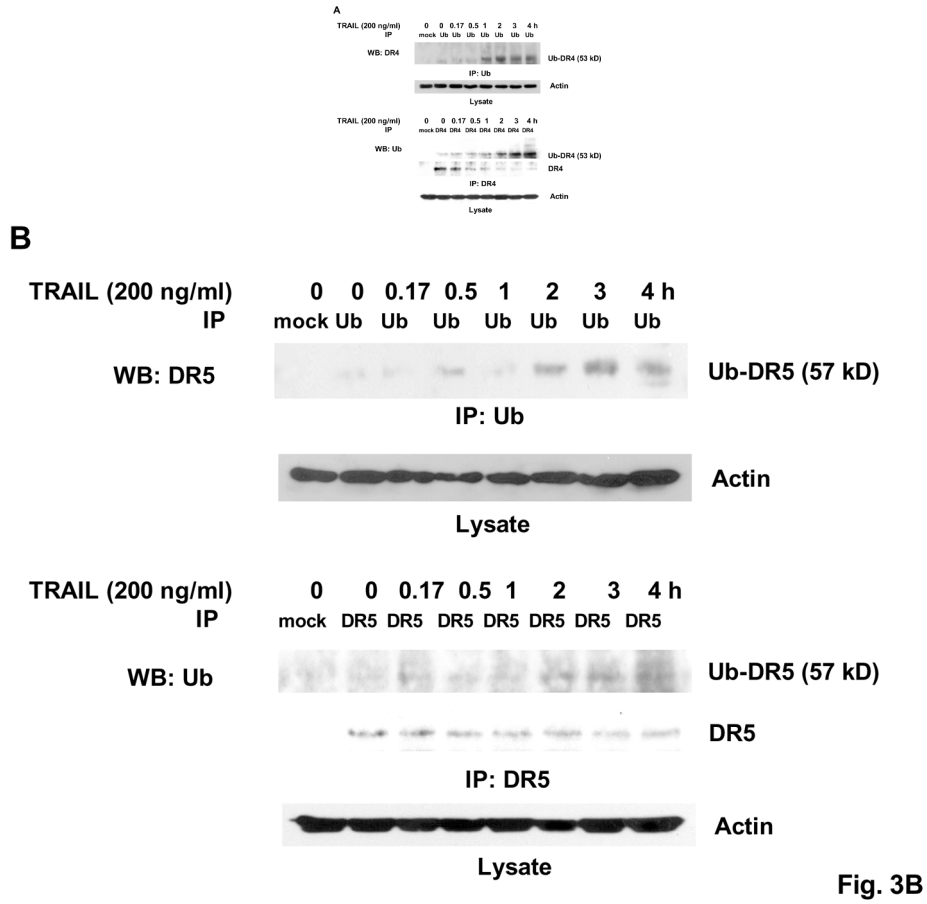


Figure 3. Ubiquitination of TRAIL receptors (DR4, DR5) during TRAIL treatment in DU-145 cells

Cells were treated with TRAIL (200 ng/ml) for various times. Cell lysates were immunoprecipitated with anti-Ub antibody and immunoblotted with anti-DR4 (H-130) antibody (**A**) or anti-DR5 antibody (N-19) (**B**) (upper panels). Cell lysates were immunoprecipitated with anti-DR4 antibody (HS-101, Alexis) (**A**) or anti-DR5 antibody (HS-201, Alexis) (**B**) and then immunoblotted with anti-Ub antibody (lower panels).

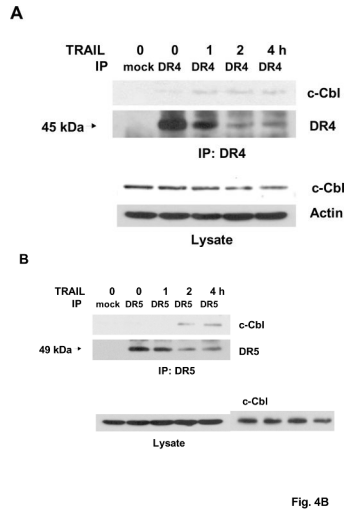


Fig. 4B

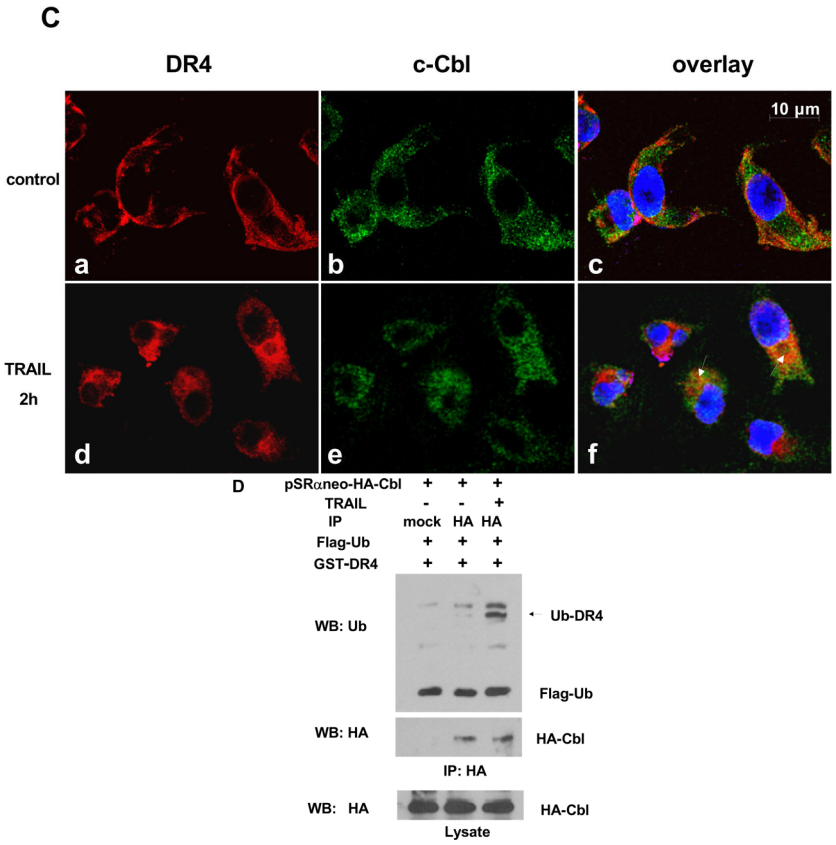


Figure 4. Interaction between c-Cbl and TRAIL receptors during TRAIL treatment in DU-145 cells

Cells were treated with TRAIL (200 ng/ml) for various times and then cell lysates were immunoprecipitated with anti-DR4 (**A**) or anti-DR5 (**B**) antibody and immunoblotted with anti-c-Cbl, anti-DR4 or anti-DR5 antibody (upper panels). The presence of c-Cbl or actin in the lysates was verified by immunoblotting (lower panels). (**C**) Cells were untreated (control) or treated with TRAIL (200 ng/ml) for 2 h and stained with anti-DR4 (red) and anti-c-Cbl (green) antibodies. Nuclei were stained with DAPI (blue). ***a,*** DR4 is expressed mainly on cell membrane; ***b,*** c-Cbl shows the cytoplasm localization; ***c,*** overlay (a) and (b) showing no co-localization of DR4 and c-Cbl in cytoplasm; ***d,*** DR4 is translocated to cytoplasm during

treatment with TRAIL; ***e,*** c-Cbl is expressed in cytoplasm; ***f,*** figures (d) and (e) are overlaid showing co-localization of DR4 and c-Cbl in cytoplasm (yellow) (arrows). (**D**) Cells were transfected with pSRα neo-HA-Cbl and then untreated (lanes 1 and 2) or treated with TRAIL (200 ng/ml) for 2 h (lane 3). Cell lystates were immunoprecipitated with mock IgG (lane 1) or anti-HA antibody (lanes 2 and 3). *In vitro* ubiquitination assay was performed as described in Experimental Procedures. Samples were immunoblotted with anti-Ub or anti-HA antibody (upper panels). Cell lysates were immunoblotted with anti-HA antibody (lower panel).

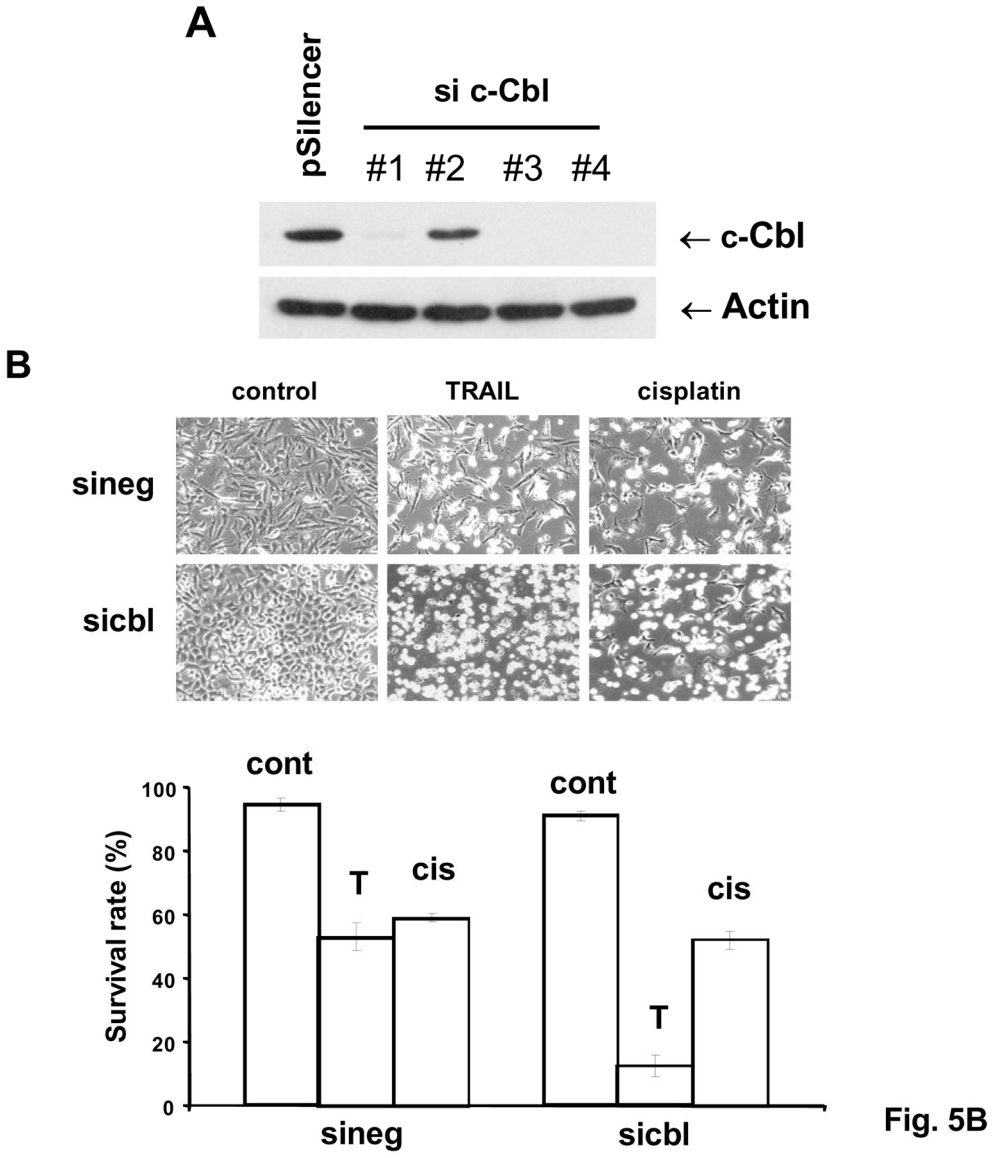
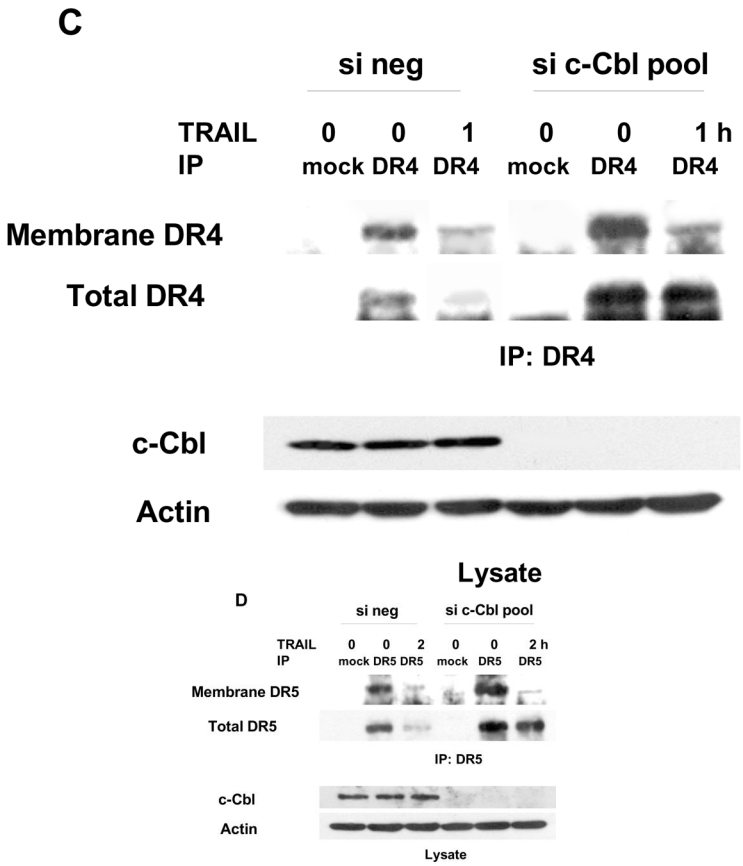
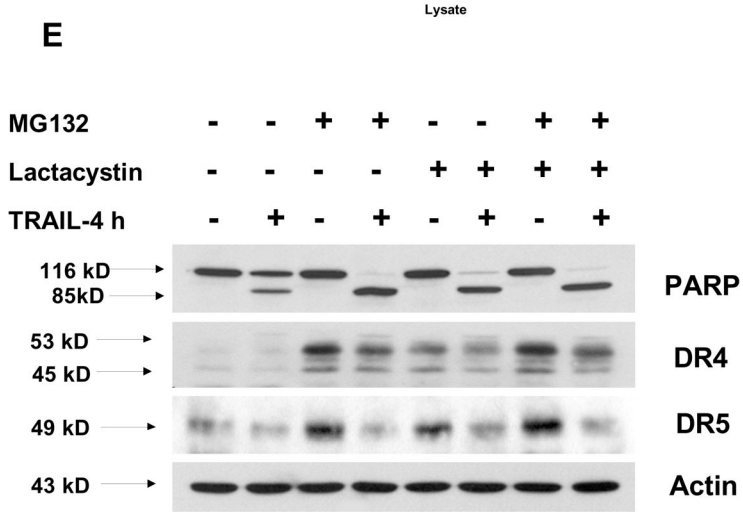
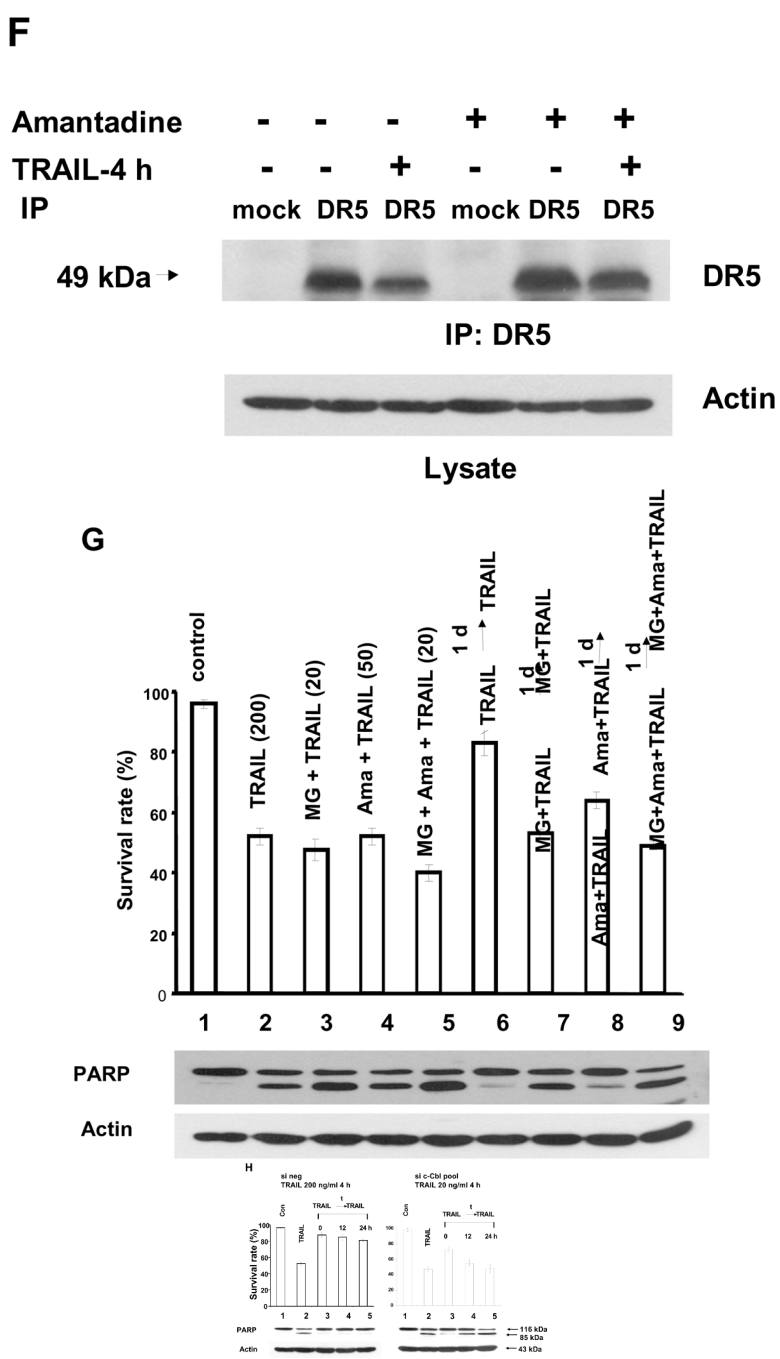


Fig. 5B







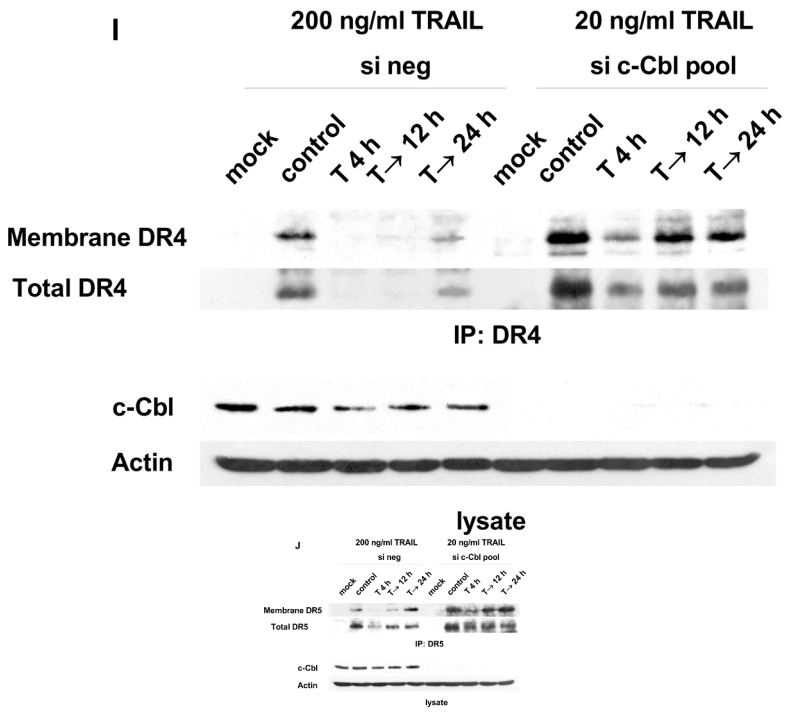


Figure 5. Role of c-Cbl in TRAIL sensitivity, level of TRAIL receptors (DR4, DR5), and development of acquired TRAIL resistance in DU-145 cells

(**A**) Intracellular level of c-Cbl was examined in control vector transfected (*pSilencer*) or pSilencer-si c-Cbl stably transfected single cell clones. (**B**) Control plasmid (si neg) or a pool of pSilencer-si c-Cbl stably transfected clones (si c-Cbl pool) were treated with TRAIL (200 ng/ml) for 4 h or cisplatin (5 μg/ml) for 24 h and then morphological features were analyzed with a phase-contrast inverted microscope (upper panels), or cell survival was determined by tryphan blue exclusion assay (lower panel). Error bars represent the S.E. from three separate experiments. (**C** and **D**) Control plasmid (si neg) or a pool of pSilencer-si c-Cbl stably transfected (si c-Cbl pool) cells were treated with TRAIL (200 ng/ml) for 1 h or 2 h, and then cells were treated with biotin-X-NHS (0.5 mg/ml) following washing with PBS. After labeling with biotin, cell lysates were immunoprecipitated with anti-DR4 or anti-DR5 antibody. Membrane-bound DR4 or DR5 was detected with peroxidase-conjugated streptavidin. Total DR4 or DR was detected with anti-DR4 or anti-DR5 antibody, respectively (upper panels), and cell lysates were subjected to immunoblotting for c-Cbl or actin (lower panels). (**E**) Cells were pretreated with 10 μM MG132 and/or 10 μM lactacystin for 4 h followed by 200 ng/ml TRAIL treatment for 4 h and then cell lysates were subjected to immunoblotting for PARP, DR4 , DR5 or actin. (**F**) Cells were pretreated with 100 μM amantadine for 4 h followed by 200 ng/ml TRAIL treatment for 4 h. Cell lystates were immunoprecipitated with mock IgG or anti-DR5 antibody and then immunoblotted with anti-DR5 antibody (upper panel). Cell lysates were subjected to immunoblotting for actin (lower panel). (**G**) **Lanes 2–5:** Cells were treated with TRAIL (200 ng/ml in lane 2, 20 ng/ml in lanes 3 and 5, 50 ng/ml in lane 4) for 4 h after pretreatment with 10 μM MG132 (MG, lane 3), 100 μM amantadine (Ama, lane 4), or MG + Ama (lane 5) for 4 h or without pretreatment (lane 2). **Lanes 6–9:** Cells were first treated as described above and then detached cells were removed by washing out with PBS. After removal of detached cells, fresh media were added onto the remaining attached cells and cells were incubated for 1 day, and then treated a second time with TRAIL (200 ng/ml in lane 6, 20 ng/ml in lanes 7 and 9, 50 ng/ml in lane 8) for 4 h after pretreatment with 10 μM MG (lane 7), 100 μM Ama (lane 8), or MG + Ama (lane 9) for 4 h or without pretreatment (lane 6). (**H**) Control plasmid (si neg) or a pool of pSilencer-si c-Cbl stably transfected (si c-Cbl pool) cells

were first treated with 200 ng/ml or 20 ng/ml TRAIL, respectively, for 4 h and then detached cells were removed by washing out with PBS. After removal of detached cells, fresh media were added onto the remaining attached cells and cell were incubated for the time indicated (0 h, 12 h, 24 h), and then treated a second time with TRAIL (200 ng/ml for si neg cells or 20 ng/ml for si c-Cbl cells) for 4 h. Cell survival was determined by trypan blue exclusion assay (upper panel) and cell lysates were subjected to immunoblotting for PARP or actin (lower panels). Con, untreated control cells. Error bars represent the S.E. from three separate experiments. (**I** and **J**) Control plasmid (si neg) or a pool of pSilencer-si c-Cbl stably transfected (si Cbl pool) cells were first treated with 200 ng/ml or 20 ng/ml TRAIL, respectively, for 4 h and then detached cells were removed by washing out with PBS. After removal of detached cells, fresh media were added onto the remaining attached cells and cells were incubated for the time indicated (0 h, 12 h, 24 h), and then cells were treated with biotin-X-NHS (0.5 mg/ml) following washing with PBS. After labeling with biotin, cell lysates were immunoprecipitated with anti-DR4 (**I**) or anti-DR5 (**J**) antibody. Membrane-bound DR4 or DR5 was detected with peroxidase-conjugated streptavidin. Total DR4 or DR5 was detected with anti-DR4 or anti-DR5 antibody, respectively (upper panels), and cell lysates were subjected to immunoblotting for c-Cbl or actin (lower panels).

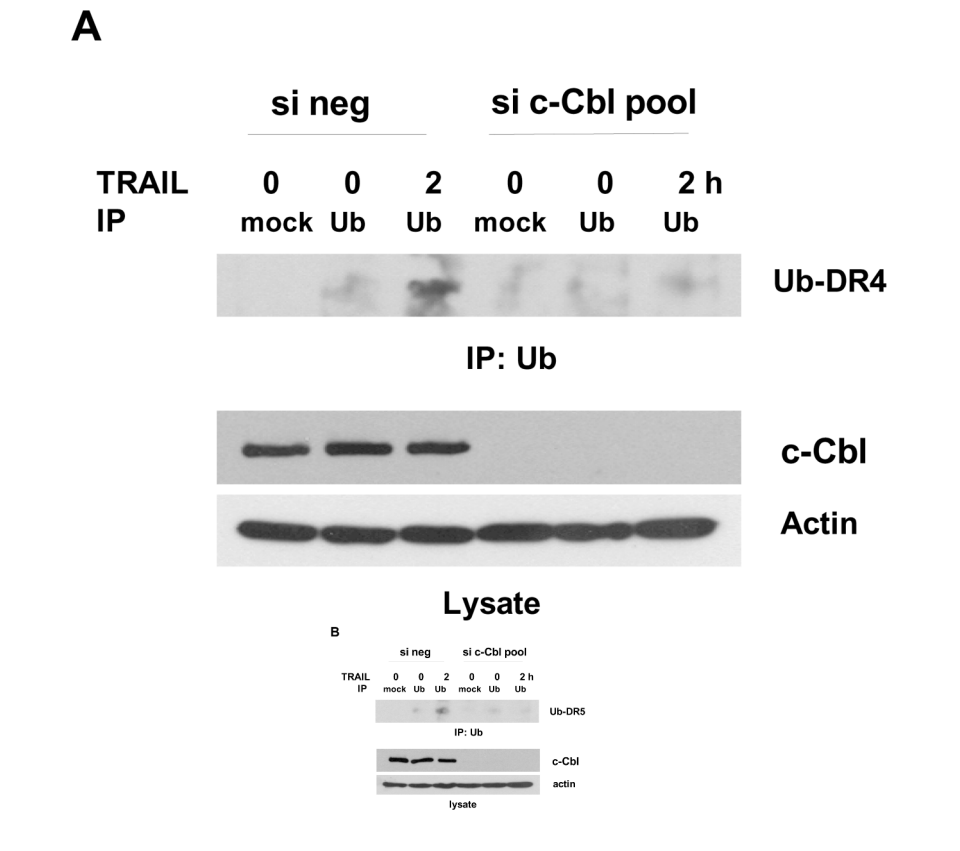
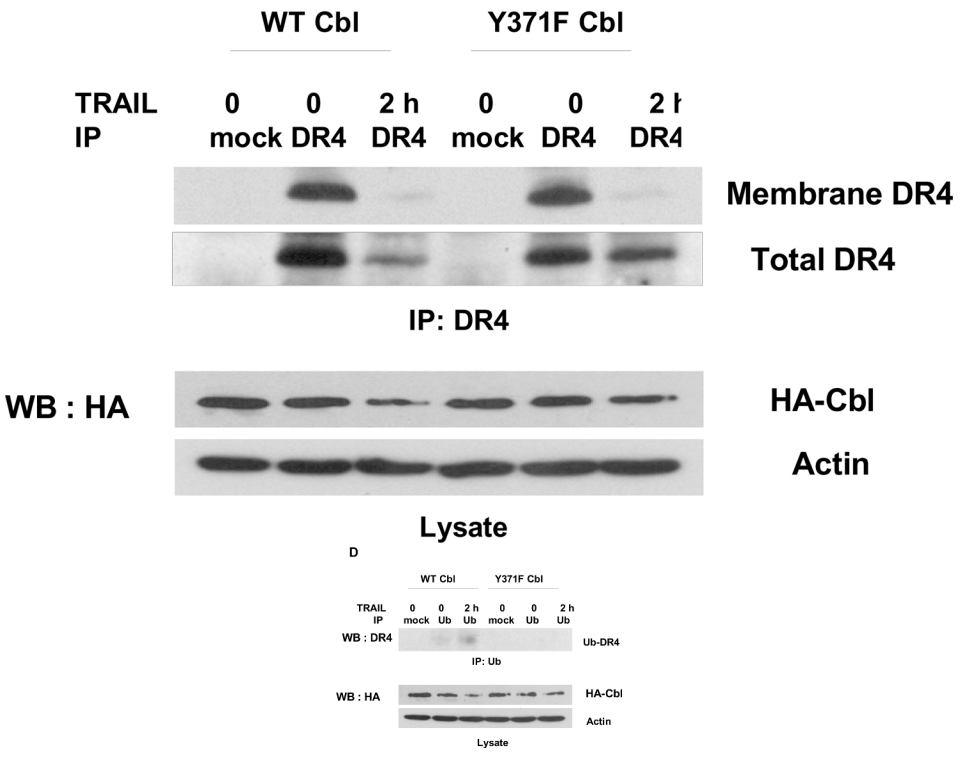


Fig. 6B



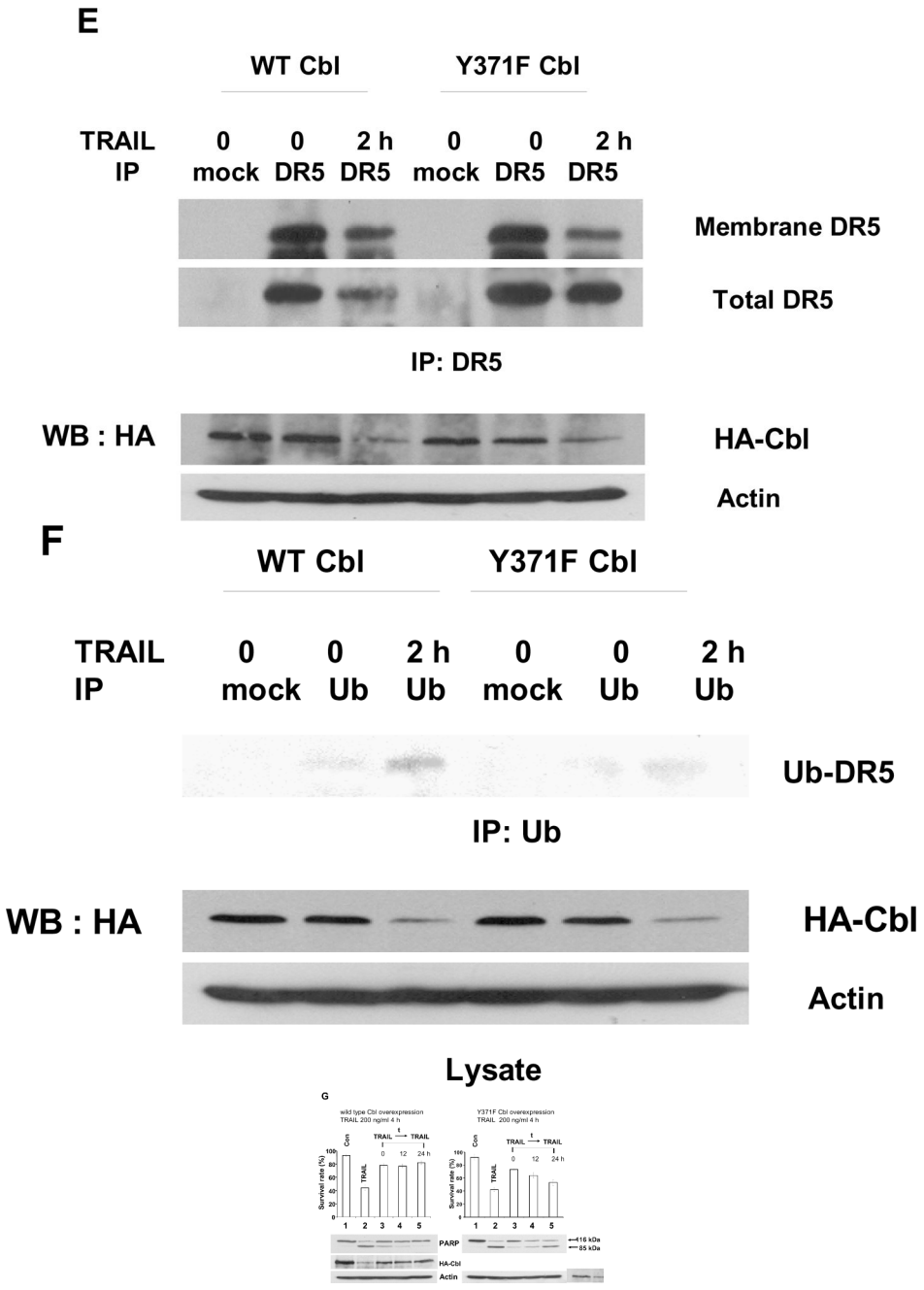


Figure 6. Biological function of c-Cbl as an E3 ligase for the ubiquitination of TRAIL receptors and the role of its ubiquitination activity in the development of acquired TRAIL resistance

(**A** and **B**) Control plasmid (si neg) or a pool of pSilencer-si c-Cbl stably transfected cells (si c-Cbl pool) were treated with TRAIL (200 ng/ml) for 2 h, and then cell lysates were immunoprecipitated with anti-Ub antibody and immunoblotted with anti-DR4 (**A**) or anti-DR5 (**B**) antibody (upper panels). Cell lysates were subjected to immunoblotting for c-Cbl or actin (lower panels). (**C** and **D**) Cells were transfected with pSRα neo-HA-Cbl (wild type) or mutant type (Y371F Cbl). **Upper panels:** After 48 h incubation, cells were treated with TRAIL (200 ng/ml) for 2 h, and then immunoprecipitated with anti-DR4 antibody after labeling with biotin (**C**) or immunoprecipitated with anti-Ub antibody (**D**). Membrane-bound DR4 was detected with peroxidase-conjugated streptavidin. Total DR4 was detected with anti-DR4 antibody.

Ubiquitinated DR4 was detected with anti-DR4 antibody. **Lower panels:** Cell lysates were subjected to immunoblotting for HA or actin. (**E** and **F**) Cells were transfected with pSRα neo-HA-Cbl (wild type) or mutant type (Y371F Cbl). **Upper panels:** After 48 h incubation, cells were treated with TRAIL (200 ng/ml) for 2 h, and then immunoprecipitated with anti-DR5 antibody after labeling with biotin (**E**) or immunoprecipitated with anti-Ub antibody (**F**). Membrane-bound DR5 was detected with peroxidase-conjugated streptavidin. Total DR5 was detected with anti-DR5 antibody. Ubiquitinated DR5 was detected with anti-DR5 antibody. **Lower panels:** Expression of wild-type or mutant-type HA-Cbl was detected with anti-HA antibody. (**G**) Cells were transfected with pSRα neo-HA-Cbl (wild type) or mutant type (Y371F Cbl). After 48 h incubation, cells were first treated with TRAIL (200 ng/ml) for 4 h, and then detached cells were removed by washing out with PBS. After removal of detached cells, fresh media were added onto the remaining attached cells and cells were incubated for the time indicated (0 h, 12 h, 24 h), and then treated a second time with TRAIL (200 ng/ml) for 4 h. Cell survival was determined by trypan blue exclusion assay (upper panel) and cell lysates were subjected to immunoblotting for PARP, HA-Cbl or actin (lower panels). Con, untreated control cells. Error bars represent the S.E. from three separate experiments.