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Cross-Resistance to Death Ligand-Induced Apoptosis in Cisplatin-Selected HeLa Cells Associated with Overexpression of DDB2 and Subsequent Induction of cFLIP

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Abbreviations: Cisplatin, *cis*-diamminedichloroplatinum (II); TNF, tumor necrosis factor; DISC, death-inducing signaling complex; DDB2, damaged DNA-binding protein 2; DAPI, 4', 6-diamidino-2-phenylindole; cFLIP, cellular FLICE like inhibitory protein

ABSTRACT

This work reports the involvement of DDB2, a component involved in the genomic repair of UV damage, in the cross-resistance of cisplatin-selected cell lines to death ligand-mediated apoptosis. The cisplatin-resistant cell line (HR3) exhibits enhanced expression of DDB2 and cross-resistance to UV-induced activation of apoptosis and caspases. This investigation further demonstrates that HR3 cells also exhibited cross-resistance to death ligands (Fas-inducing antibody and TNF-α). Depletion of the elevated DDB2 in HR3 cells sensitizes Fas-inducing antibody- and TNF-α-induced apoptosis. In contrast, the over-expression of DDB2 induces cFLIP expression and further attenuates death ligand-induced apoptosis. Moreover, RT-PCR and reporter assay indicated that DDB2 could increase both endogenous and exogenous cFLIP mRNA levels. Accordingly, the elimination of cFLIP by antisense oligonucleotides (ASO) suppresses DDB2 protection. These findings reveal that DDB2 regulates TNF signaling-mediated apoptosis via cFLIP and contributes to acquired cross-resistance. DDB2, while participating in DNA repair, functions as a negative regulator of apoptosis, and may therefore have a pivotal role in regulating immune response and cancer-therapeutic efficacy.

INTRODUCTION

Cisplatin is a widely used chemotherapeutic drug; nonetheless, the presence of resistance tumors usually limits the efficacy of cisplatin (Chao, 1996; Siddik, 2003). Accordingly, the cross-resistance of these tumors severely increases the difficulty of chemotherapy. Reduced apoptosis is one of the mechanisms by which cancer cells escape the cytotoxicity of cisplatin (Gonzalez et al., 2001). Although marked biochemical changes have been observed in cell lines that become resistant to cisplatin (Andrews et al., 1988; Chao et al., 1991b), the involvement of apoptotic signaling in the acquisition of cisplatin- and cross-resistance remains largely unknown.

The authors' earlier work revealed that a cisplatin-selected HeLa cell line (HR3) exhibited greater damaged-DNA binding activity and better DNA repair than the parental cells (Chao and Huang, 1993; Chao et al., 1991b) and showed cross-resistance to various chemotherapeutic drugs, Fas and UV (Table I; Chao et al., 1991a; Kamarajan et al., 2003). UV induces apoptosis by activating TNF signaling (Kulms and Schwarz, 2002). The trimerization of Fas, a member of TNF family, on the cell surface may cause the activated receptor to recruit the signaling molecules, FADD and caspase-8, forming the death-inducing signaling complex (DISC), and the following activation of caspases and apoptosis (Nagata, 1997). With regard to the regulation of TNF signaling, cFLIP has been identified as a blocker of TNF-induced apoptosis (Krammer, 2000). cFLIP prevents the recruitment and cleavage of caspase-8 at the DISC and subsequently suppresses apoptosis (Scaffidi et al., 1999).

Damaged DNA-binding protein (DDB) is a hetero-dimer that is composed of DDB1 and DDB2. The DDB1 protein was proposed to help to recognize DNA damage during UV-induced nucleotide excision repair (NER) (Chu, 1994; Dualan et al., 1995). However, various studies have shown that DDB1 is not crucial to this step

(Sancar, 1996). The detection of mutations in the DDB2 gene in a subset of XP-E cells that exhibit reduced or no DDB activity reveals that DDB2 may contribute to NER and the sensitivity of cells to genotoxic stress (Tang and Chu, 2002). Over-expression of DDB2 enhances NER in hamster cells (Sun et al., 2002b; Tang et al., 2000) and human cells (Fitch et al., 2003b; Wakasugi et al., 2002). *In vivo* binding studies have demonstrated that DDB2 activate the recruitment of XPC to cyclobutane pyrimidine dimers and may be the initial recognition factor in the NER pathway (Fitch et al., 2003a; Wakasugi et al., 2002). These findings reveal that DDB2 has a critical role in DNA repair. Furthermore, DDB2 has also been found to interact with the COP9 signalosome (Groisman et al., 2003), a comple x that exhibits ubiquitin ligase activity, suggesting that DDB2 may regulate the proteosomal pathway. For example, DDBs are involved in regulating HBx stability and HBx-mediated apoptosis associated with proteasome-mediated degradation (Bergametti et al., 2002; Bontron et al., 2002). DDB2 is degraded quickly following UV irradiation via the ubiquitin-mediated proteasome (Fitch et al., 2003b; Rapic-Otrin et al., 2002). Therefore, the regulation of DDB2 by the proteasome may be the rate-limiting step in NER. DDBs also exhibit a transcriptional function (Hayes et al., 1998; Shiyanov et al., 1999). DDBs associate with the C-terminal activation domain of E2F1 and cooperate with E2F1 to stimulate the transcription of an E2F1 responsive reporter (Hayes et al., 1998). E2F1, has been implicated in inducing apoptosis as well as having a well-established proliferative effect of all members of the E2F family (Phillips et al., 1999).

The depletion of DDB2 from HR3 cells restored the lines' sensitivities to UV-induced apoptosis to levels similar to those of sensitive cells (Sun et al., 2002a). Over-expression of DDB2 attenuated UV-induced apoptosis in DDB2-depleted HR18 cells (Sun et al., 2002a). More interestingly, the over-expression of 82TO, a DDB2 mutant

that does not significantly enhance DDB activity, also protected HR18 cells against UV (Sun et al., 2002a). These results reveal that DDB2 may protect cells via an undefined mechanism as well as by promoting DNA repair. This study investigates the cross-resistance of cell surface receptor death signaling and the possible involvement of DDB2 in this pathway. The results of this study have proven that (i) cisplatin-selected HeLa cells also acquired cross-resistance to TNF signaling (Fas and TNF)-mediated apoptosis, and (ii) DDB2 inhibition overcomes TNF signaling-mediated apoptotic resistance via cFLIP.

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Materials and Methods

Cell lines and culture. Human cervix carcinoma HeLa cells, cisplatin-selected HeLa cell lines (HR3) (Chao et al., 1991b), HR3 stably transfected with DDB2 antisense cDNA cells (HR18) (Sun et al., 2002a), HEK293, F9, V79 and MCF7 cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD) supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco). The cell lines were maintained at 37^{o}C in a humidified atmosphere of 5% CO_2 and 95% air. Low concentration of cisplatin (1 μM) (Farmitalia, Milano, Italy) was added to the medium to maintain the resistant phenotype. Before any experiments, resistant cells intended as source materials were cultivated in media without cisplatin for three weeks.

Western blot analysis. Cells (2×10^6) were treated without or with Fas-inducing antibody (activating) (clone CH11, Upstate Biotech., Lake placid, NY) or human recombinant TNF-α (Pharmingen, San Diego, CA) together with 1 μg/ml of cycloheximide (Fulda et al., 2000) for 24h at 37 ^{o}C. The treated cells were washed with phosphate-buffered saline (PBS) and lysed in RIPA lysis buffer (Tris-HCl: 50 mM, pH 7.4, NP- 40: 1%, sodium deoxycholate: 0.25%, NaCl: 150 mM, EGTA: 1 mM, PMSF: 1 mM, protease inhibitor cocktail (Roche, Mannheim, Germany), Na_3VO_4: 1 mM and NaF: 1 mM) on ice for 30 min. Insoluble material was removed by centrifugation at 14000 rpm for 10 min at 4^{o}C. Protein concentrations were measured using a Bio-Rad protein assay kit (Bio-Rad Lab., Hercules, CA). Proteins were separated by 12% SDS-PAGE, transferred onto PVDF membranes and incubated with antibodies reactive to caspase-8 (Cell Signaling Tech. Inc, Beverly, MA) caspase-7, caspase-3, PARP, DFF, cFLIP, β-actin (Santa Cruz Biotech., Santa

Cruz, CA), DDB1, or DDB2. Antibodies to DDB1 and to DDB2 were generated in New Zealand white rabbits using conventional procedure as previously described (Chao et al., 1991a). The antigen-antibody complexes were visualized by standard enhanced chemiluminescence reaction (Pierce, Rockford, IL).

Analysis of clonogenicity and apoptosis. For clonogenic survival, cells were seeded in 60 mm dishes and treated with various concentrations of anti-Fas antibody or human recombinant TNF-α together with 1 μg/ml of cycloheximide. After 14 days of incubation at 37°C, plates were stained with a crystal violet, and colonies with more than 50 cells were scored. For assessment of apoptosis, cells growing in six well plates were either left untreated or treated with Fas-inducing antibody or TNF-α together with 1 μg/ml of cycloheximide for 24 h at 37°C. The cells were fixed with methanol and incubated with DAPI (4-diamidino-2-phenylindole) (Sigma) solution for 30 min in darkness. Floating cells from each well were also fixed and returned to the respective wells. All cells were analyzed using an Olympus microscope at 420 nm. Apoptotic cells exhibiting morphologic features of apoptosis, including chromatin condensation and nuclear fragmentation (Sun et al., 2002a), were counted in 6-8 randomly selected fields. Approximately 500 nuclei were examined for each sample, and the results were expressed as the number of apoptotic nuclei over the total number of nuclei counted.

Generation and infection of recombinant adenoviruses. Replication-deficient recombinant adenoviruses containing DDB2 or β-Gal were generated as described previously (Sun et al., 2002a). Cells were infected with adenoviruses at 3000 MOI for 36 h or indicated time before treatment.

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Reverse trascription-polymerase chain reaction (RT-PCR). Total cellular RNA was prepared from cells with an RNeasy mini kit (Qiagen, Valencia, CA, USA). First-strand cDNA was synthesized from 1 μg of total RNA using oligo-dT primer and Omniscript reverse transcriptase (Qiagen). The primers for human cFLIP RT-PCRs were (forward primer) 5'-GCTGAAGTCATCCATCAGGT-3' and (reverse primer) 5'-CATACTGAGATGCAAGAATT-3'. The primers for glyceraldehydes-3-phophate dehydrogenase (GAPDH) RT-PCRs were (forward primer) 5'-TGGTATCGTGGAAGGACTCATGAC-3' and (reverse primer) 5' TGCCAGTGAGCTTCCCGTTCAGC-3'. PCR was performed for 32 cycles at 95^{o}C for 45 sec, 58^{o}C for 45 sec, and 72^{o}C for 1 min (Perkin-Elmer 9600, Normalk, CT, USA). The RT-PCR products were resolved on 1.5% agarose gel and stained with ethidium bromide. After electrophoresis, reaction products were visualized on a UV transilluminator and photographed.

Real-Time PCR. Real-time PCR was performed on HeLa, HR3, HR18 and HR18 infected with DDB2 or β-Gal expressing adenoviruses (at 3000 MOI for 60 h). Ten μg of total RNA, extracted with TRIzol reagent (Invitrogen), was treated with DNase and converted to cDNA using oligo-dT primers with the SuperScript First-Strand Synthesis System (Invitrogen). An ABI Prism 7700 and Sequence Detection System software was used for real-time PCR and primer design, respectively (PerkinElmer Life and Analytical Sciences, Boston, MA). Triplicate PCR amplifications of 10 ng of the cDNA were performed using the Taqman Master Mix provided by PerkinElmer. Fold change in RNA abundance was calculated using the standard curve method for quantification (ABI Prism 7700 SDS User Bulletin 2 P/N 4303859 Revision A). The

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GenBank sequence numbers (u97074, U18300, NM_000996 [GenBank]) were used for the cFLIP, DDB2 and Ribosomal protein L35a primer design. The primers are as follows: cFLIP, forward, 5'- GTGGAGACCCACCTGCTCA -3'; reverse, 5'- GGACACATCAGATTTATCCAAATCC -3'; DDB2, forward, 5'- CCCTGAACCCATGCTGTGAT -3'; reverse, 5'- TCGGGACTGAAACAAGCTGC -3'; RL35A, forward, 5'- GCTGTGGTCCAAGGCCATTTT -3'; reverse, 5'- CCGAGTTACTTTTCCCCAGATGAC -3'

Inhibition assay with antisense oligonucleotides (ASO). For antisense experiments, phosphothioated cFLIP antisense oligonucleotide (ASO) (ACTTGTCCCTGCTCCTTGAA) or control phosphothioated oligonucleotide (GGATGGTCCCCCCTCCACCAGGAGA) [synthesized by PAN Facility, Stanford University] was delivered into cells by lipofection (Invitrogen) at a final concentration of 600 nM. After 4 h, medium was removed and replaced with the appropriate cell growth medium containing the indicated concentration of oligonucleotide. Down-regulation of the relative protein level was evaluated by Western blotting after 24 h. For experiments requiring additional DDB2 or β-Gal infection, cells were first transfected with cFLIP ASO for 4 h and replaced with the appropriate cell growth medium containing oligonucleotide and respective viruses for 36 h, and then cells were stimulated with the appropriate doses of anti-Fas antibody for 24 h.

Luciferase assay. Cells were co-transfected with total 3 μg of plasmid DNA containing 1 μg pFP-1, with a potential cFLIP promoter region (flanking from -920 to +43 of cFLIP exon1 start site; accession number AF238465, a kind gift from Dr. B.M. Evers, The University of Texas Medical Branch at Galveston, Galveston, TX),

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together with the indicated amount of pcDNA3, pcDNA3-DDB2 or pcDNA3-HMG1 by lipofection (Invitrogen). After 24 h or indicated time, cells were lysed and the luciferase activities of the lysates were measured (Promega) with a β-scintillation counter.

Results

Over-expression of DDB2 in cisplatin-selected cells associated with cross-resistance to TNF signaling-mediated apoptosis. The amount of DDB2 in resistant HR3 cells was at least twice that in sensitive HeLa cells (Fig. 1A, top panel). A typical resulting cell line, HR18, was generated by stably transfecting HR3 cells using DDB2 antisense, to examine the role of DDB2 in apoptosis resistance (Sun et al., 2002a). The DDB2 level of HR18 was reduced and almost undetectable (Fig. 1A, top panel). Although both DDB1 and DDB2 are required to recognize UV-induced DNA damage (Hwang et al., 1998), the levels of DDB1 were equally high in both sensitive and resistant HeLa cell lines (Fig. 1A, bottom panel).

Fas-inducing antibody or TNF-α-mediated apoptotic signalings in HeLa, HR3 and HR18 cell lines were studied to examine the cross-resistance of cell surface receptor death signaling. Anti-Fas antibody or TNF-α induced apoptosis in a dose-dependent manner in both HeLa and resistant HR3 cells (Figs. 1B and C), but this phenomenon was less evident in cisplatin-resistant HR3 cells. The depletion of DDB2 from HR3 cells (HR18 cells) promoted apoptosis induced by either death receptor in a manner similar to the depletion of HeLa cells (Fig. 1B and C). Furthermore, the activation of caspase cascade was examined in these cell lines. Fas-inducing antibody or TNF-α activated caspase-8, -7 and -3 and induced the cleavage of DFF in a dose-dependent manner, in both HeLa and HR3 cells (Fig. 2A and B). However, stimulation-induced activation of caspases and DFF cleavage were reduced in HR3 cells. In contrast, HR18 exhibited more severe Fas-inducing antibody or TNF-α-mediated activation of caspase-8, -7 and -3 and DFF cleavage than did HR3 cells (Figs. 2A and B). These results show that cisplatin-resistant cells thus also exhibited

cross-resistance to cell surface receptor-mediated apoptosis and that the reduction of sensitivity was reversed by depletion of DDB2.

Over-expressing DDB2 suppresses TNF signaling-mediated apoptosis. DDB2 was over-expressed in HR18 cells to confirm these findings. The adenovirus-mediated over-expression of DDB2 in HR18 cells protected cells against Fas-inducing antibody- or TNF-α-induced caspase-3 activation, DFF cleavage (Fig. 3A) and apoptosis (Figs. 3B and C). These effects followed from DDB2 expression rather than viral infection, since they were not detected in HR18 cells infected with viruses that carry a β-Gal gene. Additionally, colony-forming assay demonstrated that the survival of HR18 cells against surface death factors was suppressed in a dose-dependent manner, and the over-expression of DDB2, but not β-Gal in HR18 cells blocked Fas-inducing antibody- and TNF-α-mediated cytotoxicity (Figs. 4A and B).

DDB2 promotes cFLIP expression. Next, the levels of anti-apoptotic factors in the resistant cells were investigated. The expression of cFLIP, but not of IAP1, was found to be stronger in cisplatin-resistant HR3 cells than in sensitive HeLa cells (Fig. 5A). More surprisingly, the depletion of DDB2 suppressed the expression of cFLIP (Fig. 5A, compare HR3 and HR18 cells). The ecotopic expression of DDB2 in DDB2-depleted HR18 cells was investigated to evaluate whether cFLIP accumulation in HR3 cells was caused by DDB2, whose level is also increased in the cells. Over-expression of DDB2 time-dependently increased the expression of cFLIP in HR18 cells (Fig. 5B). Adenoviruses did not affect cFLIP expression, as evidenced by the fact that a control β-Gal virus did not induce cFLIP expression. The induction of cFLIP by the over-expression of DDB2 was also observed in HeLa cells within 36 h after the

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expression of the virus infection, although less strongly than in HR18 (data not shown), probably because HeLa cells contain more endogenous DDB2. Therefore, the levels of cFLIP seemed to be directly governed by DDB2.

Inhibiting cFLIP eliminates the protection of DDB2 in TNF signaling-mediated apoptosis. Whether DDB2 protection against TNF signaling-mediated apoptosis is mediated by cFLIP was studied by cFLIP inhibition. cFLIP expression was strongly inhibited by cFLIP antisense oligonucleotides (cFLIP ASO) in HR18 cells (Fig. 6A, top panel). As shown in Fig. 6A (bottom panel), the inhibition of cFLIP markedly sensitized HR18 cells to Fas-mediated apoptosis. Moreover, cFLIP inhibition eliminates DDB2 protection against Fas-mediated apoptosis (Fig. 6A, bottom panel). These findings clearly demonstrated that DDB2 protection against TNF signaling-mediated apoptosis is mediated by cFLIP. The protective effect of DDB2 was also observed in other cell lines, F9, V79 and MCF-7 cells (Fig. 6B), that express little DDB2 (Sun et al., 2002b). Hence, the over-expression of DDB2 markedly protected cells against Fas-induced apoptosis. Protection against Fas-induced apoptosis by the over-expression of DDB2 in cells may be a general effect and is not limited to HeLa cells.

DDB2 enhances the expression level of cFLIP mRNA. How DDB2 regulates cFLIP protein level is of interest. DDB2 is a transcriptional partner of E2F1 (Hayes et al., 1998; Shiyanov et al., 1999). Additionally, microarray analysis has suggested that cFLIP may be one of the E2F1-regulated genes (Stanelle et al., 2002). The cFLIP mRNA levels in HeLa, HR3 and HR18 cells were determined by RT-PCR to assess the relationship between DDB2 and cFLIP. The cFLIP mRNA level was higher in

cisplatin-resistant HR3 than in HeLa cells, and depletion of DDB2 decreased the mRNA level of cFLIP in HR18 cells (Fig. 7A, top panel). Moreover, the over-expression of DDB2 increased cFLIP mRNA expression in HR18 cells (Fig. 7A, bottom panel). These results indicate that DDB2 may regulate the mRNA transcription of cFLIP. To test this possibility, activation of cFLIP promoter activity by DDB2 was investigated. DDB2 enhanced cFLIP promoter activity in a dose- and time-dependent manner (Figs. 7B and C, respectively). In contrast, HMG1, a nucleosomal protein, did not modulate cFLIP promoter activity (Fig. 7D). These findings demonstrated that DDB2 promotes the expression of cFLIP in both protein and mRNA levels.

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Discussion

In a previous study, we found that the cisplatin-resistant cells (HR3) exhibit cross-resistance to UV by a mechanism involving DDB2 (Sun et al., 2002a). However, the detailed mechanism remains unknown. In the present study, we have demonstrated that the HR3 cells also displayed cross-resistance to death ligands in a DDB2/cFLIP-dependent manner, by which DDB2 might protect cells against UV. Additionally, a transcriptional regulation of cFLIP by DDB2 was shown and proposed (Fig. 8), suggesting DDB2 as a potential therapeutic target. This report established that DDB2, a DNA repair protein, promotes the expression of cFLIP and the further inhibition of apoptosis. It also demonstrated that the depletion of DDB2 reverses the cross-resistance. This protective effect is initially observed in a resistant HeLa cell model. A similar protective effect was also observed in other cell lines with lower DDB2 levels, indicating that protective DDB2 in apoptosis may be evolutionally regulated. This finding explains the cross-resistance of the cisplatin-resistant HeLa cells to UV- and TNF receptor-mediated apoptosis (Chao et al., 1991a; Kamarajan et al., 2003). The authors recently showed that the resistant cells can be resensitized to UV by reducing their DDB2 level (Sun et al., 2002a). UV-triggered apoptosis occurs primarily via cell surface pathways (Kulms et al., 1999): a simple explanation is that the level of cFLIP originally adopted to suppress apoptosis is reduced by the reduction of DDB2. Accordingly, apoptotic signals such as TNFR pathway, which is inhibited by cFLIP (Krammer, 2000), are potentially regulated by the DDB2 level. Works on the over-expression of DDB2 further support the involvement of DDB2 in cell surface-mediated death signals and apoptosis. The over-expression of 82TO, a DDB2 mutant that does not significantly enhance DDB activity (Nichols, 2000), also protected HeLa cells from Fas-inducing antibody-induced cell death (data not shown),

suggesting that the protection effect of DDB2 maybe independent of its DNA repair activity. However, the over-expression or inhibition of DDB2 only slightly affected cisplatin-induced caspase-8 signaling or apoptosis (data not shown), probably because cisplatin induces primarily the mitochondrial apoptotic-signaling (Gonzalez et al., 2001). The role of DDB2 in cell surface receptor-mediated death signaling is thus unique.

Although the involvement of DDBs in the regulation of interferon-inducible RNA-dependent protein kinase promoter has been documented (Das et al., 2004), the mechanism of regulation by DDB2 of transcription, which underlies its protective effect against apoptosis, remains largely unknown. DDB2 is a transcriptional partner of E2F1; however, the target of DDBs/E2F1 has not been identified (Hayes et al., 1998; Shiyanov et al., 1999). In this study, the over-expression of DDB2 increases the expression of cFLIP in both mRNA and protein levels. Furthermore, E2F1 is shown to regulate the expression of cFLIP (Stanelle et al., 2002). Therefore, cFLIP is suggested to be the first potential target of DDB2/E2F1. E2F1 promotes TNF-α induced apoptosis by stabilizing TRAF2 protein (Phillips et al., 1999). However, the possibility that DDB2/E2F1 may co-activate cFLIP expression sheds light on the dual role of E2F1 in regulating cell survival and death. p53 transactivates DDB2 in humans, but not in mice, in response to UV (Tan and Chu, 2002), so the knockout of DDB2-rendered MEF cells' resistance to UV (Itoh et al., 2004) is likely to follow from the differential regulation of DDB2/cFLIP between humans and mice. DDB1 and DDB2 have been shown to complex with COP9 signalsome, which regulate the stability of nuclear protein by ubiquination and the proteosome pathway (Groisman et al., 2003). Although DDB2 promoted the expression of cFLIP protein through transcription, the possibility that DDB2 may modulate cFLIP stability cannot be ruled out.

p53 activates proapoptotic genes to promote p53-dependent apoptosis (Fridman and Lowe, 2003). p53 transactivates DDB2 (Hwang et al., 1999), indicating that p53 has an alternative role in regulating apoptosis. DDB2 transactivates cFLIP, enhancing its apoptosis-inhibitory function, as depicted in Fig. 8. Most cancer cells exhibit attenuated p53 activity, allowing them to escape apoptosis during cancer therapy. Although p53 activity is almost entirely attenuated by HPV-encoded antigens, most HeLa cells are vulnerable to being killed by appropriate concentrations of cisplatin, while some cells may escape p53-dependent apoptosis. These escaped cells potentially exhibit the over-expression of DDB2. Thus, the variation of the protective effect of DDB2 in the induction of apoptosis between cell lines depends on the status of p53 in the cell, and the apoptotic pathway induced by the death stimuli. Cisplatin induce a minor p53 signaling and the following apoptosis in HeLa cells (Wesierska-Gadek et al., 2002; Chao et al.'s unpublished observation). Therefore, the enhanced expression of DDB2 in cisplatin resistant cells is due to activated p53. These results represent a molecular explanation of why cancer cells that receive long-term cytotoxic treatment become resistant to cytotoxic drugs and possibly also to TNF death factors. This investigation may also suggest that DDB2 is a target in chemotherapy based on cell surface death factors.

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Figure Legends

Figure 1. Sensitization of TNF-receptor-mediated apoptosis by depleting DDB2 expression. A, Abundance of DDB2 in cisplatin-resistant cells. Whole cell extracts of HeLa (H), HR3, and HR18 cells were subjected to immunoblot analysis with antibodies to DDB2 or DDB1. Blots were reprobed with anti-β-actin to demonstrate equal protein loading. B and C, Sensitization of apoptosis by inhibiting DDB2. Cells were either left untreated or treated with indicated concentrations of anti-Fas antibody (B) or TNF-α (C) for 24 h. The percentage of apoptosis was evaluated by DAPI staining and fluorescence microscopy. Approximately 500 nuclei were examined for each sample. The plotted values represent the mean ± S.D. obtained from three independent experiments. $^{**}P<0.01$ and $^{***}P<0.001$.

Figure 2. Promotion of TNF-receptor-mediated caspase signaling by depleting DDB2 expression. HeLa (H), HR3 and HR18 cells were either left untreated or treated with indicated concentrations of anti-Fas antibody (A) or TNF-α (B) for 24 h. Immunoblot analysis of the lysates was performed using anti-caspase-8, anti-caspase-7, anti-caspase-3, anti-DFF or anti-β-actin as described in Materials and Methods.

Figure 3. Inhibition of TNF-receptor-mediated caspase signaling and apoptosis by adenovirus-mediated overexpression of DDB2. A, Overexpression of DDB2 attenuates anti-Fas antibody- and TNF-α-induced caspase-3 and DFF cleavage. Cells were either uninfected or infected for 36 h with β-Gal or DDB2 recombinant viruses and left untreated or treated with anti-Fas antibody (30 ng/ml) or TNF-α (50 ng/ml) for 24 h. Immunoblot analysis of the lysates was performed using anti-caspase-3, anti-DFF, anti-DDB2 or anti-β-actin as described in Materials and Methods. B and C,

Overexpression of DDB2 protects cells against anti-Fas antibody- and TNF-α-induced apoptosis. Cells were either left uninfected or infected with β-Gal or DDB2 recombinant virus for 36 h and left untreated or treated with indicated concentrations of anti-Fas antibody (B) or TNF-α (C) for 24 h. The percentage of apoptosis was evaluated by DAPI staining and fluorescence microscopy. The plotted values represent the mean ± S.D. obtained from three independent experiments. $^{**}P<0.01$.

Figure 4. Enhancement of cell survival by adenovirus-mediated overexpression of DDB2. Cells were either uninfected or infected for 36 h with β-Gal or DDB2 recombinant viruses, and untreated or treated with indicated concentrations of anti-Fas antibody (A) or TNF-α (B) for 12 h. After 14 days of incubation at 37^{o}C, plates were stained with crystal violet, and colonies of over 50 cells were scored. Data are expressed as percentage survival relative to the survival of the untreated cells, and are mean ± S.D. determined over three independent experiments. P values denote the significant difference between β-Gal- and DDB2- infected cells. $^{**}P<0.01$ and $^{***}P<0.001$.

Figure 5. Regulation of cFLIP level by DDB2. A, Overexpression of cFLIP in cisplatin- resistant cells (HR3). Whole cell extracts of HeLa (H), HR3 and HR18 cells were subjected to immunoblot analysis with antibodies to cFLIP, IAP1, DDB2 or β-actin. B, DDB2 enhances the cFLIP expression. Whole cell extracts of HR18 cells either uninfected (C) or infected with DDB2 or β-Gal recombinant viruses for indicated times were subjected to immunoblot analysis with antibodies to cFLIP, DDB2 or β-actin.

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Figure 6. Elimination of the DDB2 protection against TNF-receptor-mediated apoptosis by inhibiting cFLIP. A, cFLIP inhibition eliminates DDB2 protection in TNF-receptor-mediated apoptosis. (Top panel), HR18 cells were either untransfected (C) or transfected with CO ASO (600 nM) or cFLIP ASO (600 nM), as described in the experimental procedures. Whole cell extracts were subjected to immunoblot analysis with antibody to cFLIP or β-actin. (Bottom panel), cells were either untransfected or transfected with CO ASO (600 nM) or cFLIP ASO (600 nM), as described under experimental procedures, and infected for 36 h with DDB2 recombinant virus; they were then treated with indicated concentrations of anti-Fas antibody for 24 h. The percentage of apoptosis was evaluated by DAPI staining and fluorescence microscopy. The plotted values represent the mean ± S.D. obtained in three independent experiments. **P*<0.05 and ***P*<0.01. B, Overexpression of DDB2 protects cells against anti-Fas antibody-induced apoptosis. Cells were either left uninfected or infected with β-Gal or DDB2 recombinant virus for 36 h, and left untreated or treated with indicated concentrations of anti-Fas antibody for 24 h. The percentage of apoptosis was evaluated by DAPI staining and fluorescence microscopy. The plotted values represent the mean ± S.D. obtained from three independent experiments. **P*<0.05, ***P*<0.01 and ****P*<0.001.

Figure 7. DDB2 enhances the cFLIP mRNA expression. A, Overexpression of cFLIP mRNA in cisplatin-resistant cells (HR3). Total RNA was isolated from HeLa (H) HR3 and HR18 cells and RT-PCR and Real-Time PCR analysis of cFLIP, GAPDH and DDB2 were performed as described in Materials and Methods. GAPDH served as the control PCR reaction. The plotted values represent the mean ± S.D. obtained from three independent experiments. ***P*<0.01. B-D, DDB2 up-regulates

cFLIP promoter activity. HEK293 cells were co-transfected with pFP-1 (1 μg) together with indicated amount (μg) of empty vector (pcDNA3) or DDB2-expressing vector (pcDNA3-DDB2) (B), or 2 μg of pcDNA3, pcDNA3-DDB2 or pcDNA3-HMG1 (C and D) by lipofection. After 24 h (B and D) or indicated time (C), cells were lysed and the luciferase activity was measured. The plotted values represent the mean ± S.D. obtained from three independent experiments. ***P<0.001.

Figure 8. A schematic representation for the protection effect of DDB2 against death ligand-induced apoptosis through cFLIP. DDB2 transactivates cFLIP, enhancing their apoptosis-inhibitory function (this study). The death receptors-induced apoptosis is attenuated by the up-regulated cFLIP and, consequently, activation of initiator caspases (3 and 7), cleavage of cellular substrates, PARP and DFF, and apoptosis are inhibited. DL, death ligands; DR, death receptors.

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Table I. Cross-resistance to drugs in HeLa and HR3 cells

	IC_{50} (M)[a]		Fold
	HeLa	HR3	resistance
Cisplatin	$(5.5 \pm 0.4) \times 10^{-8}$	$(9.9 \pm 1) \times 10^{-7}$	18
Mitomycin C	$(9 \pm 1) \times 10^{-8}$	$(9 \pm 0.8) \times 10^{-7}$	10
EMS	$(8 \pm 0.9) \times 10^{-3}$	$(2.4 \pm 0.3) \times 10^{-2}$	5
Colchicine	$(3 \pm 0.2) \times 10^{-8}$	$(4 \pm 0.7) \times 10^{-8}$	1.3
Adriamycin	$(1.5 \pm 0.2) \times 10^{-6}$	$(1.5 \pm 0.2) \times 10^{-6}$	1

[a] The data were expressed as $IC_{50} \pm$ SE (n=5); values were calculated from the cytotoxicity curves.

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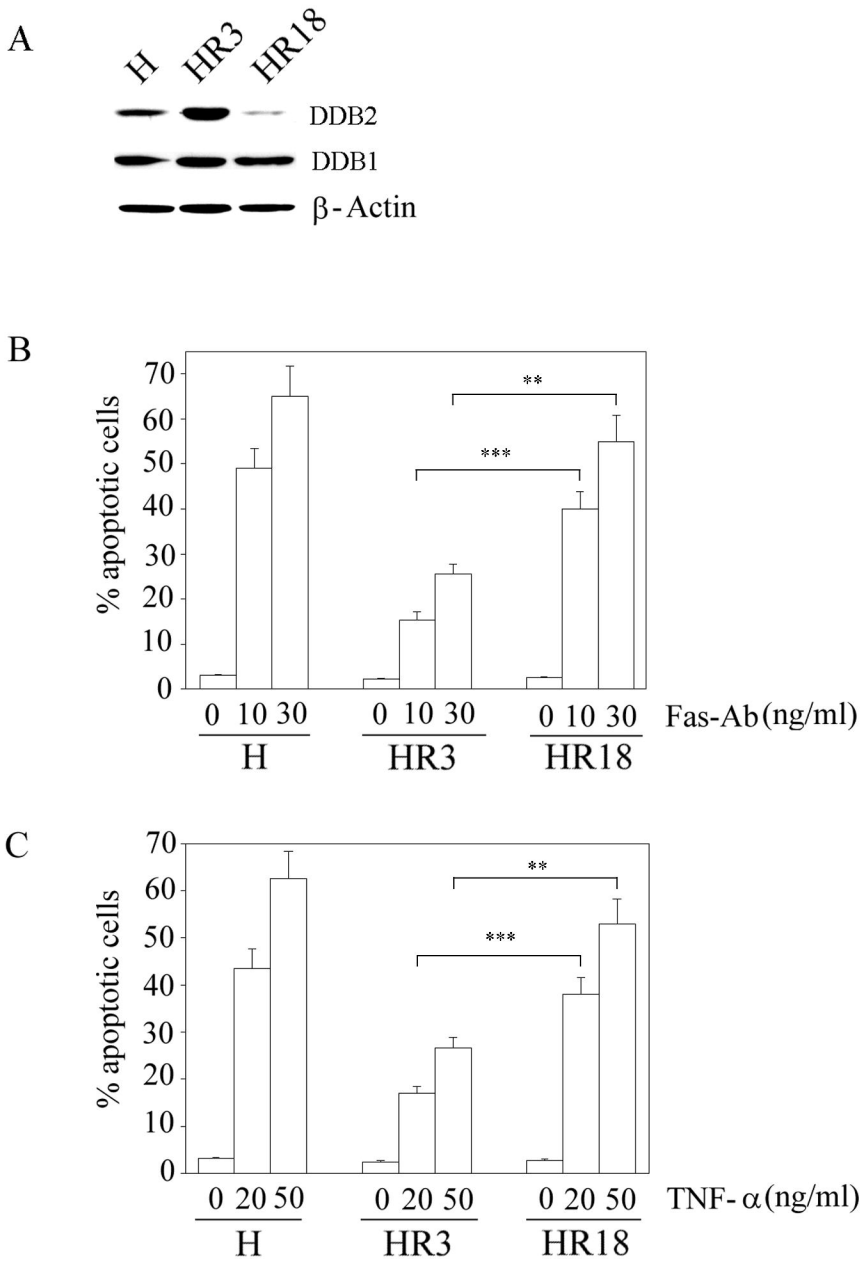

Fig. 1

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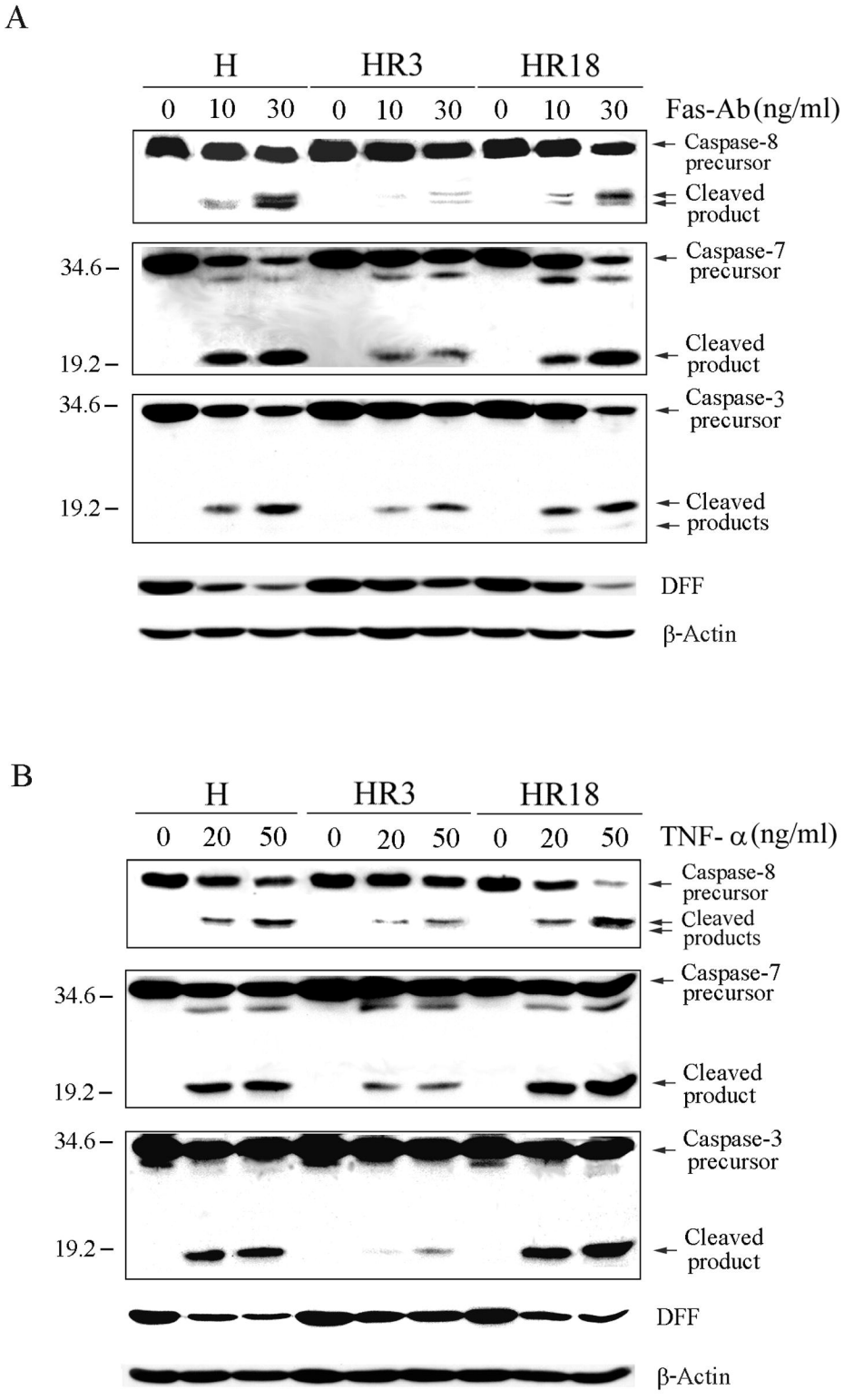

Fig. 2

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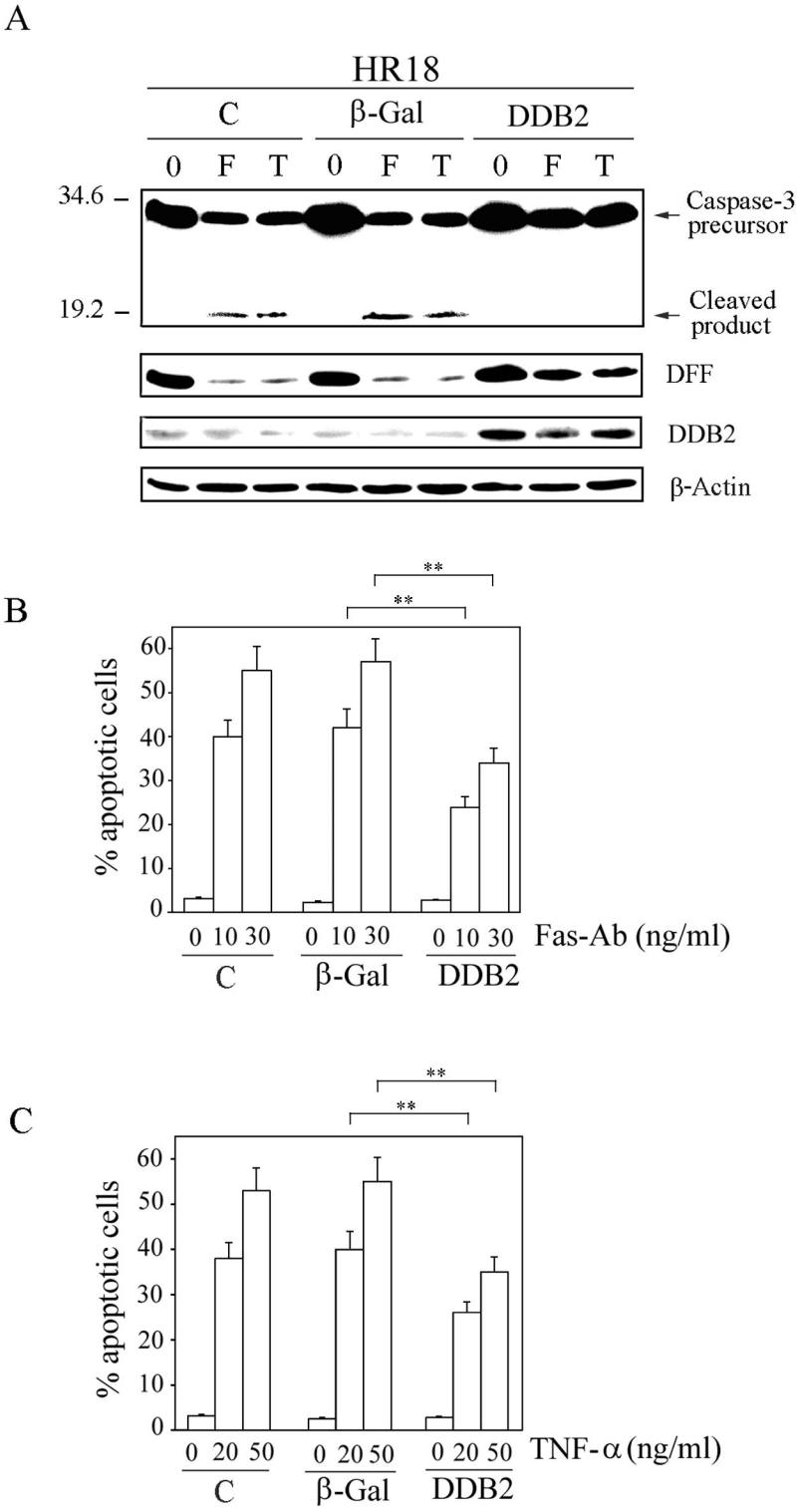

Fig. 3

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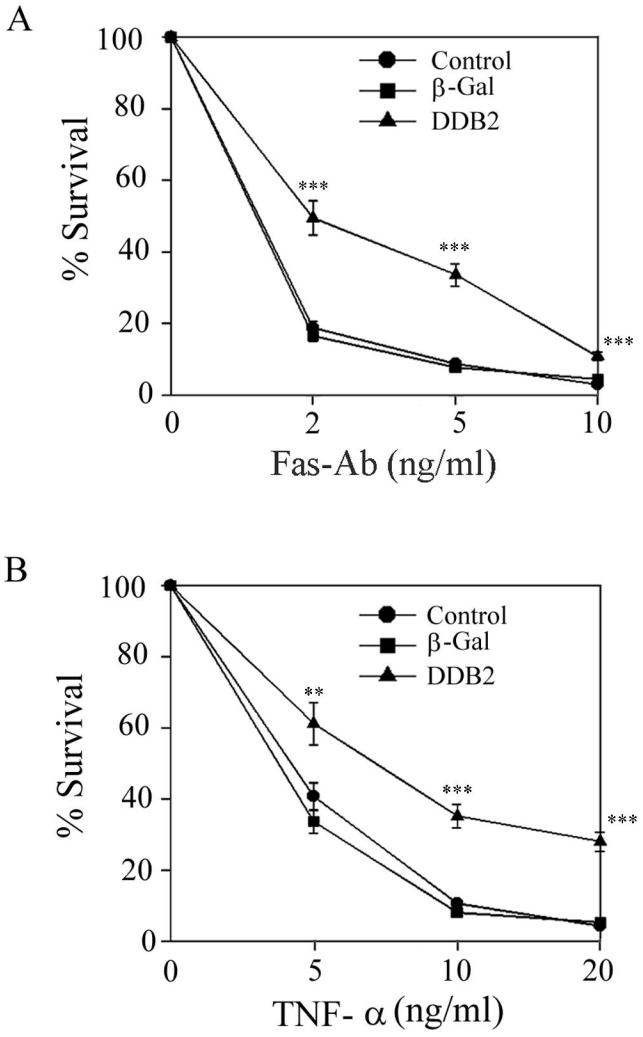

Fig. 4

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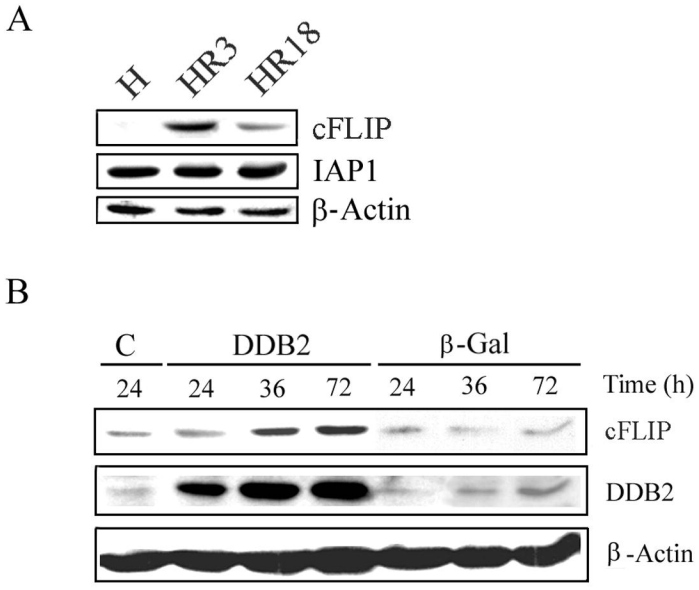

Fig. 5

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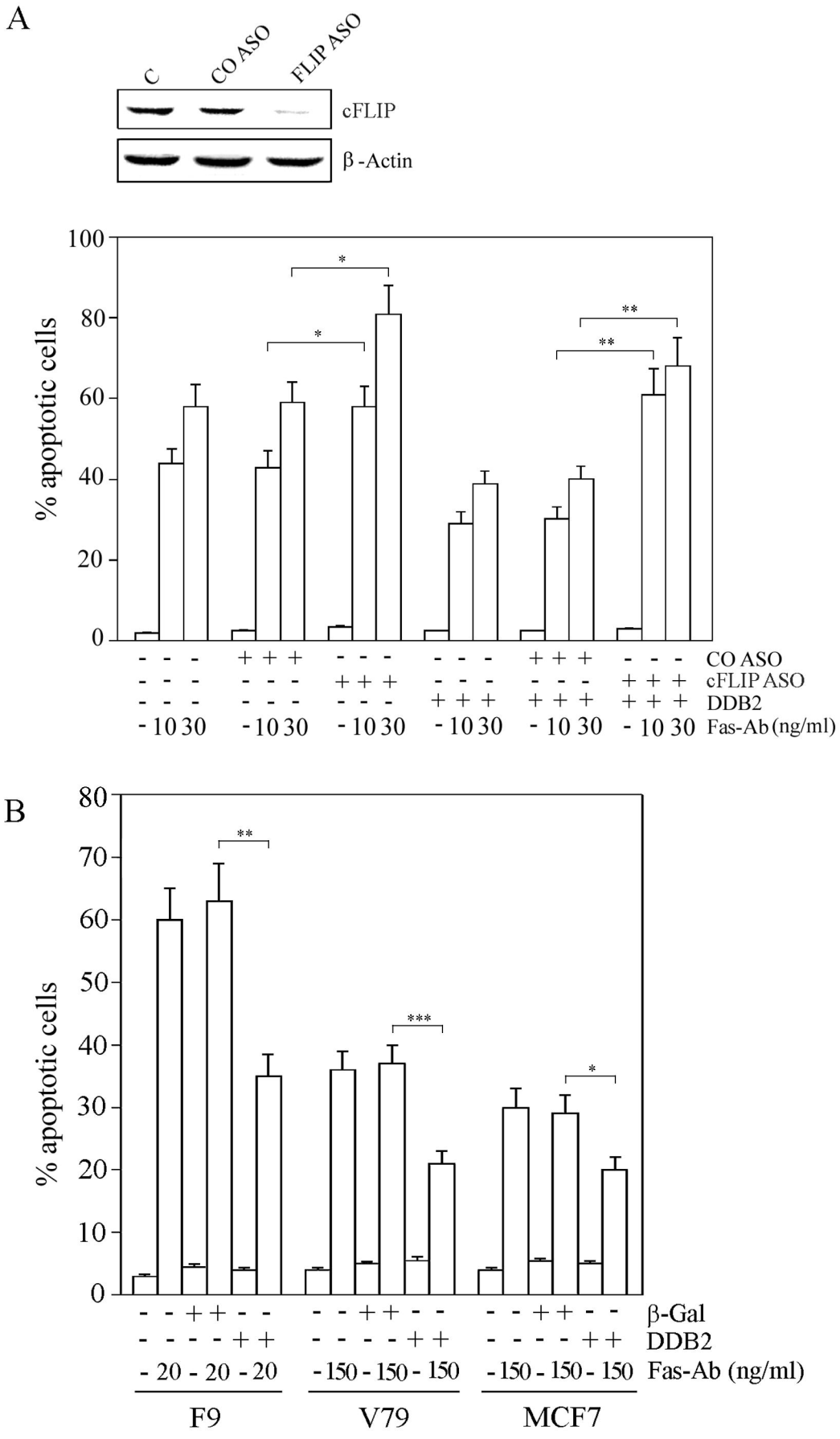

Fig. 6

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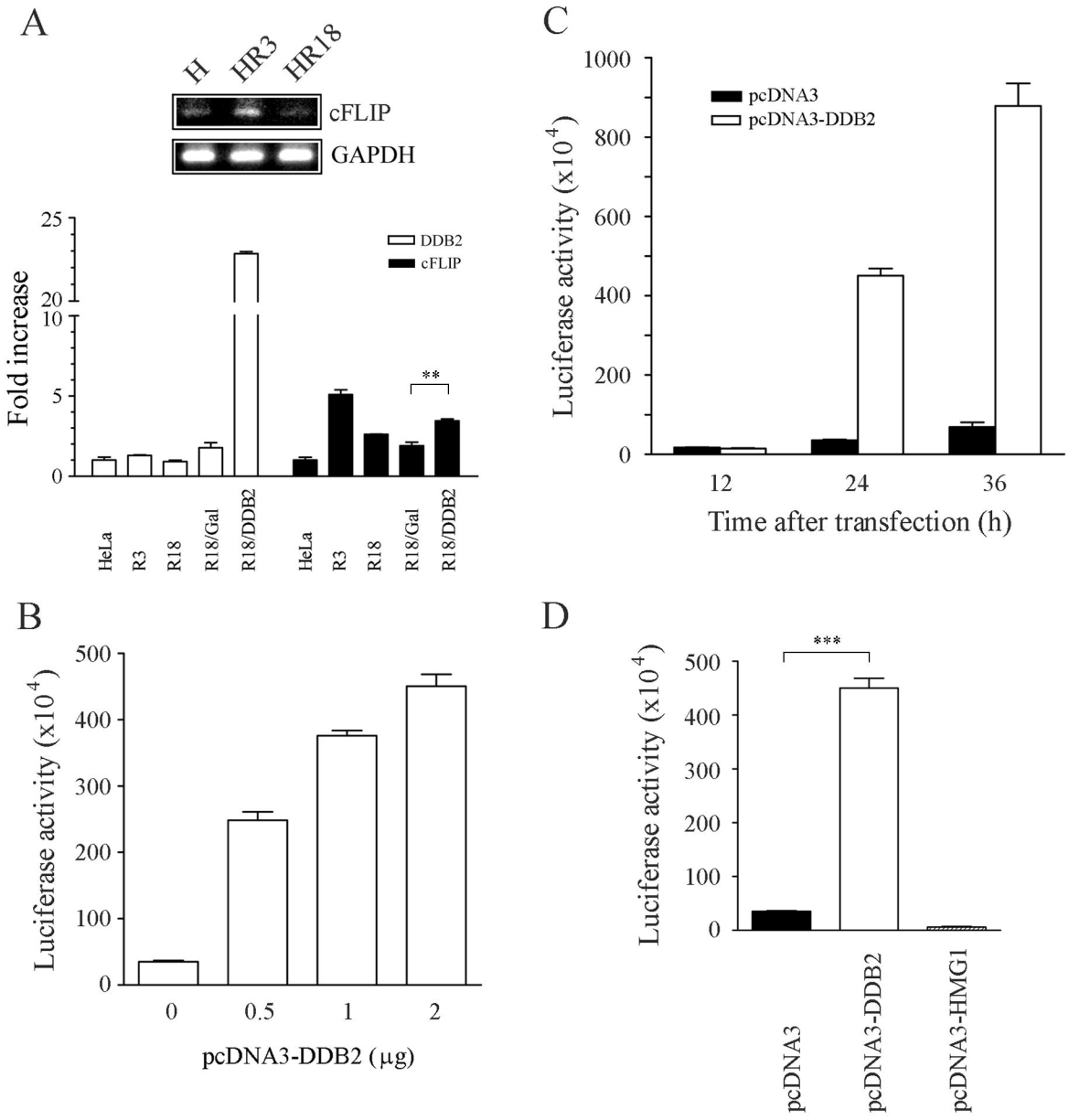

Fig. 7

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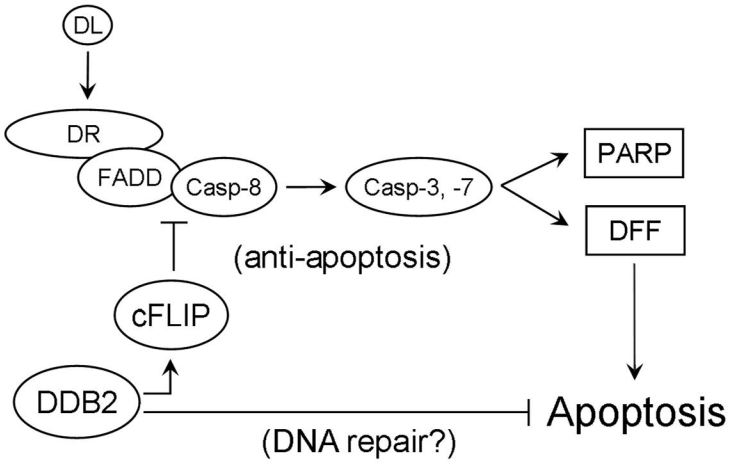

Fig. 8