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c-FLIP Knockdown Induces Ligand-independent DR5-, FADD-, Caspase-8-, and Caspase-9-dependent Apoptosis in Breast Cancer Cells

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Abstract

Cellular-FLICE inhibitory protein (c-FLIP) is an inhibitor of apoptosis downstream of the death receptors Fas, DR4, and DR5, and is expressed as long (c-FLIP$_L$) and short (c-FLIP$_S$) splice forms. We found that the knockdown of c-FLIP using small interfering RNA (siRNA) triggered ligand-independent caspase-8- and -9-dependent spontaneous apoptosis and decreased the proliferation of MCF-7 breast cancer cells. Further analysis revealed that an apoptotic inhibitory complex (AIC) comprised of DR5, FADD, caspase-8, and c-FLIP$_L$ exists in MCF-7 cells, and the absence of c-FLIP$_L$ from this complex induces DR5- and FADD-mediated caspase-8 activation in the death inducing signaling complex (DISC). c-FLIP$_S$ was not detected in the AIC, and using splice form-specific siRNAs we showed that c-FLIP$_L$ but not c-FLIP$_S$ is required to prevent spontaneous death signaling in MCF-7 cells. These results clearly show that c-FLIP$_L$ prevents ligand-independent death signaling and provides direct support for studying c-FLIP as a relevant therapeutic target for breast cancers.

1. Introduction

Apoptosis is an essential process in human development, immunity, and tissue homeostasis. One mechanism by which chemotherapeutic agents kill tumor cells is by inducing the apoptotic death pathways. The two primary apoptotic pathways are the death receptor and mithochondrial pathways [1,2]. Apoptotic signaling and execution through these two pathways is dependent on caspases, a group of cysteine proteases that degrade a critical set of cellular proteins near specific aspartic acid residues [3]. In the death receptor pathway, the apoptosis-promoting members of the tumor necrosis factor superfamily, such as Apo2L/TRAIL and Fas ligand, engage their respective death receptors, DR4/DR5 or Fas, which homotypically bind to the adaptor protein FADD. FADD then recruits the initiating caspases-8 and -10 through homophilic death effector domain (DED) interactions to form the death inducing signaling complex (DISC) [4,5]. The close proximity of the initiator caspases in the DISC facilitates their dimerization and activation. At the DISC, caspase-8 is initially processed into a 10 kDa species that is released from the DISC and an intermediate 43/41 kDa species that remains tethered to the DISC. The 43/41 kDa intermediate form is subsequently cleaved to give an 18 kDa species that associates with the 10 kDa species to form the active caspase-8 protease,

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which is released into the cytosol. The active caspase-8 cleaves downstream caspases such as caspases-3, -6, and -7, as well as the pro-apoptotic protein Bid which initiates the apoptotic process [6]. Cleaved Bid activates downstream pro-apoptotic proteins such as Bax and Bak which promote cytochrome *c* release from the mitochondria into the cytosol, thereby linking the two pathways [7]. Cytochrome *c* associates with caspase-9, dATP, and APAF-1 forming the apoptosome complex leading to the activation of caspase-9, which cleaves the downstream effector caspases-3, -6, and -7 [8,9]. Effector caspase activation promotes cellular disintegration by the cleavage of multiple cellular proteins such as fodrin, protein kinase C, gelsolin, and poly(ADP-ribose) polymerase (PARP) [7,10].

Cellular FLICE inhibitory protein (c-FLIP) is expressed in various cancers and is an inhibitior of death ligand-induced apoptosis downstream of death receptors and FADD [11]. Additionally, c-FLIP expression is associated with enhanced tumorgenicity and poor clinical outcome in many types of cancers because of chemotherapeutic drug and TRAIL resistance [12–18]. So far, 11 distinct c-FLIP splice variants have been reported, three of which are expressed as proteins: the 55 kDa long form (c-FLIP$_L$), 26 kDa short form (c-FLIP$_S$), and a 24 kDa form (c-FLIP$_R$). All c-FLIP isoforms contain two N-terminal DED domains but only c-FLIP$_L$ harbors a C-terminal caspase domain with structural similarity to caspase-8. In the caspase-like domain of c-FLIP$_L$, the catalytically active cysteine is replaced by tyrosine, rendering the molecule proteolytically inactive [19]. c-FLIP proteins are recruited to the DISC by their DED domains and compete with the initiator caspases for FADD binding sites [11]. c-FLIP$_S$ and c-FLIP$_R$ both inhibit death signaling at the DISC by inhibiting caspase-8 activation [20,21]. The function of c-FLIP$_L$ at the DISC is controversial because studies have shown that it can be anti-apoptotic like c-FLIP$_S$, or pro-apoptotic by directly activating caspase-8 [22, 23]. The pro-apoptotic role is supported by studies showing that c-FLIP-deficient mice die early in embryonic development due to heart failure, which is a defect also observed in caspase-8- and FADD-deficient mice [24,25]. However, the majority of reports encompassing diverse types of cancers indicate that the role of c-FLIP$_L$ is generally anti-apoptotic in cancer cells. For instance, the silencing of c-FLIP expression using small interfering RNAs (siRNAs) has been shown to promote spontaneous apoptosis in colorectal, lung, and lymphoma cancer cell lines and augments TRAIL- and chemotherapy-induced apoptosis in various cancer cell types [23,26–31].

In addition to c-FLIP's role in apoptosis, c-FLIP proteins have also been shown to be involved in proliferation, cell cycle progression, and carcinogenesis. The overexpression of c-FLIP$_L$ inhibited the proteosomal degradation of β-catenin and the elevated β-catenin levels were shown to either promote Wnt signaling or induce cyclin D expression, leading to the proliferation and cell cycle progression of cancer cells [32,33]. In both studies, the c-FLIP/β-catenin signals contributing to cell growth were reversed by the selective silencing of c-FLIP expression. Recently, a study showed that the caspase-8-dependent cleavage of c-FLIP produced an N-terminal p22 fragment that directly induced NF κB activation and promoted the proliferation of lymphocytic cells [34]. Furthermore, several studies have shown that c-FLIP overexpression can promote carcinogenesis and aggressiveness of endometrial and cervical cancers [35,36]. These studies highlight the functional importance of c-FLIP in the proliferation of cancer cells.

The aim of this study was to elucidate the mechanism of how the silencing of c-FLIP triggers spontaneous apoptosis in breast cancer cells. We discovered that silencing the c-FLIP gene leads to DR5-, FADD-, caspases-8- and -9-induced apoptosis in MCF-7 breast cancer cells. Furthermore, we observed that c-FLIP$_L$ interacts with DR5, FADD, and caspase-8 forming an apoptotic inhibitory complex (AIC) in these cells. Collectively, this study shows the knockdown of c-FLIP expression triggers spontaneous apoptosis by activating both the death receptor and mitochondrial pathways and inhibiting breast cancer cell proliferation.

2. Materials and Methods

2.1 Cell culture, materials, and antibodies

The MCF-7 human breast cancer cell line was obtained from American Type Culture Collection (ATTC, Manassas, VA), and was maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS) and 100 ng/ml each of penicillin and streptomycin (Invitrogen, Inc., Carlsbad, CA) at 37°C in 5% CO_2. The caspase-8 inhibitor (z-IETD-fmk) and the caspase-9 inhibitor (z-LEHD-fmk) were purchased from R&D Systems (Minneapolis, MN). The inhibitors were dissolved in DMSO and added to the cultured cells so that the final concentration of DMSO was 0.1%. In this study the following primary antibodies were used: anti-c-FLIP clone G-11 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-c-FLIP clone Dave-2 (ProSci Inc., Poway, CA), anti-DR5 clone B-D37 (IgG_{2b}) (Santa Cruz Biotechnology), anti-DR5 (Cell Signaling Technology, Danvers, MA), anti-caspase-8 clone 3-1-9 (BD Biosciences, San Jose, CA), anti-caspase-6 clone B93-4 (BD Biosciences), anti-caspase-7 clone 10-1-62 (BD Biosciences), anti-caspase-9 clone 5B4 (MBL International Corp., Woburn, MA), anti-FADD clone 1 (BD Biosciences), anti-BID clone 5C9 (Santa Cruz Biotechnology), Anti-DR4 (BD Biosciences), and anti-β-actin clone AC-74 (Sigma-Aldrich, St. Louis, MO).

2.2. Western blotting and immunoprecipitations

MCF-7 cells were harvested, rinsed in cold PBS, and lysed in M-PER (Pierce Biotechnology, Rockford, IL) containing 1% protease inhibitor cocktail (Sigma). Protein concentration was determined by using the BCA/Cu_2SO_4 protein assay (Sigma) as described by the manufacturer. One hundred micrograms of lysate were separated on a NuPAGE 4–12% or 10% Bis-Tris gel (Invitrogen) and transferred to an Immobilon-P membrane (Fisher Scientific, Pittsburgh, PA). Membranes were incubated in blocking buffer (PBS, 0.1% Tween 20, 5% skim milk) and then incubated first with specific antibodies in blocking buffer followed by the addition of horseradish peroxidase-conjugated sheep anti-mouse or anti-rabbit secondary antibodies (Amersham Biosciences, Piscataway, NJ). For detection of the anti-c-FLIP clone Dave-2 antibody, a horseradish peroxidase-conjugated goat anti-rat secondary antibody was used (Santa Cruz). Immunoreactive proteins were visualized by using SuperSignal West Pico solutions (Pierce Biotechnology).

For immunoprecipitations, MCF-7 cells were rinsed in ice cold PBS and lysed in buffer containing 20 mM Tris-HCl [37], 1% NP-40, 150 mM NaCl, 10% glycerol, and 1% protease inhibitor cocktail (Sigma). Five hundred micrograms of cell lysate were incubated overnight with 5 μg of DR5 antibody at 4°C. The following day, 50 μL of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) were added and the mixture was incubated for an additional night at 4°C. The immunoprecipitates were collected, washed in PBS, and analyzed by SDS-PAGE as described above. To eliminate the interference of heavy and light chains of the immunoprecipitating antibody, the Mouse TrueBlot ULTRA secondary antibody (eBioscience, San Diego, CA) was used.

2.3. siRNA preparation and transfection

c-FLIP, FADD, and DR5 siRNA oligonucleotides were synthesized by Invitrogen according to published sequences [27,38,39]. The siCONTROL Non-Targeting siRNA (catalog number D-001210-03) was purchased from Dharmacon Research, Inc. (Lafayette, CO). MCF-7 cells were seeded at a density of 2×10^5 cells/mL in antibiotic-free medium one day prior to transfection. For transfection, 100 nM of siRNA was mixed with DharmaFECT 1 transfection reagent (Dharmacon) according to the manufacturer's instructions. The cells were incubated with the siRNA-DharmaFECT 1 complexes for 48 or 72 h before further analysis.

2.3. Determination of apoptosis using Hoechst 33342 stain

To visualize nuclear morphology in MCF-7 cells by DNA staining, 1×10^5 cells were plated in Lab-Tek II chamber slides (Thermo Fisher Scientific, Waltham, MA) and treated with caspase inhibitors, siRNA, or caspase inhibitors and siRNA for 48 or 72 h. The cells were stained with 10 μM of Hoechst 33342 stain (Sigma) for 1 hour and viewed by fluorescence microscopy using a Nikon Diaphot 200 Inverted Microscope with a magnification of 40X. The images were captured using a Diagnostic Instruments SPOT color camera.

2.4. Annexin V binding assay to detect apoptotic cells

MCF-7 cells were plated at a density of 2×10^5 cells/mL and transfected with c-FLIP siRNA for 48 h, harvested, and stained with fluorescein isothiocyanate-labeled Annexin V (BD Biosciences) and propidium iodide according to the manufacturer's protocol. The cells were analyzed using a FACScan (BD Biosciences) flow cytometry and CellQuest software (BD Biosciences). Apoptotic and necrotic cells were analyzed by quadrant statistics on the propidium iodide-positive, annexin V-positive, and propidium iodide- and annexin V-positive cells, respectively.

2.5 Mitochondrial staining

To observe the morphological changes in the mitochondria due to c-FLIP gene knockdown, 1×10^5 MCF-7 cells were plated in Lab-Tek II chamber slides (Thermo Fisher Scientific) and transfected with c-FLIP-specific siRNA for 48 h. Cells were stained with 10 μM Hoechst 33342 stain (Sigma) and 500 nM MiTotracker Red CMXRos (Invitrogen) for 1 hour, and viewed by fluorescence microscopy using a Nikon Diaphot 200 Inverted Microscope with a magnification of 40X. The images were captured using a Diagnostic Instruments SPOT color camera.

2.6 Clonogenic assay

MCF-7 cells were transfected with c-FLIP siRNAs for 48 h, harvested, rinsed in PBS, and 1,000 cells were added to each well of a 6-well plate containing complete growth medium. The plates were incubated for 5 nights and colonies were counted.

3. Results

3.1. Knockdown of the c-FLIP gene triggers spontaneous caspase-8 and -9-dependent apoptosis in MCF-7 breast cancer cells

MCF-7 cells were transfected with c-FLIP-specific siRNA and immunoblot analysis showed the expression of both c-FLIP isoforms were silenced (FIG. 1A). An early event in the apoptotic process is the disruption of phospholipid asymmetry in the plasma membrane and the exposure of phosphatidylserine on the outer leaflet of the plasma membrane. Annexin V is a protein that binds to negatively charged phospholipids such as phosphatidylserine and is used to detect apoptotic cells. Double staining the cells with annexin V and propidium iodide allowed us to detect apoptotic cells by flow cytometry. Furthermore, we also identified apoptotic cells by fluorescence microscopy of Hoechst 33342-stained MCF-7 cells, since apoptosis is characterized by changes in nuclear morphology [40]. As shown in Figure 1B, C, and D, the silencing of c-FLIP coincided with a 45% increase in apoptosis as compared to cells treated with non-targeting siRNA. Immunoblot analysis of c-FLIP siRNA-treated whole cell lysates showed that caspase-8 was activated and Bid was cleaved forming tBid (Fig. 2A). Moreover, when the non-targeting siRNA-treated cells were co-stained with Hoechst 33342 and MitoTracker Red, the stain was sequestered in the nucleus and mitochondria, but c-FLIP siRNA-treated cells undergoing apoptosis gave diffuse staining showing that the nuclear and mitochondrial structures were disrupted, resulting in cytochrome *c* release and activation of

caspases-9, -6 and -7 (Fig. 2B and C). These results show that c-FLIP knockdown promotes signaling through both the death receptor and mitochondrial apoptotic pathways.

To determine whether the apoptosis mediated by the c-FLIP knockdown was caspase-8-dependent, we treated cells with c-FLIP siRNA in the presence or absence of the caspase-8 inhibitor z-IETD-fmk. As shown in Fig. 3A, the inhibition of caspase-8 blocked the spontaneous apoptosis induced by c-FLIP gene knockdown. These results reveal that silencing the c-FLIP gene triggers apoptosis by a caspase-8-dependent mechanism. Since c-FLIP knockdown leads to caspase-8 activation and Bid cleavage, we next examined whether c-FLIP siRNA-induced apoptosis is dependent on the activation of caspase-9. Transfection of cells with c-FLIP siRNA followed by the addition of the caspase-9 inhibitor z-LEHD-fmk protected the cells from spontaneous apoptosis, revealing that c-FLIP siRNA-triggered apoptosis occurred by a caspase-9-dependent process (Fig. 3B). Next, we determined if c-FLIP siRNA-mediated caspase-7 activation was dependent on caspase-8 or caspase-9. Transfection of cells with c-FLIP siRNA followed by the addition of the caspase-9 inhibitor z-LEHD-fmk prevented caspase-7 activation, revealing that caspase-7 was activated by caspase-9 in the absence of c-FLIP (Fig. 3C). Since MCF-7 cells do not express caspase-3, these results reveal that caspase-7 can substitute for caspase-3 and mediate apoptosis downstream of caspase-9 activation in the absence of c-FLIP. Collectively, these results show that silencing of c-FLIP induces apoptosis by activating both the death receptor (caspase-8) and the mitochondrial (caspase-9) pathways in MCF-7 cells.

3.2. Silencing of c-FLIP induces FADD-mediated apoptosis

To investigate the role of FADD in c-FLIP siRNA-triggered apoptosis, we silenced the FADD gene by using a FADD-specific siRNA (Fig. 4A). The knockdown of FADD expression reduced the c-FLIP siRNA-mediated production of the caspase-8-p18 active subunit by 60% and decreased apoptosis from 55% to 15%, revealing that FADD is involved in the processing of caspase-8 (Fig. 4B, C, and D). The spontaneous apoptosis in FADD- and c-FLIP-siRNA treated cells as compared to FADD- and non-targeting siRNA-treated cells was most likely due to incomplete silencing of the FADD gene. These results reveal that in the absence of c-FLIP, FADD is required for the processing and activation of caspase-8.

3.3. Knockdown of c-FLIP triggers DR5-mediated apoptosis

It is well documented that c-FLIP can inhibit caspase-8 activation and DISC formation induced by the TRAIL receptor DR5 [23,27,41,42]. Therefore, to investigate the role of DR5 in c-FLIP siRNA-mediated apoptosis, we knocked down the expression of DR5 using a DR5-specific siRNA (Fig. 5A). We discovered that the knockdown of DR5 gene expression decreased the c-FLIP siRNA-triggered production of the caspase-8-p18 active subunit by 60% and reduced apoptosis from 55% to 22% (Fig. 5B, C and D). These findings show that DR5 is required for the processing and activation of caspase-8 in the absence of c-FLIP. The apoptosis in DR5- and c-FLIP-siRNA treated cells as compared to DR5- and non-targeting siRNA-treated cells is probably due to the FADD-induced activation of caspase-8 (see Fig. 4), as well as the incomplete knockdown of DR5 gene expression (Fig. 5A). These data clearly show that DR5 is involved in inducing spontaneous apoptosis in MCF-7 cells following the knockdown of c-FLIP.

3.4. DR5 binds to c-FLIP$_L$, caspase-8, and FADD independently of receptor ligation

Because c-FLIP siRNA-triggered apoptosis was partially dependent on the expression of DR5, we determined whether c-FLIP$_L$ interacts with DR5 in MCF-7 cells. As shown in Figure 6, c-FLIP$_L$, caspase-8, and FADD, but not DR4 and c-FLIP$_S$, were immunoprecipitated from whole cell lysates using an anti-DR5 monoclonal antibody. These results reveal that c-FLIP$_L$ interacts with DR5, FADD, and caspase-8 to form an apoptotic inhibitory compex (AIC) that inhibits

spontaneous DISC signaling. However, when c-FLIP$_L$ is absent from the AIC, activation of caspase-8 via DR5 and FADD triggers DISC activation and apoptosis. We did not detect any DR4 in the DR5 immunocomplexes, indicating that DR5 and DR4 do not exist as a heterocomplex in MCF-7 cells. Additionally, since c-FLIP$_S$ was not detected in the immunoprecipitated complex, we determined whether c-FLIP$_S$ expression was involved in regulating apoptosis in these cells. Transfection of cells with c-FLIP$_L$- or FLIP$_S$-specific siRNAs silenced the expression of each gene (Fig. 7A), and induced apoptosis in 50% and 15% of the cells, respectively, as compared to 8% in non-targeting siRNA-treated cells (Fig. 7B). These results clearly show that c-FLIP-specific siRNA-triggered apoptosis is primarily due to the silencing of the c-FLIP$_L$ gene and not the c-FLIP$_S$ gene, and that c-FLIP$_L$ expression is required to prevent spontaneous caspase-8 activation from occurring at the DISC. Furthermore, the small increase in spontaneous apoptosis following the knockdown of the c-FLIP$_S$ gene may be due to its role in regulating the formation and activation of the DISC, even though it does not bind directly to DR5.

3.5. Knockdown of c-FLIP decreases the proliferation of MCF-7 cells

To examine whether silencing the c-FLIP gene affects the proliferation and long term survival of MCF-7 cells, we executed a colony forming assay. The results presented in Fig. 8 show that cells transfected with c-FLIP-specific siRNA, c-FLIP$_L$-specific siRNA, or c-FLIP$_S$-specific siRNA reduced the number of colonies by 67%, 63%, and 22%, respectively. These data show that both c-FLIP isoforms promote the proliferation of MCF-7 cells, but c-FLIP$_L$ appears to be more important than c-FLIP$_S$ in promoting long term survival. Collectively, these results reveal that c-FLIP$_L$ inhibits spontaneous DISC formation and promotes breast cancer cell proliferation.

4. Discussion

c-FLIP is a regulator of apoptosis that is mediated by the activation of death receptors TNFR1, Fas, DR4, and DR5 [43,44]. Following death receptor engagement, c-FLIP inhibits caspase-8 binding to FADD and prevents DISC formation and apoptosis. Several studies have shown that silencing c-FLIP expression by using c-FLIP-specific siRNA induces spontaneous apoptosis in lung, colorectal, and lymphoma cancer cell lines, and sensitizes cells to TRAIL- and chemotherapy-mediated apoptosis [23,26,28,41]. However, detailed molecular mechanisms by which downregulation of c-FLIP triggers spontaneous apoptosis have not been reported. In the present study, we found that the knockdown of the c-FLIP gene triggers ligand-independent DR5-, FADD-, caspases-8-, and -9-induced apoptosis in MCF-7 breast cancer cells. Our data revealed that a ligand-independent pre-associated apoptotic inhibitory complex (AIC) containing DR5, FADD, caspase-8, c-FLIP$_L$, but not DR4 or c-FLIP$_S$ exists in MCF-7 cells, and the absence of c-FLIP$_L$ from the complex leads to caspase-8 activation and apoptosis. In support of our findings, several studies have shown that DR5 can recruit caspase-8 and FADD independently of DR4 after receptor activation [45,46], and current studies are underway to determine if DR4 plays a role in apoptosis following the knockdown of c-FLIP. However, studies performed using neutralizing antibodies or receptor-selective mutants to DR4 and DR5, identified DR5 as having a more prominent role in death receptor-mediated apoptosis [47–49].

In agreement with a previous study, our data showed that FADD is required for activating caspase-8 and triggering apoptosis in the absence of c-FLIP [29]. c-FLIP$_L$ may inhibit FADD activity in the DISC by a similar mechanism that has been shown for the viral FLIP, M159, which cooperatively assembles with FADD and prevents FADD self-association that is required for caspase activation in the DISC [50]. By using DR5-specific siRNA, our data showed that DR5 is required for processing procaspase-8 to active caspase-8 following

knockdown of the c-FLIP gene. Previously, c-FLIP$_L$ has been shown to bind directly to DR5 by a FADD-independent mechanism; therefore, the interaction of c-FLIP$_L$ with DR5 may prevent DR5-mediated caspase-8 activation in the DISC [42]. In this paper, only caspase-8 was shown to be involved in spontaneous apoptosis due to c-FLIP$_L$ knockdown; however, caspase-10 may also be involved in triggering apoptosis in cells that express caspase-10 [51]. Together, these experiments provide direct support for the conclusion that c-FLIP$_L$ is required to prevent constitutive DISC signaling in MCF-7 cells.

These results are intriguing considering the classical model of death receptor-induced apoptosis that requires the binding of extracellular ligands, such as Fas ligand or TRAIL, to their cognate receptors, Fas and DR4/DR5, respectively, leading to the trimerization of the receptors and recruitment of cytosolic death domain containing proteins which trigger downstream signaling events. However, a recent study showed that TNFα receptors pre-associate as homotrimers on the cell surface prior to ligand binding [52]. In fact, various stimuli have been shown to mediate the clustering of pre-associated homotrimers on the cell membrane without ligand binding [53–56]. Moreover, our results show that c-FLIP$_L$ prevents the activation of pre-associated DISCs, and thereby protects the cells from spontaneous apoptosis. Therefore, c-FLIP$_L$ serves as a cytoprotective protein by preventing apoptotic signaling pathways in the absence of death-ligand engagement. The cytoprotective role of c-FLIP is supported by several studies that have shown c-FLIP can regulate the NFκB, ERK, and Wnt signaling pathways [32,34,57]. In fact, our clonogenic assay results reveal that the knockdown of c-FLIP decreases the proliferation and long term survival of MCF-7 cells.

Based on the results from this study, we have proposed an apoptotic signaling pathway following the knockdown of c-FLIP in MCF-7 cells (Fig. 9). From our findings, an apoptotic inhibitory complex (AIC) comprised of DR5, FADD, caspase-8, and c-FLIP$_L$ exists, and the knockdown of c-FLIP$_L$ triggers DISC activation leading to FADD- and DR5-mediated caspase-8 activation. Active caspase-8 cleaves Bid to form truncated Bid resulting in mitochondrial disintegration and caspase-9 activation. Activation of caspase-9 leads to the activation of the downstream effector caspases-6 and -7 triggering apoptosis.

There does not appear to be a “handle” to inhibit c-FLIP function with small molecule ligands, since its cytoprotective DISC binding is mediated by highly conserved DEDs which function by homotypic binding. However, small molecules that induce c-FLIP downregulation and sensitize neoplastic cells to apoptosis induction by the cytokine TRAIL have been identified [58–61]. Moreover, several studies have shown that silencing c-FLIP expression by using c-FLIP-specific siRNA induces apoptosis in lung, colorectal, and lymphoma cancer cell lines, and sensitizes cells to TRAIL- and chemotherapy-mediated apoptosis. Therefore, the development of gene therapy strategies to knockdown c-FLIP expression should overcome the barrier of dose-limiting toxicity associated with cancer chemotherapy. In conclusion, this study adds key mechanistic insight into how c-FLIP prevents ligand-independent death signaling and validates c-FLIP as a relevant therapeutic target for breast cancers.

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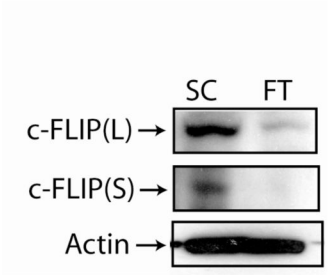

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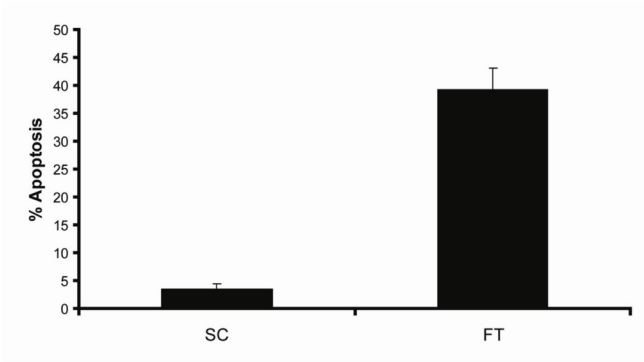

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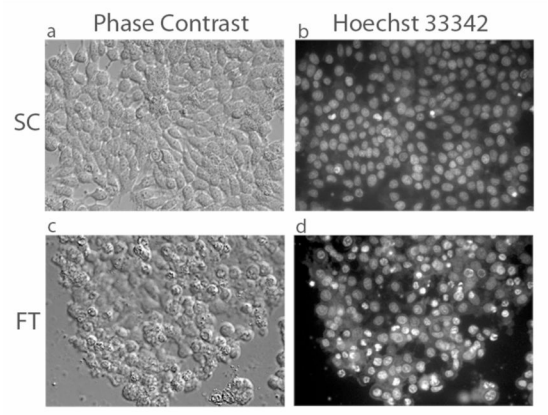

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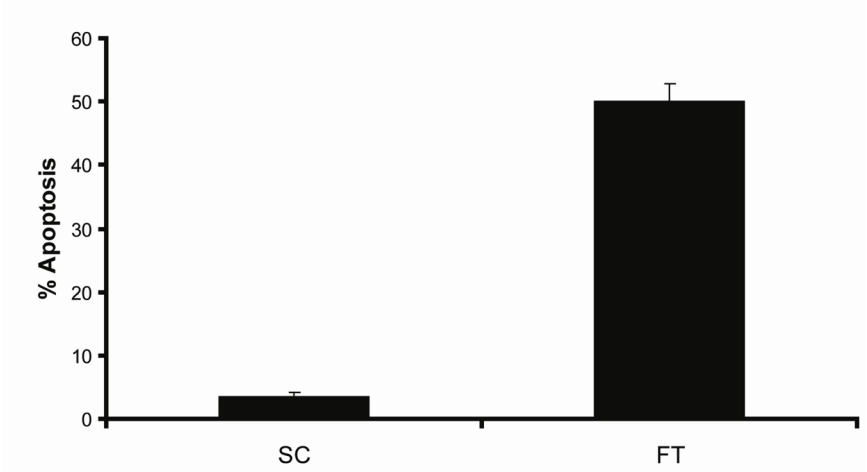

Fig.1. c-FLIP gene silencing triggers spontaneous apoptosis in MCF-7 cells

A, Immunoblot analysis of c-FLIP$_L$, c-FLIP$_S$, and β-actin in cell lysates following the treatment with 100 nM of non-targeting siRNA (SC) or c-FLIP-targeting siRNA (FT) for 48 h. *B*, Cells were transfected with 100 nM non-targeting siRNA (SC) or c-FLIP-specific siRNA (FT) for 48 h, and cells were harvested and apoptosis was determined by FACS analysis as described in the Materials and Methods section. Compensation was executed for each experiment using untreated cells stained with Annexin V and propidium iodide. Error bars show standard deviation from duplicate measurements. *C*, Cells were transfected with 100 nM non-targeting siRNA (SC) or c-FLIP-specific siRNA (FT) for 48 h, and Phase contrast (a and c) and fluorescence microscopy images (b and d) are shown after staining the DNA with Hoechst 33342. *D*, Cells were transfected with 100 nM non-targeting siRNA (SC) or c-FLIP-specific siRNA (FT) for 48 h, and the percentage of apoptotic cells was determined by counting fragmented nuclei stained with Hoechst 33342 among 200 cells. All values are representative of three independent experiments, and error bars show standard deviation from triplicate counts.

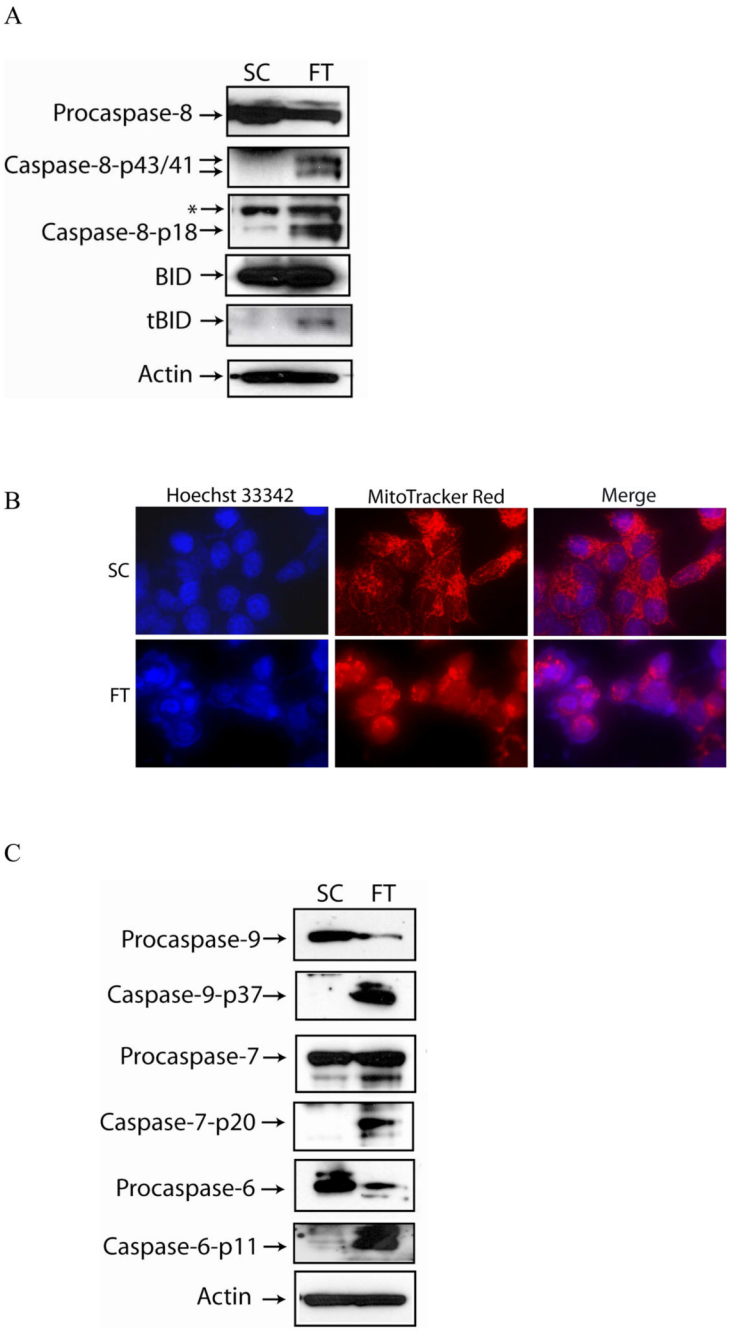

Fig. 2. c-FLIP knockdown induces the activation of caspases, Bid, and mitochondrial disintegration

A, Immunoblot analysis of caspase-8, BID, and β-actin in cell lysates following the transfection with non-targeting siRNA (SC) or c-FLIP-specific siRNA (FT) for 48 h. The asterisk (*) represents a non-specific protein that is recognized by the capase-8-specific antibody. B, Cells were transfected with non-targeting siRNA (SC) or c-FLIP-specific siRNA (FT) for 48 h, stained with 10 μM of Hoechst 33342 and 500 nM MitoTracker Red, and visualized by fluorescent microscopy. *C*, Immunoblot analysis of caspase-9, caspase-7, caspase-6, and β-actin in cell lysates following the transfection of MCF-7 with non-targeting siRNA (SC) or c-FLIP-specific siRNA (FT) for 48 h.

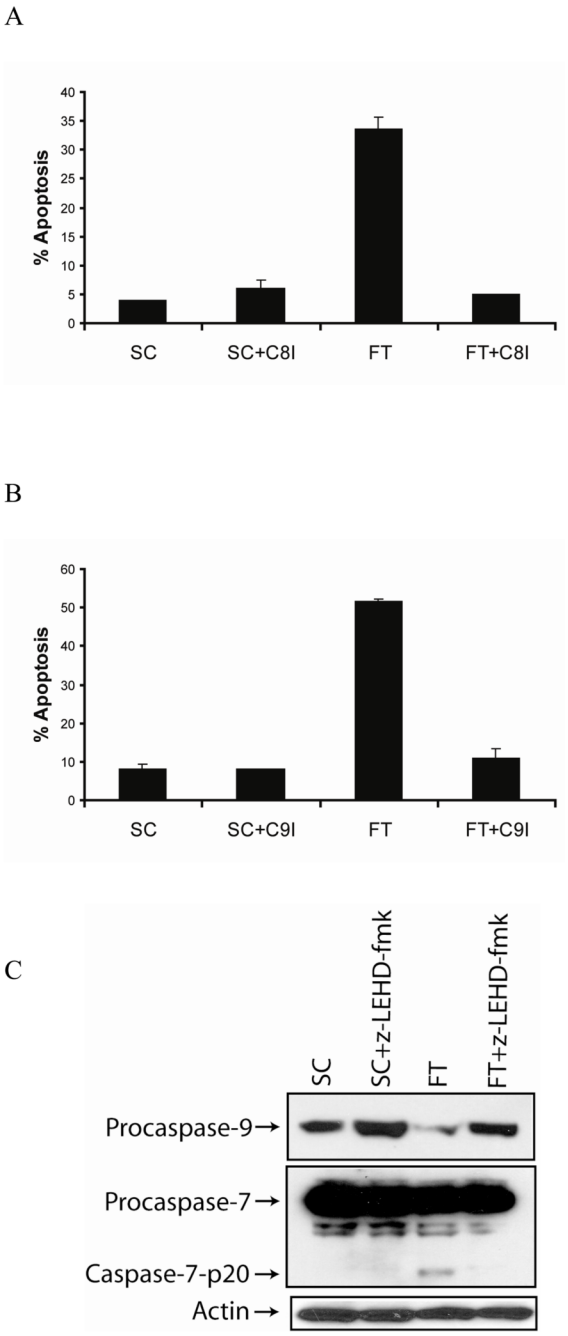

Fig. 3. c-FLIP knockdown triggers caspase-8- and caspase-9-dependent apoptosis

A, Cells were transfected with 100 nM non-targeting siRNA (SC) or c-FLIP-specific siRNA (FT) and treated with or without 20 μM of caspase-8 inhibitor (z-IETD-fmk) for 48 h, and apoptosis was determined by counting fragmented nuclei after staining with Hoecsht 33342 among 200 cells. All values are representative of three independent experiments, and error bars show standard deviation from triplicate counts. *B*, Cells were transfected with 100 nM non-targeting siRNA (SC) or c-FLIP-specific siRNA (FT) and incubated with or without 20 μM of caspase-9 inhibitor (z-LEHD-fmk) for 48 h, and apoptosis was determined by counting fragmented nuclei after staining with Hoecsht 33342 among 200 cells. All values are representative of three independent experiments, and error bars show standard deviation from

triplicate counts. *C*, Immunoblot analysis of caspase-9, caspase-7, and β-actin in cell lysates after transfecting with 100 nM non-targeting siRNA (SC) or c-FLIP-specific siRNA (FT) and incubated with or without 20 μM of the caspase-9 inhibitor (z-LEHD-fmk) for 48 h.

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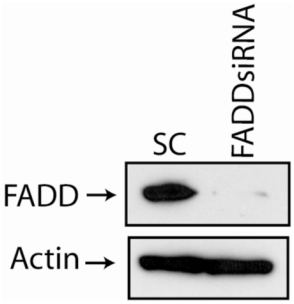

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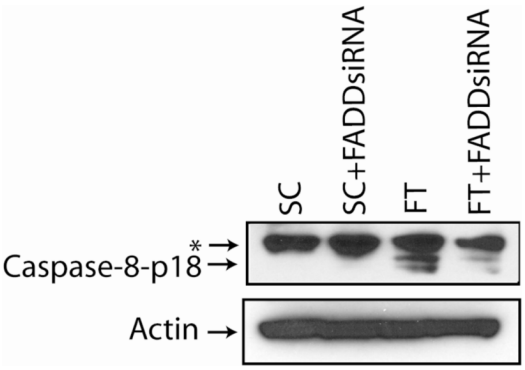

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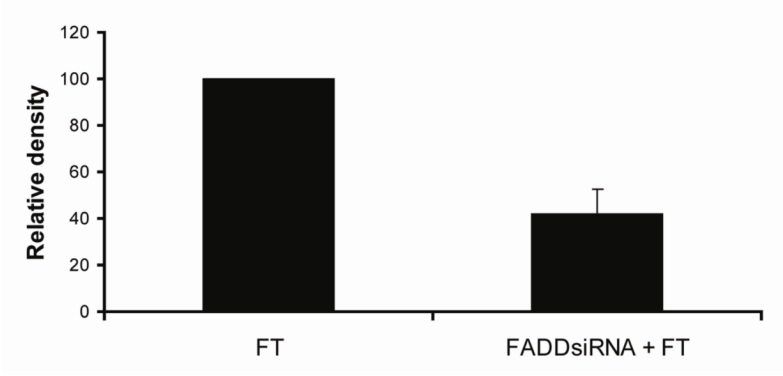

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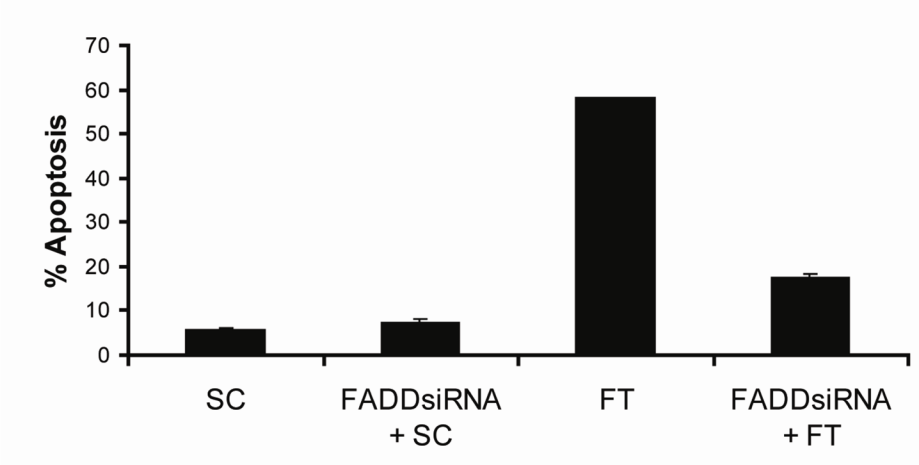

Fig. 4. Silencing of c-FLIP triggers FADD-dependent apoptosis

A, Immunoblot analysis of FADD and β-actin in cell lysates following the transfection with 100 nM of non-targeting siRNA (SC) or FADD-specific siRNA for 72 h. *B*, Immunoblot analysis of caspase-8 and β-actin in cell lysates treated with 100 nM FADD-specific siRNA for 24 h, and treated for an additional 48 h with 100 nM of non-targeting siRNA (SC) or c-FLIP-specific siRNA (FT). The asterisk (*) represents a non-specific protein that is recognized by the capase-8-specific antibody. *C*, The density of the caspase-8-p18 band in the c-FLIP-specific siRNA (FT) or FADD siRNA and FT siRNA treated cell lysates was quantified using the Image J 1.37c software (values +/− s.d.). *D*, Cells were treated with 100 nM FADD-specific siRNA for 24 h and treated for an additional 48 h with 100 nM non-targeting siRNA (SC) or c-FLIP-specific siRNA (FT), and apoptosis was determined by counting fragmented nuclei after staining with Hoecsht 33342 among 200 cells. All values are representative of three independent experiments, and error bars show standard deviation from triplicate counts.

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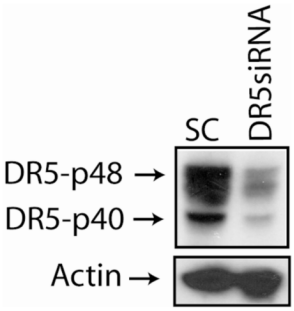

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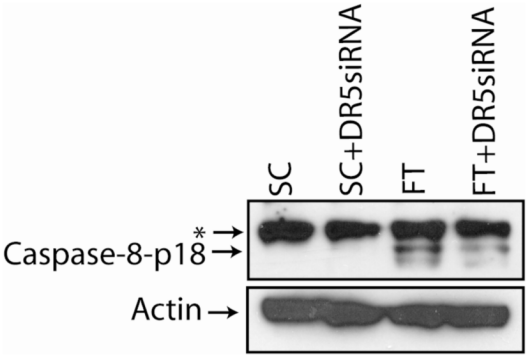

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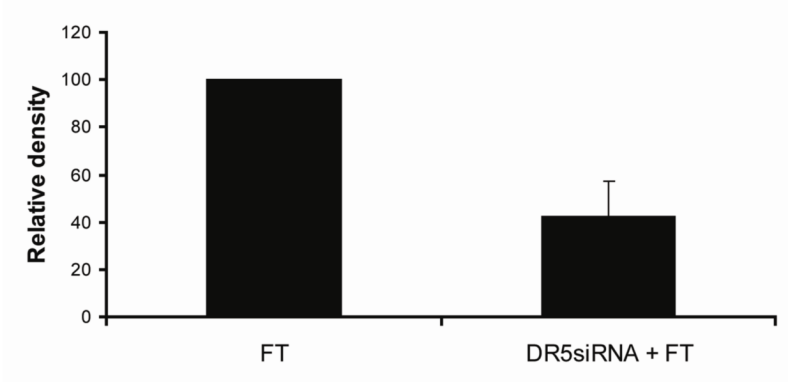

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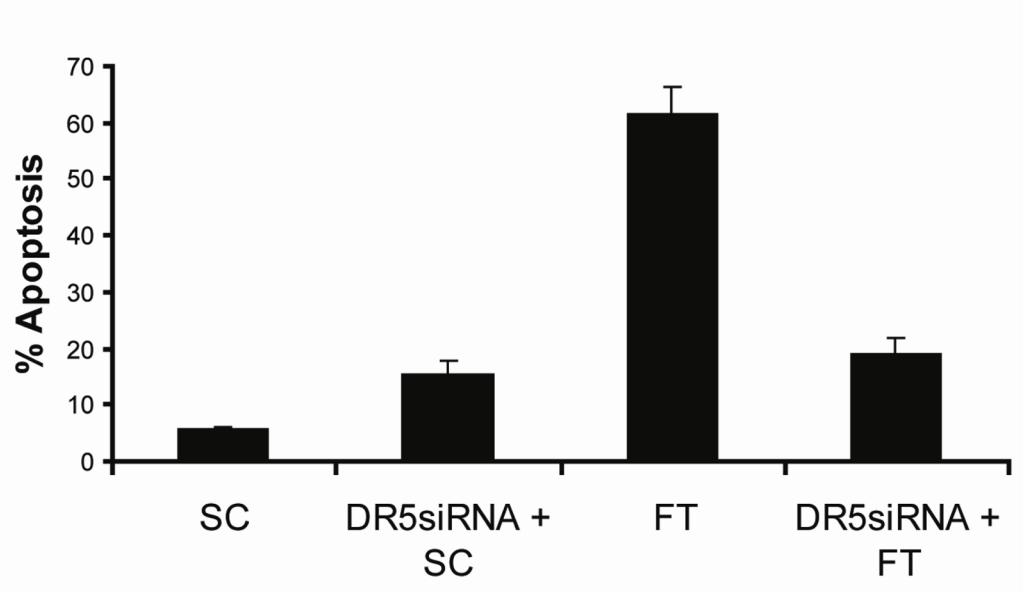

Fig. 5. The knockdown of c-FLIP triggers DR5-dependent apoptosis

A, Immunoblot analysis of DR5 and β-actin in cell lysates following the transfection with 100 nM of non-targeting siRNA (SC) or DR5-specific siRNA for 72 h. *B*, Immunoblot analysis of caspase-8 and β-actin in cell lysates treated with 100 nM DR5-specific siRNA for 24 h, and treated for an additional 48 h with 100 nM of non-targeting siRNA (SC) or c-FLIP-specific siRNA (FT). The asterisk (*) represents a non-specific protein that is recognized by the capase-8-specific antibody. *C*, The density of the caspase-8-p18 band in the c-FLIP-specific (FT) siRNA or DR5-specific siRNA and FT siRNA treated cell lysates was quantified using the Image J 1.37c software (values +/− s.d.). *D*, Cells were treated with 100 nM DR5-specific siRNA for 24 h and treated for an additional 48 h with 100 nM non-targeting siRNA (SC) or c-FLIP-specific siRNA (FT), and apoptosis was determined by counting fragmented nuclei after staining with Hoecsht 33342 among 200 cells. All values are representative of three independent experiments, and error bars show standard deviation from triplicate counts.

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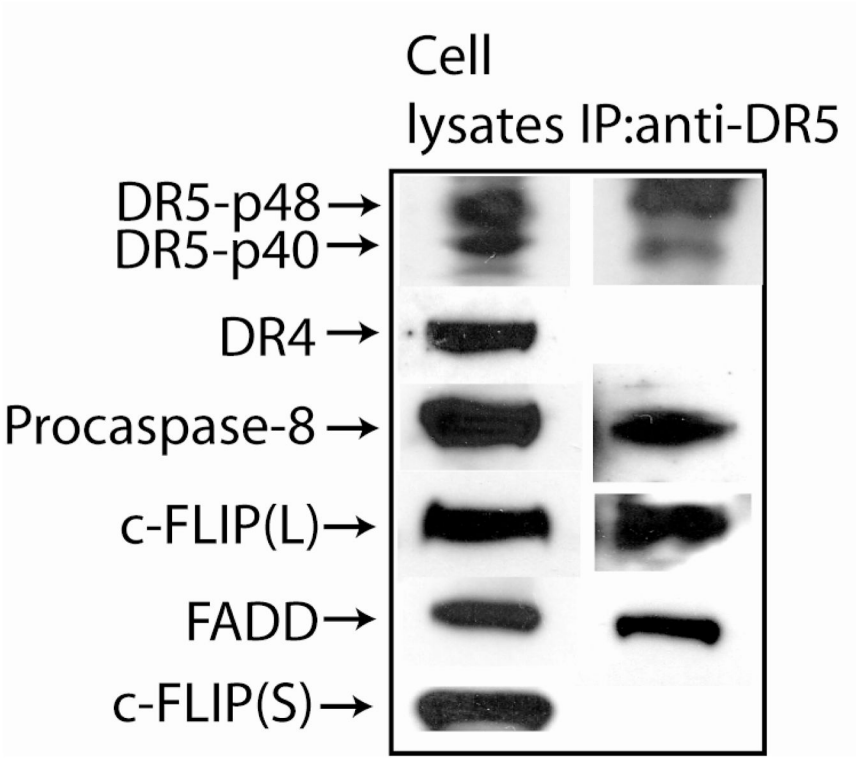

Fig. 6. DR5 binds to FADD, caspase-8, and c-FLIP

Input cell lysates and anti-DR5 immunoprecipitates were analyzed by Western blotting using DR5-, DR4-, caspase-8-, c-FLIP-, and FADD-specific antibodies. No proteins were immunoprecipitated using a control isotype-specific antibody.

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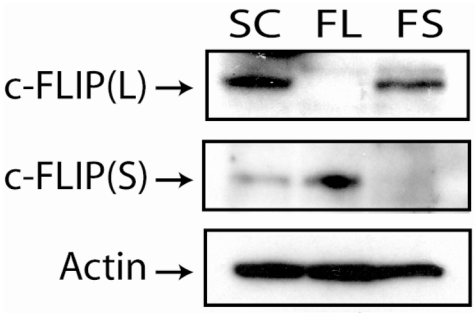

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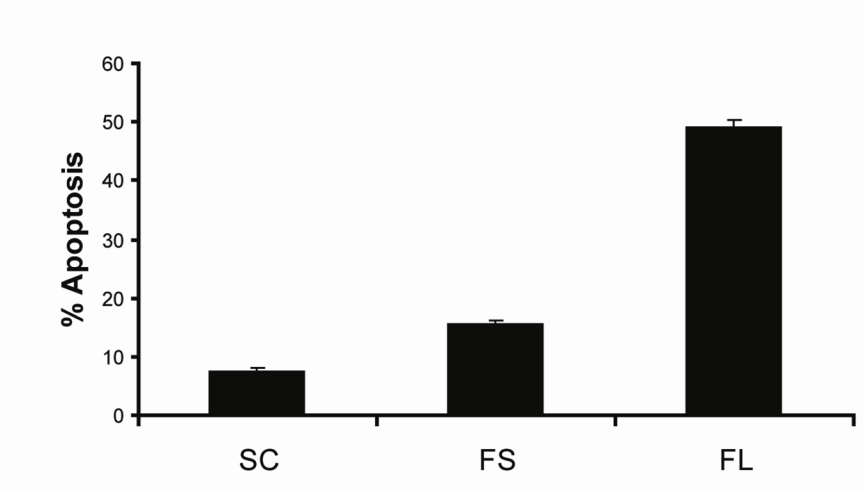

Fig. 7. c-FLIP$_L$ inhibits spontaneous apoptosis

A, Immunoblot analysis of c-FLIP$_L$, c-FLIP$_S$, and β-actin in cell lysates treated with 100 nM of non-targeting siRNA (SC), c-FLIP$_L$-targeting siRNA (FL), or c-FLIP$_S$-targeting siRNA (FS) for 48 h. *B*, Cells were treated with 100 nM of non-targeting siRNA (SC), c-FLIP$_L$-targeting siRNA (FL), or c-FLIP$_S$-targeting siRNA (FS) for 48 h, and apoptosis was quantified by counting fragmented nuclei after staining with Hoecsht 33342 among 200 cells. All values are representative of three independent experiments, and error bars show standard deviation from triplicate counts.

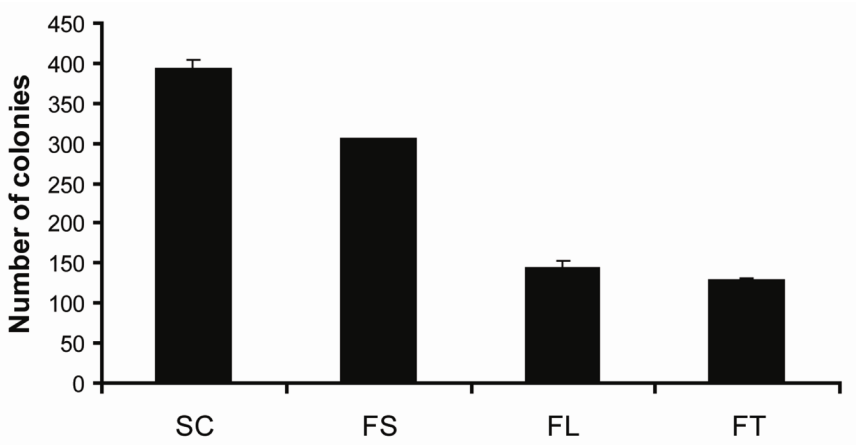

Fig. 8. Knockdown of c-FLIP decreases the proliferation of MCF-7 cells

Cells were transfected with 100 nM of non-targeting siRNA (SC), c-FLIP-specific siRNA (FT), c-$FLIP_L$-targeting siRNA (FL), or c-$FLIP_S$-targeting siRNA (FS) for 48 h. The cells were harvested and 1,000 cells were transferred to 6-well plates containing complete media. Colonies were counted after incubation for 5 days. The values are representative of three independent experiments, and error bars show standard deviation from triplicate counts.

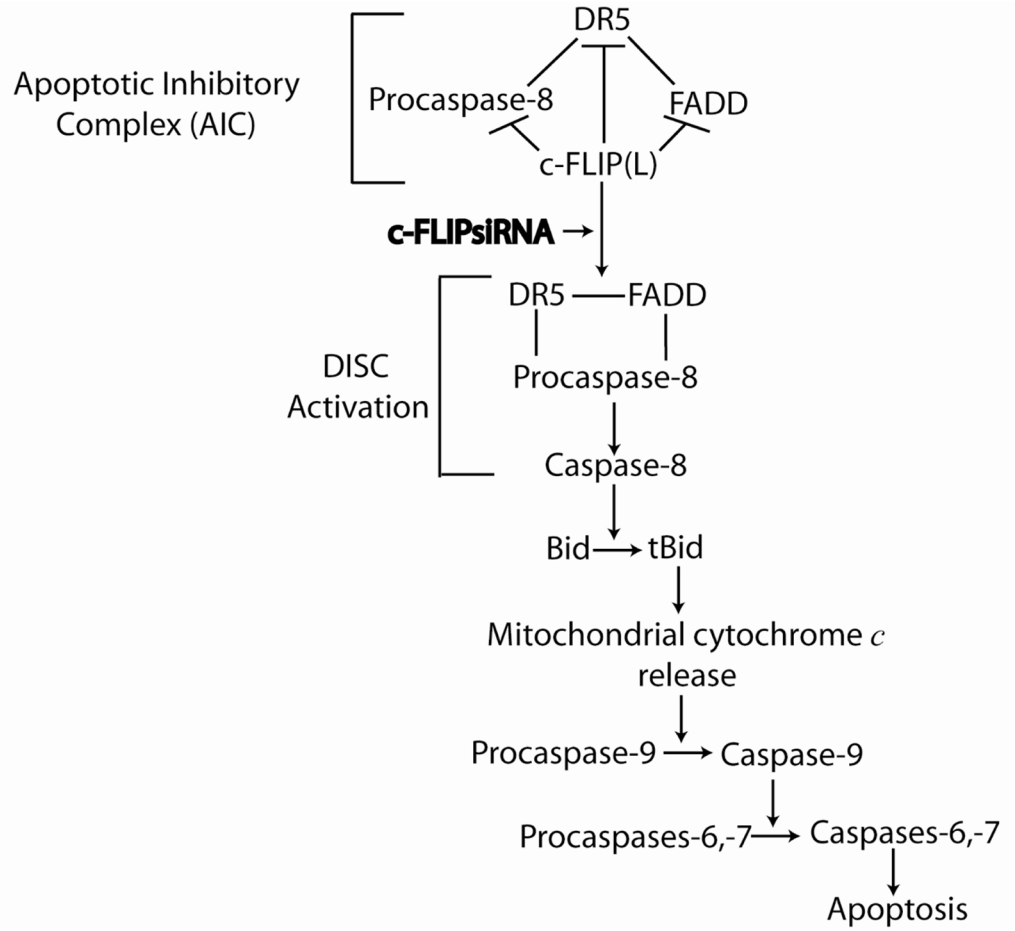

Fig. 9. Proposed signaling pathway of c-FLIP siRNA-triggered spontaneous apoptosis in MCF-7 cells

In this signaling pathway, we propose that an apoptotic inhibitory complex (AIC) comprised of DR5, FADD, caspase-8, and c-FLIP$_L$ exists, and c-FLIP$_L$ inhibits caspase-8 activation. The knockdown of c-FLIP$_L$ using a c-FLIP-specific siRNA induces DISC activation by triggering DR5- and FADD-mediated activation of procaspase-8, forming active caspase-8. Active caspase-8 cleaves Bid to form tBid, leading to the release of cytochrome *c* from the mitochondria. Mitochondrial cytochrome *c* release activates procaspase-9 producing active caspase-9, which activates downstream caspases-6 and -7 thereby inducing apoptosis. The solid lines indicate protein binding partners and the brackets show protein complexes that comprise the AIC and DISC.