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PRINCIPAL INVESTIGATOR: Aria F. Olumi, M.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, MA 02115

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Aria F. Olumi, M.D.

E-Mail: aolumi@bidmc.harvard.edu

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14. ABSTRACT: Abnormalities in programmed cell death (apoptosis) machinery play a crucial role in initiation, progression and metastasis of prostate cancer. Therefore, molecules that initiate pro-apoptotic pathways are excellent therapeutic agents in prostate cancer. However, some prostate cancer cells develop resistance to pro-apoptotic agents. In this proposal we are examining the regulatory mechanisms of c-FLIP(L), which is an important modular of apoptosis in prostate cancer.

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Table of Contents

Progress Report

INTRODUCTION

Abnormalities in apoptotic machinery play a crucial role in initiation, progression and metastasis of prostate cancer. c-FLIP(L), an anti-apoptotic molecule, has been suggested to play a role in developing resistance to pro-apoptotic agents like tumor necrosis factor (TNF) and TNF related apoptosis inducing ligand (TRAIL). In this proposal we have demonstrated that expression of c-FLIP(L) is necessary and sufficient to account for developing resistance to pro-apoptotic agents like TRAIL. Silencing expression of c-FLIP(L) is adequate to overcome other alternative mechanisms of resistance to TRAIL. Therefore, changing the expression of c-FLIP(L) successfully converts the phenotype of resistant to sensitive prostate cancers in response to pro-apoptotic agents. In addition, the expression of c-FLIP(L) is partially regulated at the transcriptional level.

Two pro-agonist antibodies to the TRAIL receptors have been developed by the Human Genome Science, Inc. (Rockville, MD) designated HGS-ETR1 and HGS-ETR2. HGS-ETR and HGS-ETR2 target the DR4 and DR5 TRAIL receptors. Both of these newly developed drugs are presently in phase I and phase II clinical trials in other carcinomas. We have obtained approval from Human Genome Science's scientific committee review board to use HGS-ETR1 and HGS-ETR2 in our prostate cancer studies outlined below. Therefore, knowledge gained from this proposal directly translates to identifying prostate cancer patients who may benefit the most from the pro-apoptotic effects of HGS-ETR1 and HGS-ETR2. This proposal is focused on identifying the molecular mechanisms of resistance to the pro-apoptotic effects of HGS-ETR1 and HGS-ETR2.

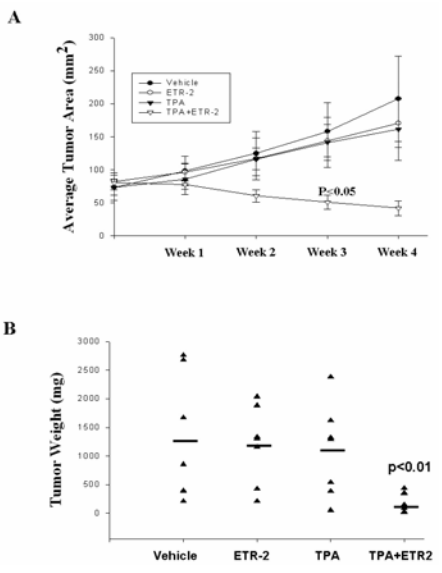

Fig. 1. HGS-ETR2 – resistant prostate cancer cells, LNCaP, were treated with HGS-ETR2, TPA or combination of the two. Evaluation of the average tumor area (A) demonstrates that combination of HGS-ETR2 + TPA effectively reduces the tumor area. In addition, average tumor weight (B) is significantly lower when HGS-ETR2 is used in combination with TPA.

BODY

Specific Aim #1: To examine the efficacy of HGS-ETR1 and HGS-ETR2 in an orthotopic prostate cancer model.

Last year we successfully developed orthotopic prostate cancer xenografts, and demonstrated that HGS-ETR2 is effective in treating orthotopic prostate cancers in an in-vivo model. However, as is the case for many cancers, some tumors are resistant to the therapeutic effects of pro-apoptotic agents. This year, we have extended our findings by focusing on mechanisms that can convert the phenotype of HGS-ET2 resistant tumors to a more sensitive phenotype.

Previously we have shown that c-Fos/AP-1 promoted TRAIL(or HGS-ETR2)-induced apoptosis by repressing the anti-apoptotic molecule c-FLIP(L). We have found that activation of c-Fos is necessary, but insufficient for apoptosis. In this portion of our

studies we investigated whether synthetic induction of c-Fos/AP-1 by low-dose 12-O-Tetradecanoylphorbol-13-acetate (TPA) [1, 2] converts the phenotype of TRAIL-resistant prostate cancer cells to a TRAIL-sensitive phenotype. In this report we will demonstrate our in-vivo xenografts results, which matches our in-vitro findings.

As shown in figure 1, the HGS-ETR2 resistant prostate cancer cells, LNCaP, are ineffectively treated with either HGS-ETR2 or TPA alone. However, combination of HGS-ETR2 and TPA can effectively reduce the tumor area (Fig. 1A) and tumor weight (Fig. 1B).

Specific Aim #2: To determine and examine the gene expression profile differences between TRAIL-sensitive (PC3) and TRAIL-resistant (PC3-TR) prostate cancer cells after treatment with HGS-ETR1 or HGS-ETR2. In our report last year, we demonstrated that we had identified the oncogene, c-Fos, to be a key regulator of TRAIL-induced (or HGS-ETR2) induced apoptosis. This year we have examined the regulatory mechanisms that involve c-Fos in the pro-apoptotic pathway. Two manuscripts that have been submitted for publication are attached. One of the manuscripts is conditionally accepted. Please see the appendix for details.

Fos proteins are basic region-leucine zipper (bZIP) transcription factors that bind to Jun or other bZIP proteins and create the AP-1 dimer complex, which regulates gene expression [3]. c-Fos has been shown to play an important role in development, inflammation and oncogenic processes. For example, TNF-family induction of c-Fos plays an important role in proper bone development [4] and regulation of inflammatory response in arthritis [5]. c-Fos, a well-established oncogene, is considered to play a critical role in tumorigenesis [6], proliferation and transformation [3], angiogenesis [7], tumor invasion and metastasis [8, 9], and its expression is associated with poor clinical outcomes [10]. Therefore, c-Fos has preferentially been considered an anti-apoptotic molecule. However, recent evidence suggests that c-Fos may also have a pro-apoptotic role [8]. The first indication of such pro-apoptotic function of c-Fos comes from the observation that c-Fos expression preceded apoptosis [11], and also observed during mammary gland involution [12], and other systems [13]. However, pro-apoptotic down-stream molecular targets of c-Fos are poorly understood.

In the attached manuscripts, we have demonstrated that nuclear c-Fos primes cancer cells to undergo apoptosis, and its expression is necessary but insufficient for TRAIL-induced apoptosis. Spatial and temporal expression of c-Fos is critical for cancer cells to undergo apoptosis after treatment with TRAIL/Apo-2L. We demonstrate that c-Fos as a pro-apoptotic molecule represses the anti-apoptotic gene, c-FLIP(L), by direct binding to c-FLIP(L)'s promoter region.

The next goal in this specific aim is to investigate the mechanisms that c-Fos "primes" cancer cells to undergo apoptosis.

Specific Aim #3: To determine the expression of DR4 and DR5 TRAIL receptors in early and advanced prostate cancer. We have obtained IRB approval to use our prostate tissue cancer bank for assessment of expression of DR4 and DR5 in early and advanced prostate cancer. Our initial results, which utilized paraffin embedded specimens, demonstrated variable intensity of DR4 and DR5 cellular membrane expression without any specific correlation to tumor stage or tumor grade. In a recent report on stage III colon cancer, investigators have found that expression level of DR4

and not DR5 correlates with disease free survival [14]. To further investigate whether there is any correlation between tumor stage and grade, we plan to also utilize fresh frozen prostate cancer samples to evaluate the expression of DR4 and DR5

KEY RESEARCH ACCOMPLISHMENTS

1. Identified that c-Fos is a key regulator of apoptotic pathway
2. Demonstrated that c-Fos binds and represses c-FLIP(L), a key anti-apoptotic molecule.
3. Demonstrated that in in-vitro and in-vivo experiments, activation of c-Fos can convert resistant cancer cells to become sensitive to the pro-apoptotic effects of TRAIL or HGS-ETR2.
4. Have submitted two manuscripts for publication. One of these manuscripts is provisionally accepted, pending minor changes.

REPORTABLE OUTCOMES

With support we received from DoD, we have submitted two manuscripts for publication based on our findings and the support received from the DoD.

CONCLUSIONS

Apoptotic pathways are altered in initiation and progression of most cancers, including prostate cancer, therefore, targeting apoptotic pathways for treatment of advanced prostate cancer is a rational approach. TRAIL-agonist compounds, like HGS-ETR2, which are effective against cancer cells but spare normal cells are ideal agents to fight cancer, because they have minimal associated cytotoxicity. Currently, HGS-ETR2 is in clinical trials for treatment of various malignancies. Therefore, it is important to differentiate between patients who harbor tumors that are sensitive as opposed to those with resistant tumors to pro-apoptotic agents like HGS-ETR2

In our progress report we have shown that activation of c-Fos plays a crucial role in mediating the response to TRAIL (or HGS-ETR2) by repressing the transcription of the c-FLIP(L) molecule. We plan to investigate the molecular mechanisms that differentiate between regulation of c-Fos in resistant and sensitive prostate cancer cells.

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c-Fos Promotes TRAIL-induced Apoptosis by Repressing c-FLIP(L)

Xiaoping Zhang[1], Hongmei Yang[1], Liang Zhang[1], Xu Huang[1], Hasan Otu[2], Towia Libermann[2], William C. DeWolf[1], Roya Khosravi-Far[3], Aria F. Olumi[1,4]

Division of Urologic Surgery[1], Center for Genomics[2] and Department of Pathology[3]

Beth Israel Deaconess Medical Center,

Harvard Medical School

Boston, MA 02215

[4]Corresponding Author:
330 Brookline Ave., Rabb 440
Boston, MA 02215
aolumi@bidmc.harvard.edu
(617) 667-4075 (phone)
(617) 975-5570 (fax)

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ABSTRACT

TRAIL/Apo-2L promotes apoptosis in cancer cells, while sparing normal cells. Although many cancers are sensitive to TRAIL-induced apoptosis, some evade the pro-apoptotic effects of TRAIL. Therefore, differentiating molecular mechanisms that distinguish between TRAIL-sensitive and TRAIL-resistant tumors are essential for effective cancer therapies. Here we show that c-Fos functions as a pro-apoptotic agent by repressing the anti-apoptotic molecule c-FLIP(L). c-Fos binds the c-FLIP(L) promoter, represses its transcriptional activity, and reduces c-FLIP(L) mRNA and protein levels. Therefore, c-Fos is a key regulator of c-FLIP(L), and activation of c-Fos determines whether a cancer cell will undergo cell death after TRAIL treatment. Strategies to activate c-Fos or inhibit c-FLIP(L) may potentiate TRAIL-based pro-apoptotic therapies.

INTRODUCTION

Aberrant apoptotic pathways contribute to initiation and progression of neoplasia, therefore pro-apoptotic agents can be used for treatment of various malignancies (1). Although many cancers are sensitive to pro-apoptotic agents like tumor necrosis factor (TNF), Fas ligand (FasL), and TNF-related apoptosis inducing ligand (TRAIL) (2), some develop resistance and apoptotic stimuli become ineffective (3). While, many apoptotic stimuli are associated with severe systemic cytotoxicity, limiting their clinical utility, TRAIL/Apo-2L has the unique feature of inducing apoptosis in cancer cells, with minimal cytotoxicity. Differentiating between cancers that are sensitive to TRAIL-induced apoptosis and cancers that are resistant to TRAIL-induced apoptosis can improve the efficacy of TRAIL-related compounds that are currently in clinical trials (4).

TRAIL-induced apoptosis may involve both extrinsic and intrinsic pathways and can be regulated by many important factors such as nuclear factor-κB (NF-κB), Akt, Bcl-2, Bax, XIAP, IAPs, Smac/DIABLO and c-FLIP (FLICE-like inhibitory protein) (5, 6). We previously demonstrated that expression of the anti-apoptotic molecule, c-FLIP(L), is necessary and sufficient to maintain resistance to TRAIL-induced apoptosis (7). Although expression of c-FLIP(L) can be regulated at the translational and post-translational levels, we have found that expression of c-FLIP(L) can also be partially regulated at the transcriptional level. In this report we demonstrate that transcription of c-FLIP(L) is repressed by the c-Fos oncoprotein.

Fos proteins are basic region-leucine zipper (bZIP) transcription factors that bind to Jun or other bZIP proteins and create the AP-1 dimer complex, which regulates gene expression (8). c-Fos has been shown to play an important role in development, inflammation and oncogenic processes. For example, TNF-family induction of c-Fos plays an important role in proper bone

development (9) and regulation of inflammatory response in arthritis (10). c-Fos, a well-established oncogene, is considered to play a critical role in tumorigenesis (11), proliferation and transformation (8), angiogenesis (12), tumor invasion and metastasis (13, 14), and its expression is associated with poor clinical outcomes (15). Therefore, c-Fos has preferentially been considered an anti-apoptotic molecule. However, recent evidence suggests that c-Fos may also have a pro-apoptotic role (13). The first indication of such pro-apoptotic function of c-Fos comes from the observation that c-Fos expression preceded apoptosis (16), and also observed during mammary gland involution (17), and other systems (18). However, pro-apoptotic down-stream molecular targets of c-Fos are poorly understood.

In this report we demonstrate that c-Fos has a novel pro-apoptotic function in TRAIL-induced apoptosis. We demonstrate that nuclear c-Fos primes cancer cells to undergo apoptosis, and its expression is necessary but insufficient for TRAIL-induced apoptosis. Spatial and temporal expression of c-Fos is critical for cancer cells to undergo apoptosis after treatment with TRAIL/Apo-2L. We demonstrate that c-Fos as a pro-apoptotic molecule represses the anti-apoptotic gene, c-FLIP(L), by direct binding to c-FLIP(L)’s promoter region.

MATERIALS AND METHODS

Materials

Horseradish peroxidase-conjugated secondary antibody (Ab) (goat-anti-mouse, goat-anti-rabbit, goat-anti-rat antibodies), biotinylated goat-anti-rabbit secondary antibodies, Oct-1 Ab, c-Fos Ab (D1 and D4), c-Jun Ab, Fos B Ab, Fra 1 Ab, Fra 2 Ab, Jun B Ab, Jun D Ab and c-Fos SiRNA were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Recombinant human TRAIL/TNFSF10 and caspase-8 inhibitor (Z-IETD) were obtained from R&D Systems Inc. (Minneapolis, MN). Caspase-8 Ab was obtained from Cell Signaling (Beverly, MA). Monoclonal Anti-FLIP Abs (Dava II) was obtained from Apotech Corp. (San Diego, CA). α-Tubulin Ab, Actinomycin D and 12-O-tetradecaoylphorbol-13-acetate (TPA) were purchased from Sigma (St. Louis, MO). GAPDH Ab was from Abcam, Inc. (Cambridge, MA). γ-^{32}p labeled ATP was purchased from Perkin Elmer (Boston, MA).

Cell Culture

PC3, LNCaP, A-498, 786-O, 769-P, MDA-MB231 and MDA-MB453 were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). PC3-TR is a TRAIL resistant subline of PC3 cells (7). SN12-PM6 was supplied by Dr. I. J. Fidler, M.D. Anderson Cancer Center (Houston, TX).

Cell viability

Cell viability was determined by MTT method (Roche Diagnostics, Indianapolis, IN) as previously described (7). Cells were then treated with various concentrations of TRAIL, or first

treated with Actinomycin D (5ug/ml) for one hour or TPA (100ng/ml) for 24 hours, then treated with TRAIL (100ng/ml).

Apoptosis assay

Apoptosis was detected by using fluorescein-conjugated Annexin V (Annexin V-FITC) kit according to manufacturer's protocol (BD Biosciences, Franklin Lakes, NJ).

Microarray assay

Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Chatsworth, CA). Micro-array analysis was performed using the Affymetrix HG-U133A chips according to previously described protocols (19). When comparing two groups of samples to identify genes enriched in a given group, we used the lower confidence bound (LCB) of the fold change (FC) since LCB has been shown to be a stringent estimate of differentially expressed genes (20).

Real-time quantitative RT-PCR, Western Blot Analyses and Immunofluorescence

Real-time quantitative RT-PCR, Western blot and immunofluorescence experiments were carried out as previously described (7, 21).

Xenograft Orthotopic Implantations and Immunohistochemical Analysis

Prostate and renal orthotopic implantations were carried out by injection of $1x10^6$ cells in either the postero-lateral lobe of the prostate or beneath the kidney capsule of athymic nude mice at 6-8 weeks of age. Mice were implanted with the following cells (Untreated group/ Treated group): PC3 (6/5), PC3-TR (7/5), LNCaP cells (5/6), SN12-PM6 (8/11**),** and A-498 (9/11)**.** After implantation of the xenografts 10 weeks for prostate cancer cells and 3 weeks for renal cancer

cells, the athymic nude mice were either untreated or treated with TRAIL-receptor 2 agonist antibody, HGS-ETR2 (Human Genome Science, Inc. (Rockville, MD)). HGS-ETR2 was injected via tail vein twice a week (10 mg/kg). Thirty days after treatment, the animals were euthanized and xenografts were harvested, and assessed for tumor size, metastasis, apoptosis and immunoreactivity for c-Fos.

Tumor tissues were fixed in 10% formalin and embedded in paraffin routinely. Histologic and immunohistochemistry were carried out as previously described (21).

Luciferase Assay

c-FLIP(L) promoter (–1179 to +281) luciferase structure was provided by Dr. W.S. El-Deiry, University of Pennsylvania (22). c-FLIP(L) promoter with the deletion of AP-1-(f) site was prepared by our lab. Briefly, c-FLIP(L) promoter (-1700,+300) was cloned from Bac-IP11-536I18 (Children's Hospital, Oakland Research Institute, Oakland, CA) with appropriate primers for PCR amplification. The primers used were: sense, 5'-CTC GAG TGA ACC TGG GAG GTT AAG GC-3'; antisense, 5'-AGA TCT GAG GCA AAG AAA CCG AAA GC-3', which contained an XhoI site and BglII site, respectively. The PCR products were inserted into pGEMT-Easy vector (Promega, Madison, WI). Once the sequence of the construct had been verified, it was subcloned into the PGL3-enhancer vector (Promega, Madison, WI) at XhoI and BglII sites. AP-1-(f) site binding site was deleted from the above c-FLIP(L)/PGL3-enhancer construct by QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The primers used were: sense, 5´-gag gcc gag gcg ggc aag gac cag cag ttg gag acc agc c-3'; antisense, 5'-ggc tgg tct cca act gct ggt cct tgc ccg cct cgg cct c-3'. The sequence of "***TCACTTGAGG***" was deleted and verified by DNA sequencing. Cells were seeded into 24-well plates. When cells

reached 80% confluence, both AP-1 luciferase reporter (25ng/well) and Ranilla reporter (5ng/well) from Stratagene (La Jolla, CA) or c-FLIP(L) reporter and Ranilla reporter were co-transfected into cells. In other experiments, when cell reached 70% confluence, c-Fos SiRNA, c-Fos or A-Fos were transfected into cells for 24 hours before transfection of luciferase and Renilla. Here Renilla served as an internal control for transfection efficiency. After 16 hours of transfection, cells were treated with TRAIL (100 ng/ml), and then both attached and floating cells were collected, prepared and further detected by using Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Samples were stored at -20°C until detection. All results represent average of at least three independent experiments ± SD.

Cell Extracts and Electrophoretic Mobility Shift Assay (EMSA)

Frozen cell pellets were resuspended in 4 volume of lysis buffer: 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 0.2 mM EGTA, 10% glycerol, 10 mM Na molybdate, 2 mM Na pyrophosphate, 2 mM Na orthovanadate, 0.5 mM spermidine, 0.15 mM spermine, 50 μM TPCK, 25 μM TLCK, 1 μg/mL each of aprotinin, pepstatin A, and leupeptin, 0.5 mM benzamidine, 1 mM DTT, and 0.5 mM PMSF. KCl was added to 400 mM final, and the extracts were incubated at 4°C for 30 min and centrifuged at 10,000 g for 5 min. The supernatant is whole cell extracts. The reactions were made using 3 μl of whole cell extract and 0.1–0.5 ng of ^{32}P-labeled double-stranded specific oligonucleotides (5,000–25,000 cpm) in the presence of 2 μg of poli dI–dC (Sigma, St. Louis, MO) in binding buffer (25 mM Tris, pH 8.0; 50 mM KCl; 6 mM MgCl2; 10% v/v glycerol). The reaction was incubated at room temperature for 20 min and run on 5-7% polyacrylamide gels containing 0.5x Tris glycine EDTA. For supershift EMSA experiments, the reaction was incubated for 15 minutes first, then 1 μl specific concentrated antibody (2 μg) was

added to each reaction and incubated for another 5 minutes. Gels were dried with Bio-Rad gel dryer (Hercules, CA) and imaged using Kodak BioMax MR Film (Fisher Scientific, Atlanta, GA). General AP-1 gel shift oligonucleotide was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Wild-type and mutant oligonucleotides with four-tandem repeats of the c-FLIP(L)'s AP-1-(f) site were designed as: 5'- at***ca***cttgagg at***ca***cttgagg at***ca***cttgagg at***ca***cttgagg -3' (wild-type) and 5'- at***tg***cttgagg at***tg***cttgagg at***tg***cttgagg at***tg***cttgagg -3' (mutant).

Transfection of c-Fos, A-Fos and SiRNA

Full length human c-Fos cDNA was provided by Dr. L Shenshedini, University of Toledo, OH (23). A-Fos vector was obtained from Dr. Charles Vinson (NCI) (24). The plasmids with or without c-Fos or A-Fos were transfected with Lipofectamine^{TM} 2000 (Invitrogen Life technologies, Carlsbad, CA). SiRNA of c-Fos was transfected into cells by TransMessenger^{TM} (QIAGEN, Valencia, CA). Non-specific SiRNA was used as control (QIAGEN, Valencia, CA). After transfection with the c-Fos or A-Fos vector for 24 hours or c-Fos SiRNA for 36-48 hours, the cells were seeded in 96-well plates for cell viability assays or treated with TRAIL.

CHIP Assay

ChIP assay was performed by the ChIP Assay Kit (Upstate Cell Signaling Solutions, Lake Placid, NY). Cells were cultured in 10 cm dishes, treated with or without TRAIL for 4 hours. Cross-linking of DNA and proteins was carried out by adding formaldehyde for final concentration of 1% and incubated for 10 minutes at 37°C. Both attached and floating cells were collected, washed, and resuspended in 200 µl of SDS lysis buffer for 10 minutes and then

sonicated. Samples were centrifuged for 10 minutes at 13,000rpm at 4°C and the supernatant was harvested. Concentration of each sample was quantitated using BCA protein assay. Positive controls were 10% of each DNA sample which did not included the IP step. The remainder of the samples was equally divided into two groups. The experimental group was immunoprecipitated with specific c-Fos (D-1) antibody, while the negative control group was immunoprecipitated with general IgG antibody. After IP, protein-DNA crosslinking was reversed. The isolated DNA was first purified, then amplified by PCR, using specific primers encompassing the c-FLIP(L) AP-1-(f) binding site (GeneBank). The primers of Figure 3G are 5'-cctgtgatcccagcactttg-3' (Forward primer) and 5'-caccatgcccgactaatttt-3' (Reverse primer).

RESULTS

c-Fos is differentially expressed in TRAIL-sensitive and TRAIL-resistant cancer cells

In order to determine whether alterations in transcription can affect TRAIL-induced apoptosis, TRAIL-resistant (PC3-TR and LNCaP) and TRAIL-sensitive (PC3) prostate cancer cells (Fig. 1A) were treated with TRAIL/Apo-2L (100 ng/ml) in the presence or absence of Actinomycin D (fig. S1), a general inhibitor of transcription. We found that Actinomycin D could affect the sensitivity of prostate cancer cells to TRAIL-induced apoptosis. Most notably, combination of Actinomycin D and TRAIL/Apo-2L enhanced apoptosis in TRAIL-resistant PC3-TR and LNCaP, suggesting that inhibition of cellular transcription can modulate TRAIL-induced apoptosis (fig. S1). Microarray analysis demonstrated that expression of c-Fos was significantly upregulated in the TRAIL-sensitive PC3 cells after treatment with TRAIL/Apo-2L for 4 hours. In contrast, expression of c-Fos was significantly down-regulated in the TRAIL-resistant PC3-TR and LNCaP cells (Table 1). Up-regulation of c-Fos mRNA was confirmed by quantitative RT-PCR. c-Fos mRNA was up-regulated in TRAIL-sensitive PC3 cells after treatment with TRAIL/Apo-2L for 4 hours or 8 hours, but not 24 hours, because most of the PC3 cells were dead after 24-hour treatment (Fig. 1B).

Spatial and temporal expression of *nuclear* c-Fos in TRAIL-sensitive prostate cancer cells

Although we found that c-Fos mRNA was significantly increased in the TRAIL-sensitive PC3 cells (Fig. 1B), we did not observe different total c-Fos protein levels in either TRAIL-sensitive or TRAIL-resistant cancer cells after TRAIL/Apo-2L treatment (Fig. 1C). Since c-Fos is a well-known transcription factor, we examined for the expression of nuclear c-Fos. In contrast to our findings for total c-Fos, we found that expression of *nuclear* c-Fos was increased spatially and temporally in the TRAIL-sensitive PC3 cells (Fig. 1D). Nuclear c-Fos was

increased after 10 minutes of TRAIL/Apo-2L treatment and reached its peak after 4 hours of treatment in the TRAIL-sensitive PC3 cells, however, nuclear c-Fos levels decreased at 24 hour after treatment because majority of PC3 cells were dead at this time point. However, there was no rise in the nuclear c-Fos levels in the TRAIL-resistant PC3-TR and LNCaP cells after TRAIL/Apo-2L treatment (Fig. 1D). Since the majority of the PC3 cells had undergone apoptosis after one hour of TRAIL/Apo-2L treatment, we wished to determine if nuclear c-Fos expression was maintained in the cells that had undergone apoptosis. We harvested the floating PC3 cells that had undergone apoptosis from the tissue culture medium after one and four hours of TRAIL/Apo-2L treatment, and determined that nuclear c-Fos was strongly expressed in the cells that had already undergone apoptosis (fig. S2). Therefore, *nuclear* c-Fos expression precedes initiation of apoptosis in TRAIL-sensitive cells, and is maintained in the cells after they have undergone apoptosis.

Immunofluorescence assessment of c-Fos expression in intact cells demonstrated that nuclear c-Fos was also expressed spatially and temporally in TRAIL-sensitive PC3 cells (Fig. 1E). After treatment with TRAIL/Apo-2L, nuclear c-Fos was increased immediately in the TRAIL-sensitive PC3 cells, but not in the TRAIL-resistant PC3-TR and LNCaP cells. Increased nuclear c-Fos in the PC3 cells was most pronounced at 10 minutes and 30 minutes after TRAIL/Apo-2L treatment, a time before the cells had undergone apoptosis as detected by Annexin V-FITC assay in flow cytometry (Fig.1F) and observed by microscopic evaluation (data not shown).

Spatial and temporal expression of nuclear c-Fos also happens in other TRAIL-sensitive cancer cells

To assure that the increased nuclear c-Fos we observed was not a PC3 cell-line or prostate cancer specific phenomenon, we evaluated other TRAIL-sensitive and TRAIL-resistant renal and breast cancer cells. We found that expression of nuclear c-Fos was also changed spatially and temporally prior to initiation of apoptosis in the TRAIL-sensitive renal (A-498) and breast (MDA-MB231) cancer cells, but not in the TRAIL-resistant renal and breast cancer cells (Fig. 2A-2B, and data not shown). Therefore, spatial and temporal expression of nuclear c-Fos appears to occur before the cells undergo apoptosis, and it is a common finding in different cancer cell types sensitive to TRAIL-induced apoptosis.

c-Fos "primes" cancer cells to undergo apoptosis

Increased expression of c-Fos has been observed during stress stimuli in many other systems (25). But it is unclear if increased c-Fos levels merely reflect a stress response or if activated c-Fos has a *direct* role in regulating apoptosis. We found that increased expression of nuclear c-Fos in the TRAIL-sensitive PC3 cells correlated with increased AP-1 luciferase activity (fig. S3) and AP-1 DNA binding detected by electrophoretic mobility gel-shift assay (fig. S4). The increased AP-1 DNA binding in the TRAIL-sensitive PC3 cells was associated specifically with increased binding of c-Fos and other c-Fos-related proteins (Fos B and Fra-2) but not with other members of the AP-1 family proteins like c-Jun, Fra-1, JunB and JunD (fig. S4B). Silencing expression of nuclear c-Fos by SiRNA (Fig. 3A) reduced AP-1 activity (Fig. 3B) and changed PC3 cells from TRAIL-sensitive to a more TRAIL-resistant phenotype (Fig. 3C). In addition, a dominant negative form of c-Fos, A-Fos (26), also reduced the AP-1 activity

in the PC-3 cells (Fig. 3D), and converted the viability of the PC3 cells from a TRAIL-sensitive to a TRAIL-resistant phenotype (Fig. 3E). On the other hand, ectopic expression of wt-c-Fos in PC3-TR and LNCaP cells enhanced AP-1 activity (fig. S5), led to nuclear localization of c-Fos (fig. S6), but did not promote apoptosis (Fig. 3F). However, the TRAIL-resistant cells (LNCaP and PC3-TR) were sensitized to TRAIL when c-Fos was ectopically expressed (Fig. 3F). Therefore, nuclear localization of c-Fos by itself is insufficient to promote apoptosis in cancer cells. This data demonstrates that c-Fos "primes" cancer cells to undergo cell death, and nuclear localization of c-Fos is necessary but insufficient for TRAIL-induced apoptosis.

c-Fos represses the expression of c-FLIP(L) directly

c-Fos expression and activity increases in the TRAIL-sensitive PC3 cells after TRAIL/Apo-2L treatment (Fig. 1, fig. S3-S4), whereas c-FLIP(L) expression in PC3 cells decreases after TRAIL/Apo-2L treatment (7). This inverse correlation between c-Fos and c-FLIP(L) proteins in the TRAIL-sensitive cells prompted us to examine whether the temporal and spatial expression of c-Fos in TRAIL-sensitive cells may affect the expression of the anti-apoptotic molecule, c-FLIP(L). After treatment with TRAIL/Apo-2L, c-FLIP(L) luciferase activity was reduced in the TRAIL-sensitive PC3 cells, whereas c-FLIP(L) luciferase activity was potentiated in the TRAIL-resistant PC3-TR and LNCaP cells (Fig. 4A). The reduced c-FLIP(L) luciferase activity correlated with reduced c-FLIP(L) protein levels in the TRAIL-sensitive cells (Fig. 4B). Silencing expression of c-Fos by SiRNA potentiated the c-FLIP(L) luciferase activity after treatment with TRAIL/Apo-2L in the TRAIL-sensitive PC3 cells, whereas the c-FLIP(L) luciferase activity was reduced when c-Fos expression was not silenced (Fig. 4C). Moreover, protein expression of c-FLIP(L) persisted when c-Fos expression was

either reduced (Fig. 3A and Fig. 4D) or its activity was suppressed by a c-Fos dominant negative, A-Fos (Fig. 4E). In contrast, c-FLIP(L) protein expression decreased when c-Fos was increased (fig. S7). This data suggests that c-Fos represses the expression of the anti-apoptotic molecule, c-FLIP(L).

To determine whether c-Fos has any direct transcriptional activity in regulating c-FLIP(L), we examined the potential AP-1 binding sites in the putative c-FLIP(L) regulatory region (17,000 base pairs upstream of c-FLIP(L)'s ATG start codon (Fig. 4F)). We identified and examined binding of c-Fos to 14 AP-1 binding sites in the putative c-FLIP(L) regulatory region, which were designated sites "a" through "n". We only detected binding of c-Fos protein to c-FLIP(L)'s AP-1-(f) site (Fig. 4G), a region of c-FLIP(L) shown to be regulated by other oncogenes (22). Chromatin immunoprecipitation (ChIP) assays demonstrated that binding of c-Fos to the c-FLIP(L) AP-1-(f) site increased in the TRAIL-sensitive PC3 cells, while c-Fos binding to the c-FLIP(L) AP-1-(f) site was reduced in the TRAIL-resistant PC3-TR and LNCaP cells after treatment with TRAIL/Apo-2L. In order to confirm the importance of c-Fos/AP-1 binding AP-1-(f) site on regulating c-FLIP(L) expression, we deleted this AP-1-(f) site in our c-FLIP(L) promoter luciferase reporter. We found that deletion of c-FLIP(L)'s AP-1-(f) site abolished the ability of c-Fos to suppress c-FLIP(L) expression (Fig. 4H).

To further determine whether binding to the c-FLIP(L)'s AP-1-(f) site was specific to AP-1-(f) DNA sequence, we designed a wild-type and mutant oligonucleotide with four-tandem repeats of the AP-1-(f) binding site. Electrophoretic mobility gel shift assays demonstrated that binding to the wild-type AP-1-(f) sequence was increased in the TRAIL-sensitive PC3 cells after treatment with TRAIL/Apo-2L, whereas there was minimal to no binding to the wild-type AP-1-(f) site in the TRAIL-resistant PC3-TR and LNCaP cells (fig. S8). In contrast, binding to the

mutant AP-1-(f) site was abolished — regardless of whether the cells were TRAIL-sensitive or TRAIL-resistant. This data further confirms that c-Fos protein binding to c-FLIP(L)'s AP-1-(f) site represses expression of the c-FLIP(L) gene and sensitizes cancer cells to undergo TRAIL-induced apoptosis. Deletion and mutations of the c-FLIP(L) AP-1(f) promoter region abrogates c-Fos' ability to repress the anti-apoptotic molecule, c-FLIP(L).

Expression of c-Fos is caspase-8 dependent

Since caspase-8 is one of the early activated and essential caspase proteins in death domain receptor induced apoptosis, we wished to determine if nuclear localization of c-Fos is caspase-dependent. We found that caspase-8 is activated and nuclear c-Fos is increased, after treatment with TRAIL/Apo-2L in the TRAIL-sensitive PC3 cells. Nevertheless, inhibition of caspase-8 by Z-IETD-fmk converted the TRAIL-sensitive PC3 cells to become TRAIL-resistant, and abrogated nuclear expression of c-Fos (fig. S9-S10). Therefore, nuclear expression of c-Fos is caspase-8 dependent.

Synthetic induction of c-Fos promotes apoptosis

Next, we wished to determine if synthetic induction of c-Fos promotes nuclear localization of c-Fos and potentiates the pro-apoptotic activity of TRAIL/Apo-2L — possibly improving its therapeutic efficacy in cancer cells resistant to TRAIL-induced apoptosis. 12-O-Tetradecanoylphorbol-13-acetate (TPA) has been shown to activate protein kinase-C (PKC), JNK pathways, c-Fos and other AP-1 family proteins in order to promote differentiation, cell cycle arrest and apoptosis in a variety of cell model systems (27-29). Moreover, TPA has been shown to promote apoptosis (30) and enhance the therapeutic effects of radiation in LNCaP

prostate cancer cells (31). Because TPA activates the c-Fos/AP-1 family of genes, we wished to determine whether TPA could sensitize the TRAIL-resistant PC3-TR and LNCaP cells to undergo apoptosis. We found that c-Fos protein and AP-1 activity of the LNCaP cells, and not PC3-TR cells, were upregulated by a non-toxic dose of TPA (Fig. 5A & B, and fig. S11). Treatment of LNCaP cells with TPA alone induced nuclear localization of c-Fos (Fig. 5C), but did not induce a significant amount of cell death (Fig. 5D). This data, combined with our earlier results (Fig. 3F and fig. S6), again suggests that nuclear localization of c-Fos "primes" cancer cells to undergo apoptosis. Further, our data suggest that nuclear localization of c-Fos is necessary for cancer cells to undergo TRAIL-induced apoptosis, but nuclear localization of c-Fos by itself is insufficient to promote apoptosis in cancer cells.

We observed cell death in LNCaP cells when TRAIL/Apo-2L was used in combination with TPA (Fig. 5D), a finding that was not observed when LNCaP cells were treated with TRAIL/Apo-2L alone. *Spatial* increase of *nuclear* c-Fos after TPA treatment was specific only to the LNCaP cells, and was not observed in the PC3-TR cells. Spatial increase of nuclear c-Fos was associated with converting the phenotype of the TRAIL-resistant LNCaP cells to a more sensitive phenotype (Fig. 5D). However, combination of TPA and TRAIL/Apo-2L had little effect on the phenotype of PC3-TR cells to undergo apoptosis. TPA, therefore, is capable of converting the phenotype of some, but not all, cancer cells from TRAIL-resistant to a more TRAIL-sensitive phenotype by inducing c-Fos/AP-1.

c-Fos translocates to the nucleus in TRAIL-sensitive orthotopic xenografts

To determine whether the spatial and temporal expression of c-Fos that we observed in-vitro in the TRAIL-sensitive cancer cells was also found in in-vivo models, we orthotopically implanted prostate cancer cells (PC3, PC3-TR and LNCaP) and renal cancer cells (SN12-PM6, A-498) in the postero-lateral lobe of the prostate and under the kidney capsule of athymic nude mice, respectively. After orthotopic implantation of the xenografts (21), the athymic mice were treated with a TRAIL-receptor 2 agonist antibody (HGS-ETR2) which is currently in clinical trials for advanced malignancies (4). Primary tumors were harvested and assessed for expression of c-Fos (Fig. 6). We found that expression of nuclear c-Fos was pronounced in the HGS-ETR2-sensitive PC3, SN12-PM6 and A-498 xenografts, but not in the HGS-ETR2-resistant PC3-TR and LNCaP xenografts. These findings suggest that nuclear expression of c-Fos is found not only in TRAIL-sensitive in-vitro models, but also in orthotopic in-vivo models after treatment with TRAIL-receptor agonist compounds. Potentially, nuclear localization of c-Fos could be used to identify human cancers that are sensitive to TRAIL-induced apoptosis.

DISCUSSION

Since the AP-1 family member protein, c-Fos, plays a crucial role in a variety of biological processes, identifying the down-stream targets of c-Fos has significant implications in understanding of normal development, inflammation and oncogenesis (10). In this report we demonstrate that c-Fos, in addition to its well-known oncogenic function, has a novel pro-apoptotic function in TRAIL-induced apoptosis. c-Fos exerts its pro-apoptotic function by repressing c-FLIP(L) in a caspase dependent manner (Fig. 7). We define Fos-dependent priming (FDP) as *spatial* and *temporal* expression of *nuclear* c-Fos after treatment with TRAIL/Apo-2L. Clinical implications of these results include the possibility of using FDP as a marker in cancer patients being treated with pro-apoptotic agents. Presence of FDP may identify tumors that are sensitive to pro-apoptotic stimuli, whereas lack of FDP identifies resistant tumors. Strategies to potentiate FDP or inhibit c-FLIP(L) can enhance the efficacy of pro-apoptotic agents for treatment of various malignancies.

Caspases are important modulators of apoptosis (for review see (32)). Activation of specific death domain receptors, like DR4 and DR5 by their ligand, TRAIL, promotes formation of death-inducing signaling complex (DISC). DISC recruits an adaptor molecule, Fas-associated death domain (FADD), which in turn interacts with and activates caspase-8 and/or caspase-10, leading to initiation of the extrinsic pro-apoptotic signalling pathway. Because of its sequence homology with caspase 8, c-FLIP(L) has been shown to competitively inhibit the interaction between FADD and caspase 8, and thus inhibiting the initiation of pro-apoptotic stimuli. We and others (7, 33, 34) have shown that persistent expression of c-FLIP(L) is necessary and sufficient to maintain resistance to TRAIL-induced apoptosis. Here, we demonstrate that c-Fos represses expression of c-FLIP(L) in a caspase-8 dependent manner. Therefore, for a cancer cell to

undergo apoptosis after TRAIL treatment is dependent on a feedback loop mechanism as determined by activation of c-Fos, c-FLIP(L) and caspase 8 (Fig. 7).

Activation of c-Fos by other TNF family signalling pathways, besides TRAIL, has been implicated in the past. For example, the TNF receptor member, Receptor Activator of NF-κB Ligand (RANKL), is a key regulator of bone homeostasis. RANKL induces expression of c-Fos, an important step in proper bone development. In order to maintain proper balance in bone development, c-Fos activates its own inhibitor, interferon-beta, to reduce RANKL signalling. Thus, an auto-regulatory mechanism involving c-Fos, the TNF receptor family member, RANKL, and interferon-beta play a crucial role in proper bone development (9). In the present study we identified a similar auto-regulatory mechanism that involves c-Fos in cancer cells undergoing TRAIL-induced apoptosis.

c-Fos is one of immediate-early response and inducible transcription factors. Its level is increased after many stress stimuli including some pro-apoptotic stimuli. For example, c-Fos levels have been increased after chemotherapy (35), ultraviolet radiation (36, 37) and TNF-α exposure (38). Few studies have noted increased levels of c-Fos after TRAIL treatment (39, 40), however, the function of c-Fos in these biological settings have not been clearly defined.

We postulate that post-translational modifications of c-Fos play an important role which determines whether cancer cells are sensitive or resistant to TRAIL-induced apoptosis. In our in-vitro and orthotopic in-vivo studies, we demonstrated that nuclear translocation of c-Fos and repression of c-FLIP(L) gene is an important process in promoting TRAIL-induced apoptosis in cancer cells. Cellular localization and activation of c-Fos can depend on its phosphorylation, protein stability and other chaperone proteins. Recent work has suggested that phosphorylation of c-Fos, which is an important determinant of its activity and expression, is tightly regulated by

a variety of kinases such as MAPK (41), FRK (42), RSK2 (43), CKII (44) and PDK1 (45). Protein stability of c-Fos, another regulator of its physiologic function, has been shown to be dependent on its C-terminal PEST3 domain which modulates c-Fos' proteosome mediated degradation (46). Associated proteins in the form of chaperone proteins or hetero-dimers can also regulate c-Fos' structure and function. For example, we have found that in the presence of the proteosome inhibitor, MG-132, c-Fos forms more stable hetero-dimers with c-Jun, another member of the AP-1 family, and sensitizes TRAIL-resistant cancer cells to become TRAIL-sensitive (manuscript in preparation). Therefore, we believe that c-Fos' post-translational modifications can significantly affect its ability to regulate c-FLIP(L) gene expression and TRAIL-induced apoptosis, and it's an area under investigation in our laboratory.

The c-FLIP family of proteins is homologous to pro-caspase 8 (for review see (32)). Both c-FLIP(L) and c-FLIP(s), and perhaps the newly detected c-FLIP(r) (47), can bind to the DED domains of FADD and caspase 8 and regulate apoptosis through their interference with the recruitment of caspase 8 to FADD. Most reports suggest that c-FLIP(L) has an anti –apoptotic role, largely due to results from experiments utilizing ectopic expression of c-FLIP(L). Moreover, c-FLIP(L) -/- mouse embryonic fibroblasts are more sensitive to pro-apoptotic agents, which strongly suggests that c-FLIP(L) has an anti-apoptotic function (48). However, some recent reports suggest that c-FLIP(L) may have a dual function, a pro-apoptotic function at low physiologic concentrations and an anti-apoptotic function at high cellular concentrations (49). In accordance with c-FLIP(L)'s role as an anti-apoptotic molecule, we have found that persistent expression of c-FLIP(L) is necessary and sufficient to maintain resistance to TRAIL-induced apoptosis. Although regulation of c-FLIP(L) can occur at the translational and post-translational levels, we found that transcriptional regulation of c-FLIP(L) may also affect cancer cells'

sensitivity to TRAIL-induced apoptosis (7). Other investigators have shown that NF-κB (50, 51), c-Myc (22), nuclear factor of activated T cells (NFAT) (52) and even androgen receptor response elements (53) may regulate expression of c-FLIP(L) through direct or indirect mechanisms. Here, we show that c-Fos directly binds the AP-1-(f) site of the c-FLIP(L) gene (Fig. 4G) and represses expression of c-FLIP(L) and promotes TRAIL-induced apoptosis. In contrast, deletion of the AP-1-(f) site abrogates binding of c-Fos, leading to enhancement of c-FLIP(L) gene expression and resistance to TRAIL-induced apoptosis. The AP-1-(f) site lies within a CpG island (Fig. 4F), therefore, methylation patterns in this site may regulate the direct interaction between c-Fos protein and the c-FLIP(L) gene, particularly since we have found that the transcriptional start site of c-FLIP(L) gene includes the AP-1-(f) site (data not shown).

In conclusion, we have demonstrated that c-Fos has a pro-apoptotic function by repressing the anti-apoptotic molecule, c-FLIP(L). FDP is necessary but insufficient for TRAIL-induced apoptosis. We believe that presence of FDP identifies cancers that are sensitive, while lack of FDP identifies cancers that are resistant to TRAIL-induced apoptosis. Strategies to enhance FDP or repress c-FLIP(L) can improve the efficacy of TRAIL mediated cancer therapies that are currently in clinical trials.

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Figure Legends

Fig. 1. Nuclear c-Fos is upregulated spatially and temporally in TRAIL-sensitive cancer cells after TRAIL treatment. **(A)** Cell viability of prostate cancer cells was detected by MTT assay. Cells were treated with TRAIL/Apo-2L with different doses for 24 hours. **(B)** Real-time quantitative RT-PCR of c-Fos. mRNA levels of control samples were set at 1, and mRNA of experimental samples were compared to the each corresponding control mRNA. " ***** " denotes significant differences compared with control ($p<0.01$). **(C)** Western blot of c-Fos in whole cell lysates after treatment with TRAIL in prostate cancer cells. GAPDH is used as loading control. **(D)** Western blot of nuclear c-Fos after treatment with TRAIL in prostate cancer cells. "Control" represents the whole cell lysate extract. Oct-1 is used as loading control of nuclear extracts. GAPDH is used as an index of quantity and quality of nuclear extracts, since majority of GAPDH exists in cytoplasm. **(E)** Time-course immunofluorescence of c-Fos in prostate cancer cells. Arrowheads show examples of nuclear c-Fos staining. FITC immunostaining assesses for c-Fos (Green), Hoechst (Blue) for nuclear staining, and merged pictures represent overlap of c-Fos and nuclear staining images. Negative control samples exclude the c-Fos primary antibody. " **'** " and " **h** " stand for minute and hour, respectively. Cells were imaged at 600 X magnification. **(F)** Time-course analysis of apoptosis in prostate cancer cells by Annexin V-FITC staining. Cells that stain positive for Annexin V-FITC and negative for Propidium Iodide (PI) are set as apoptotic cells. Error bars in above figures represent average of at least three independent experiments ± SD.

Fig. 2. c-Fos nuclear translocation also occurs in TRAIL-sensitive breast cancer cells. **(A)** Dose-dependent cell viability of breast cancer cells was detected by MTT assay. Cells were treated

with TRAIL/Apo-2L for 24 hours. Data represents average of at least three independent experiments ± SD. **(B)** Time-course immunofluorescence of c-Fos in breast cancer cells.

Fig. 3. Expression of c-Fos affects sensitivity to TRAIL-induced apoptosis. **(A)** Nuclear c- Fos in PC3 cells was analyzed by Western blot after using SiRNA of c-Fos in the absence or presence of TRAIL. Cells were transduced with c-Fos SiRNA for 36 hours then treated with TRAIL (100ng/ml) for 4 hours. Oct-1 is loading control for nuclear extracts. **(B)** AP-1 luciferase activity and **(C)** cell viability assays, with control non-specific SiRNA (-) or with c-Fos SiRNA (+), before and after TRAIL treatment. **(D)** AP-1 luciferase activity and **(E)** cell viability assays with transfection of dominant negative c-Fos, A-Fos, and TRAIL treatment. **(F)** Cell viability of the TRAIL-resistant PC3-TR and LNCaP cells without (-) and with (+) c-Fos ectopic expression, and treatment with TRAIL. "*****" denotes significant differences between control and experimental samples. Error bars indicate SD of at least three replicate experiments. c-Fos transfection (-) or A-Fos transfection (-) refer to empty vector control transfections for figures D and F.

Fig. 4. c-Fos represses expression of c-FLIP(L) by direct binding to its promoter region. **(A)** Fold change in luciferase activity of c-FLIP(L) promoter region. Luciferase activity of control samples was set arbitrarily at 1 and fold increases or fold decreases are represented accordingly. **(B)** Western blot of c-FLIP(L) level in prostate cancer cells after TRAIL treatment for 24 hours. α-Tubulin is used as loading control. **(C)** Fold change in luciferase activity of c-FLIP(L) promoter region in the absence or presence of c-Fos SiRNA and treatment with TRAIL. **(D)** & **(E)** Expression of c-FLIP(L) protein in the absence or presence of c-Fos SiRNA (D) or dominant negative c-Fos (A-Fos) (E). **(F)** AP-1 binding sites on putative promoter region of c-FLIP(L)

before the ATG start-codon. “a ” through “ n” are 14 AP-1 binding sites we analyzed by CHIP assay. **(G)** Result of AP-1-(f) binding to c-FLIP(L) promoter analyzed by CHIP assay. Negative controls are samples using non-specific IgG, positive controls are whole cell lysates without the immunoprecipitation step, and experimental samples include the CHIP assay using c-Fos antibody. **(H)** c-FLIP(L) promoter luciferase activity after deletion of AP-1-(f) in the presence and absence of TRAIL/Apo-2L. “Wt” and “Del” stand for wild-type c-FLIP(L) promoter luciferase reporter and deletion of AP-1-(f) site from c-FLIP(L) promoter luciferase reporter, respectively. “ ***** ” and “ **#** ” denote significant difference between control and experimental samples.

Fig. 5. TPA induces the expression of c-Fos, activates AP-1 activity and sensitizes the TRAIL-resistant LNCaP cells. **(A)** & **(B)** c-Fos protein and AP-1 activity in PC3-TR and LNCaP cells was analyzed by Western blot and luciferase assay, respectively. Cells were treated with either TPA or TRAIL for 24 hours alone, or pretreated with TPA for 24 hours followed by TRAIL treatment for another 24 hours. **(C)** Immunohistochemical studies demonstrate that nuclear c-Fos is detectable in LNCaP cells 24 hours after pretreatment with TPA regardless of TRAIL treatment. **(D)** Cell viability in PC3-TR and LNCaP cells after pretreatment with TPA for 24 hours with or without TRAIL. Cell viability of each control was set at 100% prior to adding TPA or TRAIL, and the cell viability of each experimental sample was compared to the control. “ * ” indicates significant differences between the control and the experimental samples.

Fig. 6. Immunohistochemical analysis of orthotopically implanted prostate cancer cells (PC3, PC3-TR and LNCaP) and renal cancer cells (SN12-PM6 and A-498). SN12-PM6 and A-498

cells are TRAIL-sensitive cells and xenografts based on in-vitro and in-vivo experiments (data not shown). TRAIL-sensitive PC3 (prostate), SN12-PM6 and A-498 (renal) xenografts demonstrate increased c-Fos nuclear levels after treatment with the TRAIL-receptor 2 agonist antibody, HGS-ETR2. Treatments of xenografts with HGS-ETR2, effectively reduced the size of the tumors in the PC3, SN12-PM6 and A-498 xenografts (PC3 xenografts' median weight: 887 mg for controls vs. 104 mg for treated group, $p<0.004$; SN12-PM6 xenografts' median weight: 713 mg for controls vs. 340 mg for treated group, $p<0.05$; A-498 xenografts' median weight: 53 mg for controls vs. 0mg for treated group, $p<0.05$). In contrast, neither the tumor size nor nuclear localization of c-Fos was affected in the TRAIL-resistant LNCaP and PC3-TR xenografts after treatment with HGS-ETR2 (LNCaP xenografts' median weight: 3570 mg in controls vs. 2250 mg for treated group, p – N.S.; PC3-TR xenografts' median weight: 120 mg for controls vs. 124 for controls, p – N.S.). Black bar represents approximately 50 μm.

Fig. 7. Model depicting the feedback-loop mechanism between c-Fos and c-FLIP(L) that is caspase 8-dependent. Arrows and "+" represent activation, while block arrows and "- " represent inhibition of the downstream molecules. Dashed line represents "priming" of cancer cells to undergo TRAIL-induced apoptosis.

Table 1. Differentially expressed genes in TRAIL-sensitive and TRAIL-resistant cells. cDNA microarray analysis of genes upregulated in TRAIL-sensitive PC3 cells, but down-regulated in TRAIL-resistant PC3-TR and LNCaP cells. Genes expressed in cells treated (T) with TRAIL (100 ng/ml) for 4 hours were compared with untreated (U) controls. Lower confidence bound (LCB) was used as a measure of gene expression. Remainder of the gene expression analysis data available for uploading in the supplement.

Genes	PC3 U	PC3 T	LCB	PC3-TR U	PC3-TR T	LCB	LNCaP U	LNCaP T	LCB
SOCS box-containing WD protein SWiP-1	91.61	155.32	1.54	210.78	102.14	-1.85	307.6	103.79	-2.67
Notch homolog 3 (Drosophila)	95.16	231.15	2.22	90.97	37.69	-1.63	126.91	49.19	-2.2
v-fos FBJ murine osteosarcoma viral oncogene homolog	-0.07	53.02	**2.57**	93.08	-3.34	**-4.55**	61.81	7.13	**-2.2**
collagen, type VI, alpha 1	718.18	1790.2	2.16	235.85	136.6	-1.55	401.67	240.52	-1.51
myosin VIIB	33.06	92.81	2.53	32.59	17.05	-1.52	52.44	20.92	-1.92
hypothetical protein FLJ14360	14.36	66.69	3.79	27.88	5.79	-2.26	25.29	9.5	-1.53

Figure 1

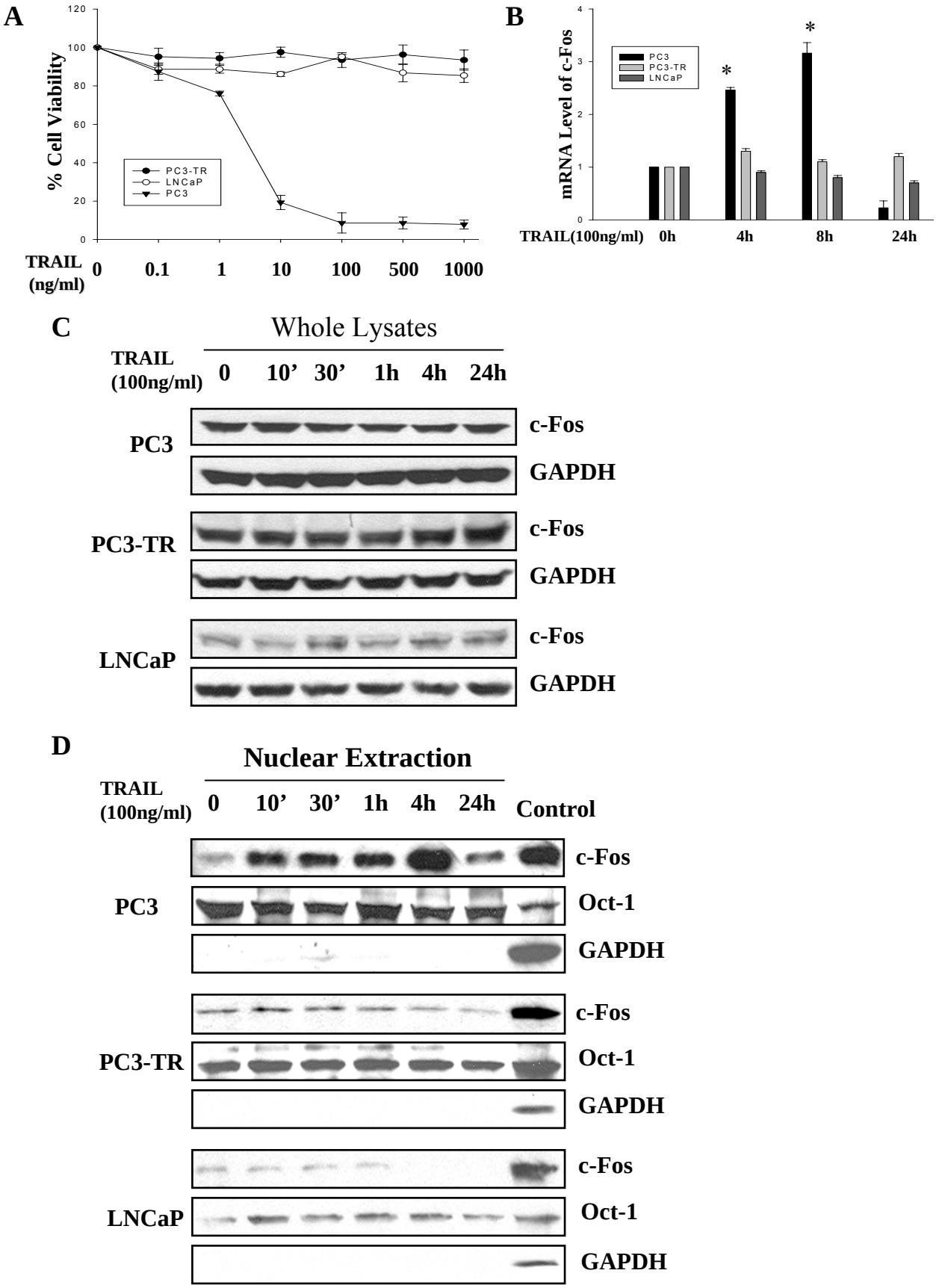

A
% Cell Viability
120
100
80
60
40
20
0
PC3-TR
LNCaP
PC3
TRAIL (ng/ml)
0
0.1
1
10
100
500
1000
B
mRNA Level of c-Fos
4
3
2
1
0
PC3
PC3-TR
LNCaP
*
*
TRAIL(100ng/ml)
0h
4h
8h
24h
C
Whole Lysates
TRAIL (100ng/ml)
0
10'
30'
1h
4h
24h
PC3
c-Fos
GAPDH
PC3-TR
c-Fos
GAPDH
LNCaP
c-Fos
GAPDH
D
Nuclear Extraction
TRAIL (100ng/ml)
0
10'
30'
1h
4h
24h
Control
PC3
c-Fos
Oct-1
GAPDH
PC3-TR
c-Fos
Oct-1
GAPDH
LNCaP
c-Fos
Oct-1
GAPDH

Figure 1 (Cont.)

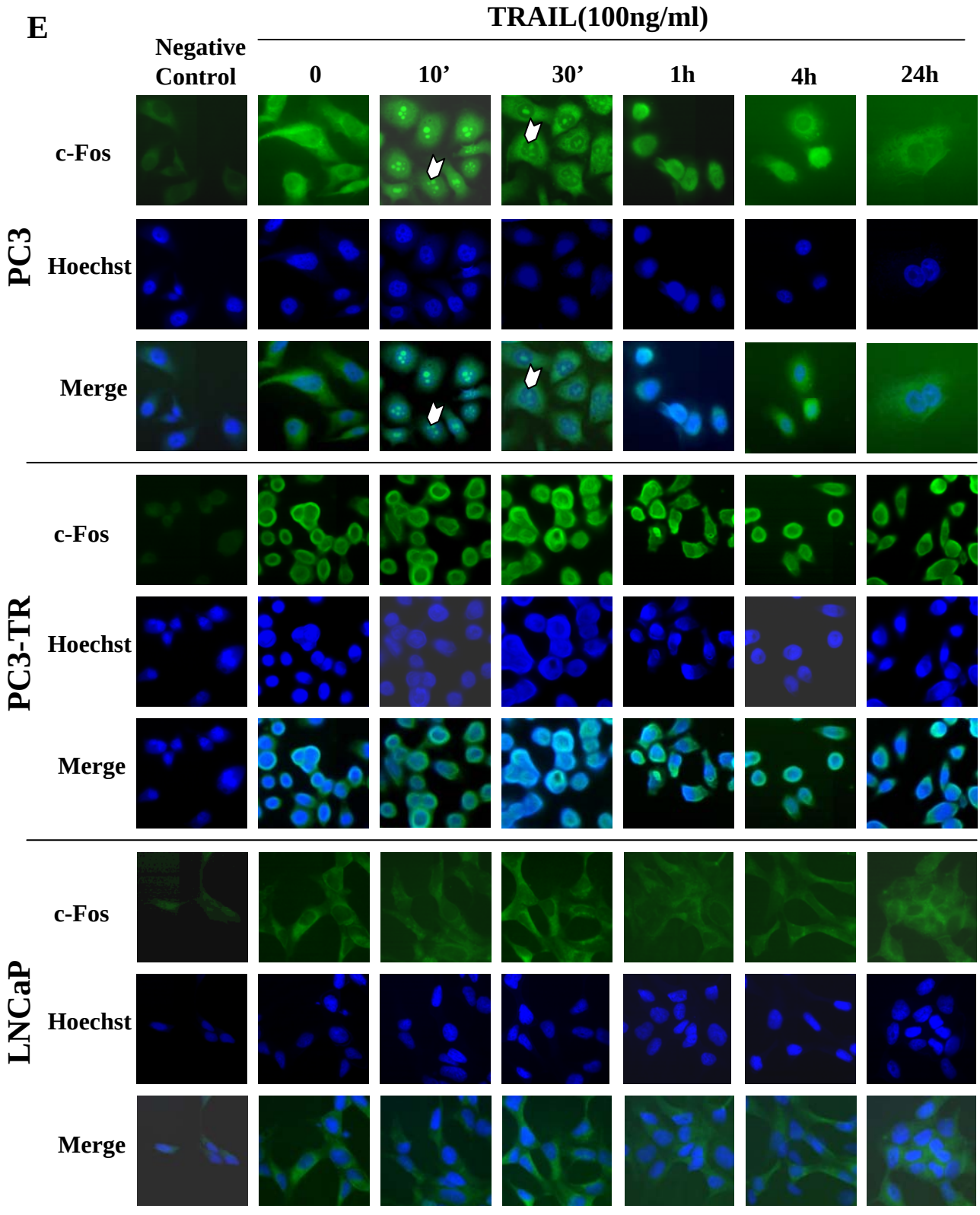


Figure 1 (Cont.)

F

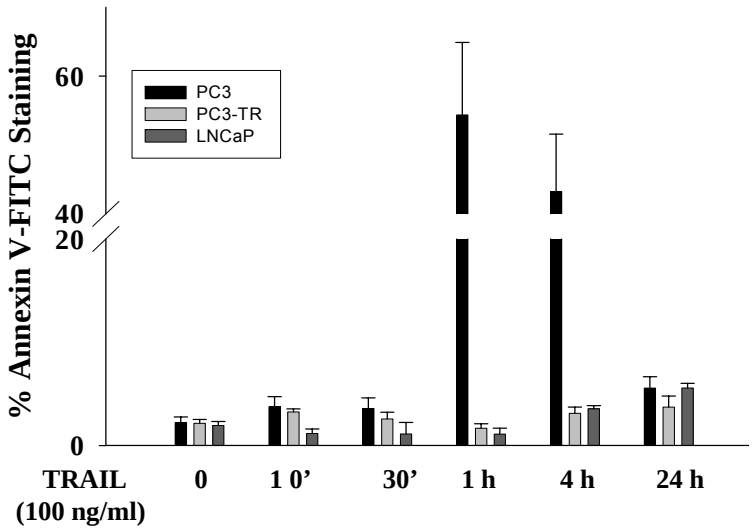
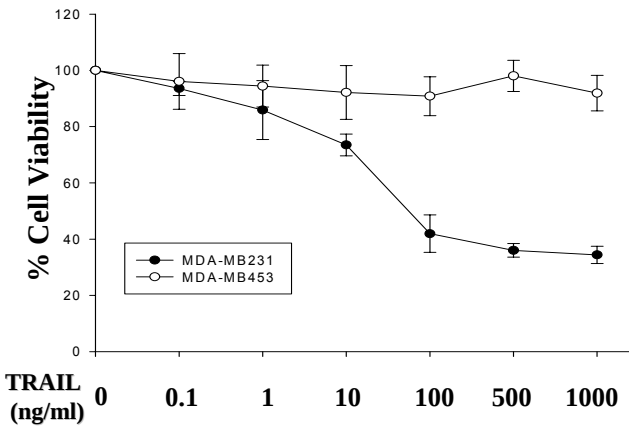


Figure 2

A


% Cell Viability
120
100
80
60
40
20
0
MDA-MB231
MDA-MB453
TRAIL (ng/ml)
0
0.1
1
10
100
500
1000

B

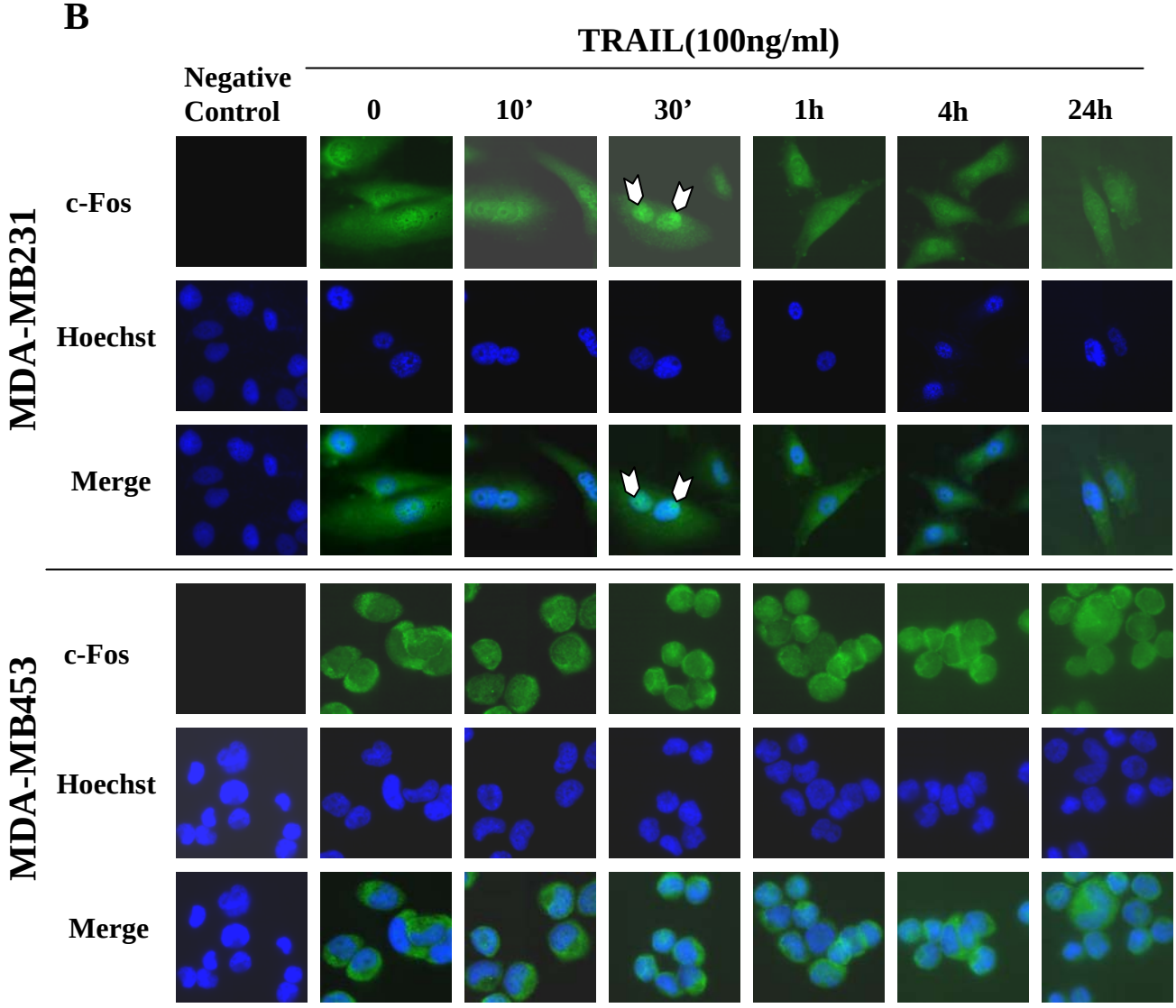

TRAIL(100ng/ml)
Negative Control
0
10'
30'
1h
4h
24h
MDA-MB231
c-Fos
Hoechst
Merge
MDA-MB453
c-Fos
Hoechst
Merge

Figure 3

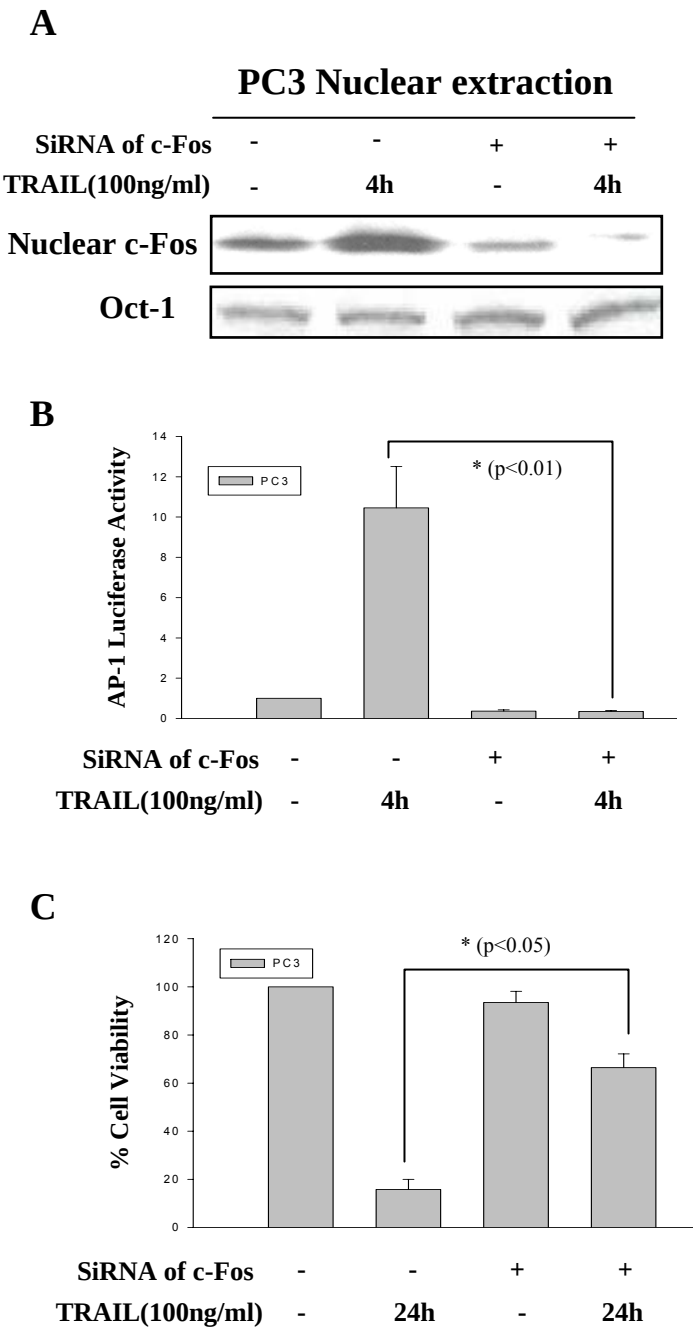
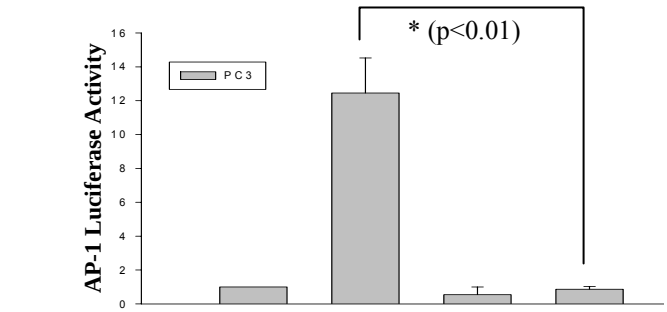

A
PC3 Nuclear extraction
SiRNA of c-Fos - - + +
TRAIL(100ng/ml) - 4h - 4h
Nuclear c-Fos
Oct-1
B
AP-1 Luciferase Activity
PC3
* (p<0.01)
SiRNA of c-Fos - - + +
TRAIL(100ng/ml) - 4h - 4h
C
% Cell Viability
PC3
* (p<0.05)
SiRNA of c-Fos - - + +
TRAIL(100ng/ml) - 24h - 24h

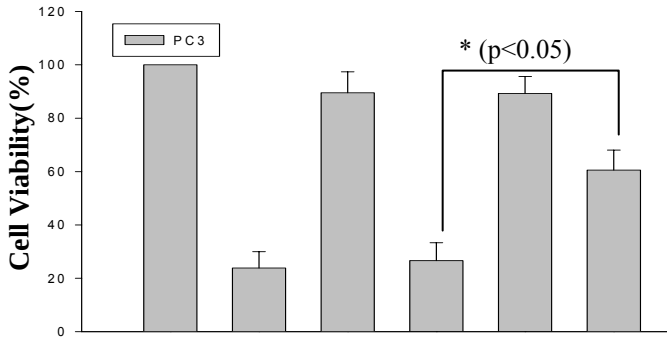
Figure 3 (Cont.)

D



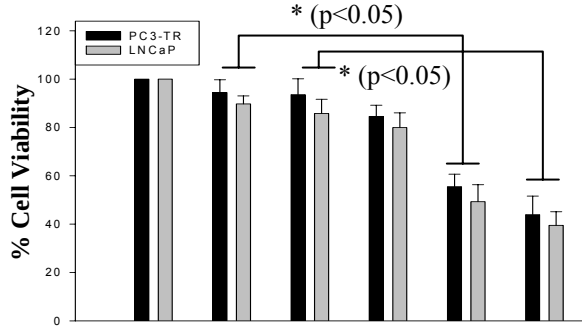
A-Fos Transfection	-	-	+	+
TRAIL(100ng/ml)	-	4h	-	4h

E



Vector	-	-	+	+	-	-
A-Fos Transfection	-	-	-	-	+	+
TRAIL(100ng/ml)	-	24h	-	24h	-	24h

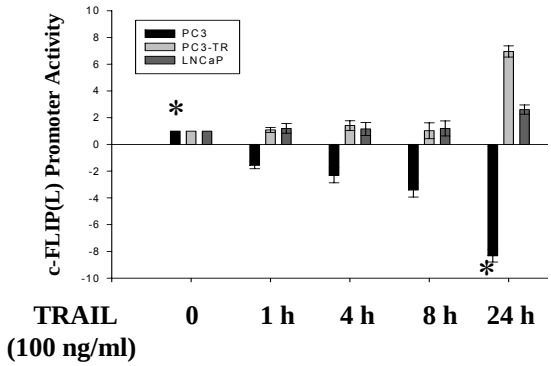
F



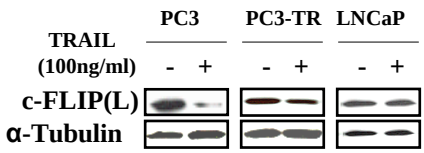
c-Fos Transfection	-	-	-	+	+	+
TRAIL(100ng/ml)	-	24h	48h	-	24h	48h

Figure 4

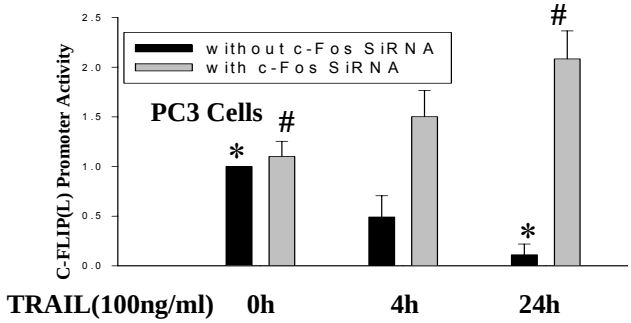
A



B



C



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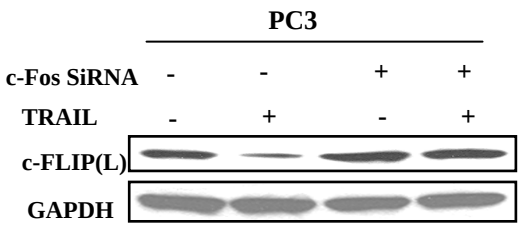
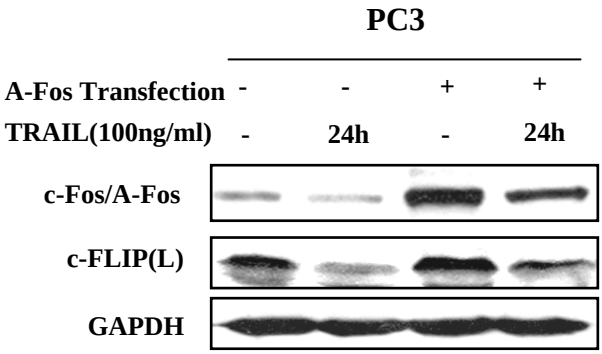


Figure 4 (Cont.)

E



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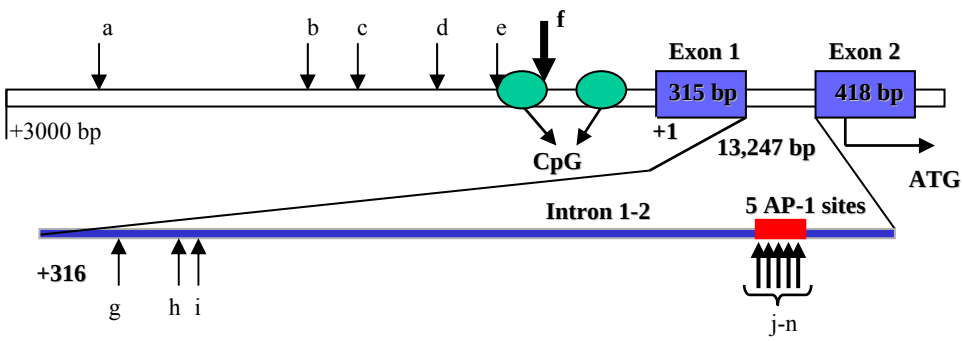
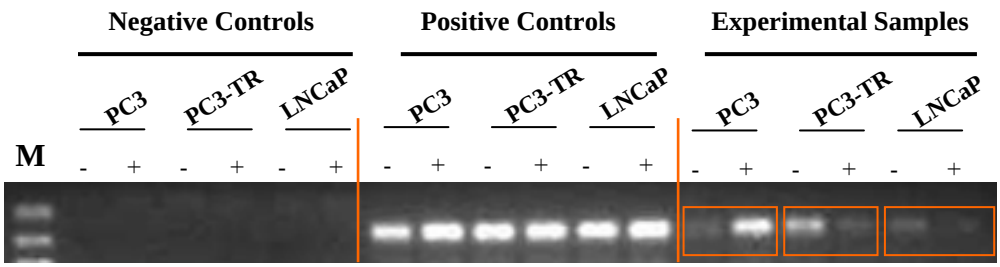


Figure 4 (Cont.)

G



H

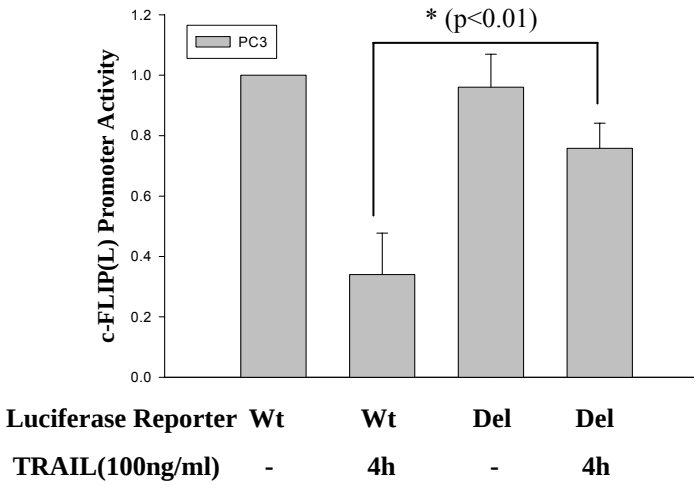
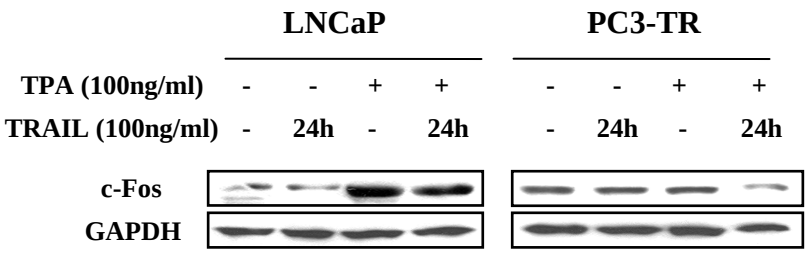


Figure 5

A


LNCaP
PC3-TR
TPA (100ng/ml) - - + + - - + +
TRAIL (100ng/ml) - 24h - 24h - 24h - 24h
c-Fos
GAPDH

B

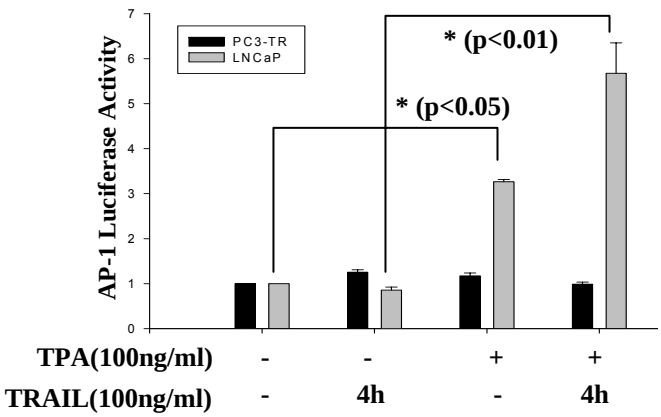
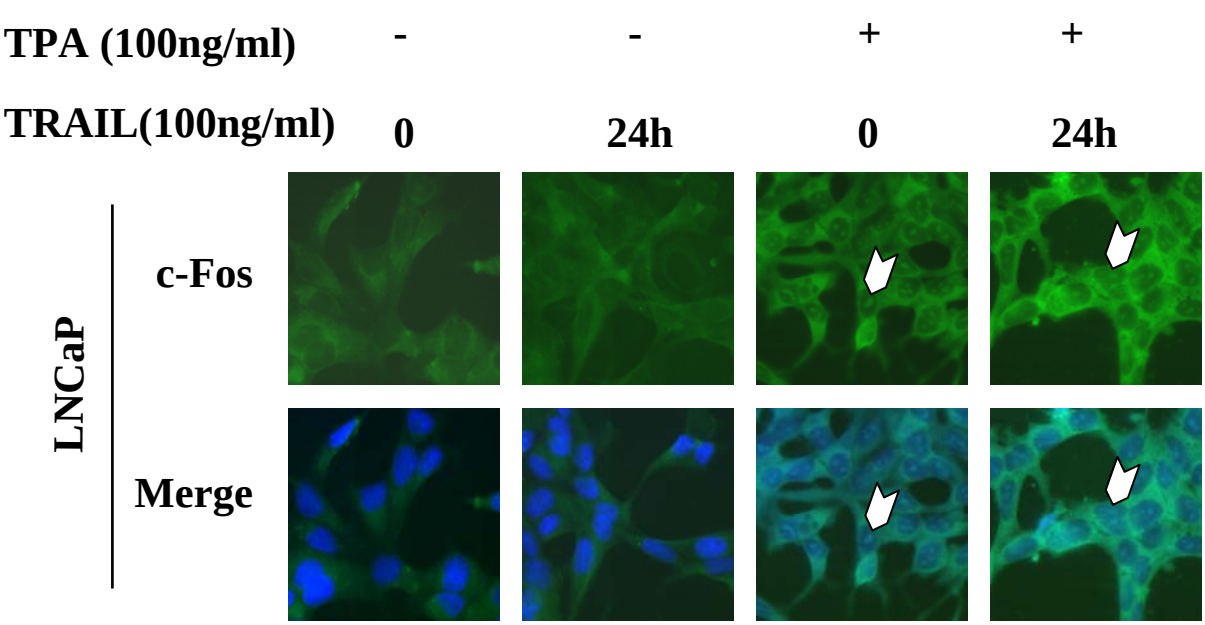

PC3-TR
LNCaP
AP-1 Luciferase Activity
0
1
2
3
4
5
6
7
* (p<0.01)
* (p<0.05)
TPA(100ng/ml) - - + +
TRAIL(100ng/ml) - 4h - 4h

Figure 5 (Cont.)

C



D

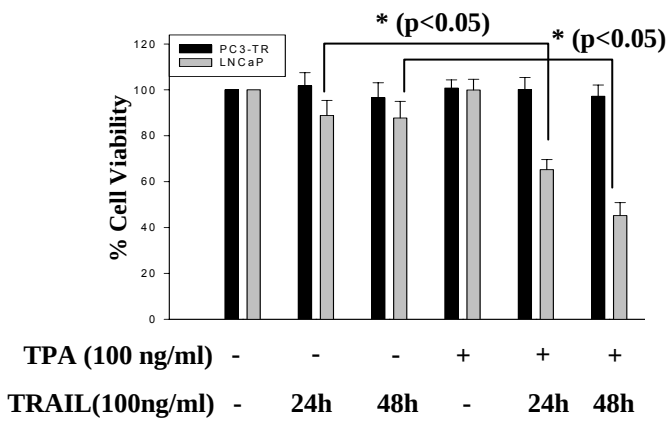


Figure 6

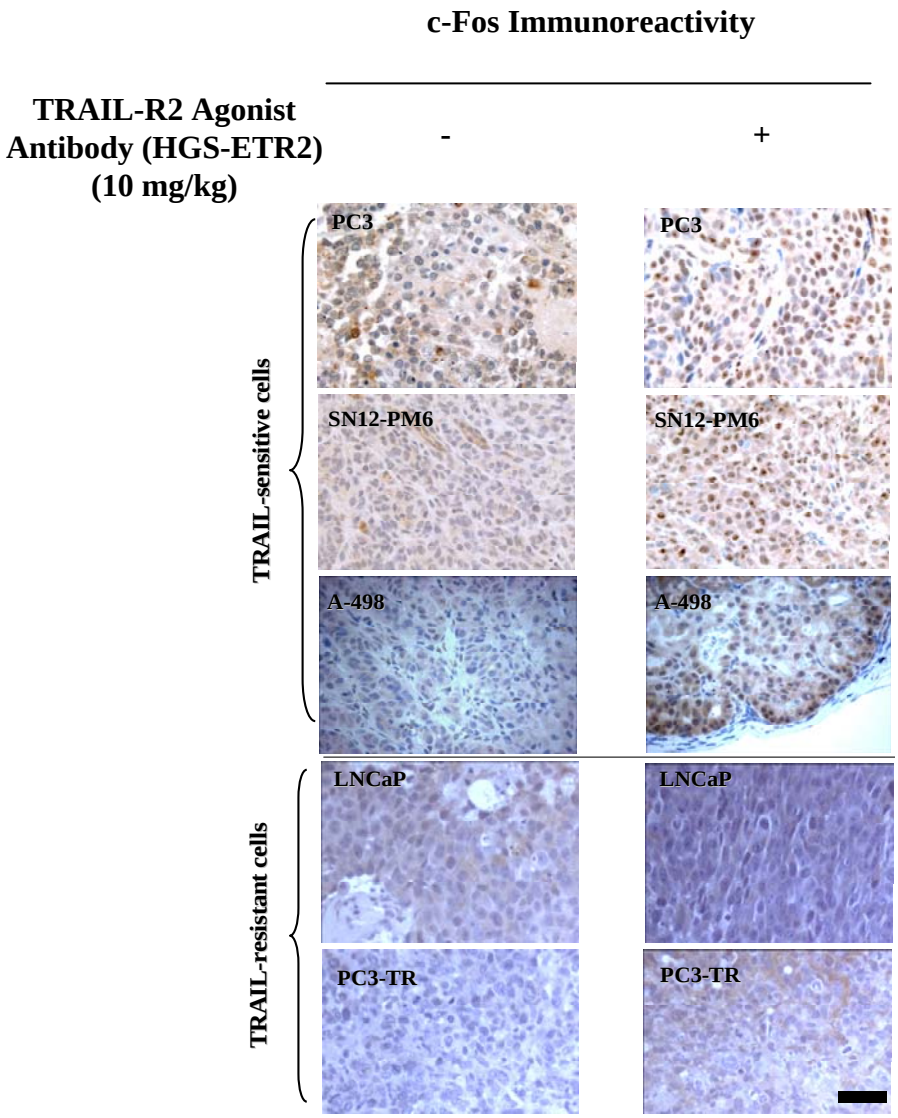
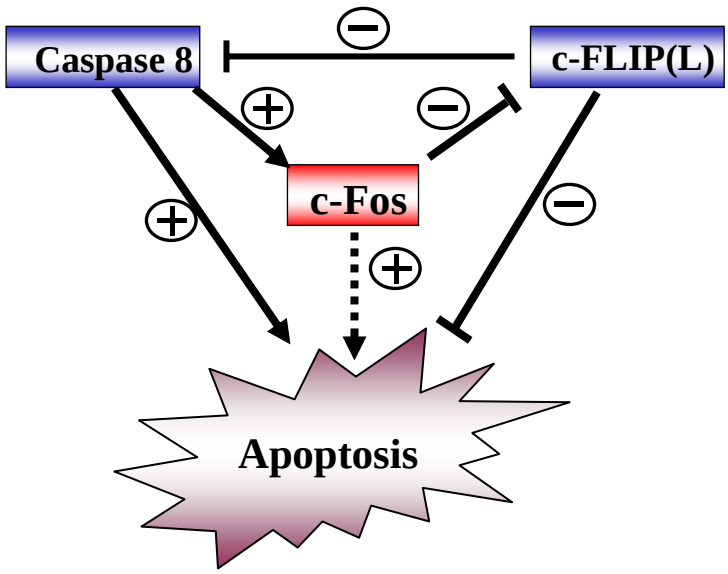

c-Fos Immunoreactivity
TRAIL-R2 Agonist Antibody (HGS-ETR2) (10 mg/kg)
-
+
TRAIL-sensitive cells
TRAIL-resistant cells
PC3
PC3
SN12-PM6
SN12-PM6
A-498
A-498
LNCaP
LNCaP
PC3-TR
PC3-TR

Figure 7



Supplementary Data

c-Fos Promotes TRAIL-induced Apoptosis by Repressing c-FLIP(L)

Xiaoping Zhang, Hongmei Yang, Liang Zhang, Xu Huang, Hasan Otu, Towia Libermann, William C. DeWolf, Roya Khosravi-Far, Aria F. Olumi

Fig. S1

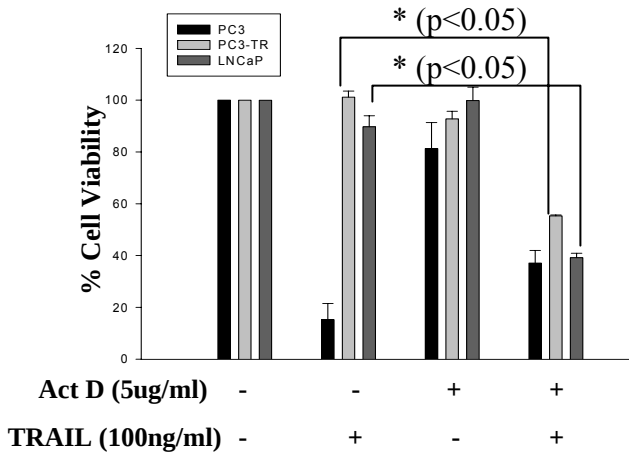


Fig. S1. Cell viability was assessed in prostate cancer cells that were pretreated with RNA synthesis inhibitor, Actinomycin D (Act D), for 1 hour followed by treatment with TRAIL (100ng/ml) for another 24 hours. Error bars indicate SD of at least three independent experiments. " ***** " represents significant differences between controls and experimental samples.

Fig. S2

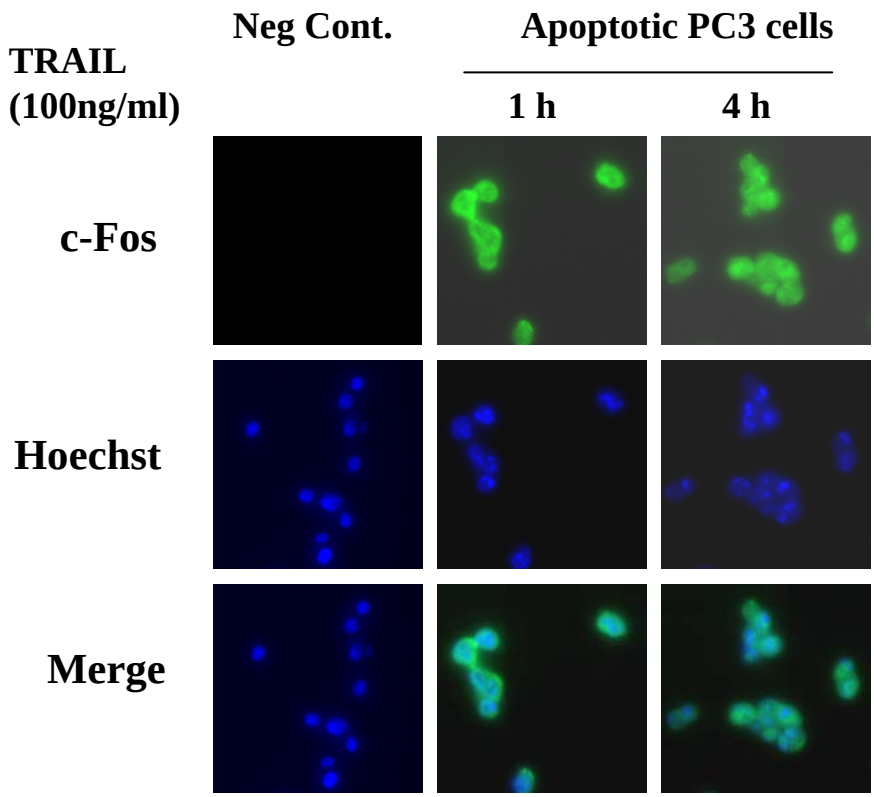


Fig. S2. Cytoimmuochemistry of c-Fos in apoptotic PC3 cells floating in tissue culture media after treatment with TRAIL for one or four hours. The floating cells harvested from the tissue culture media appeared shrunken but maintained a high intensity of nuclear c-Fos expression, which indicates apoptotic cells have high level of c-Fos staining. Negative control (Neg Cont.) is floating PC3 cells immunotained with non-specific IgG.

Fig. S3

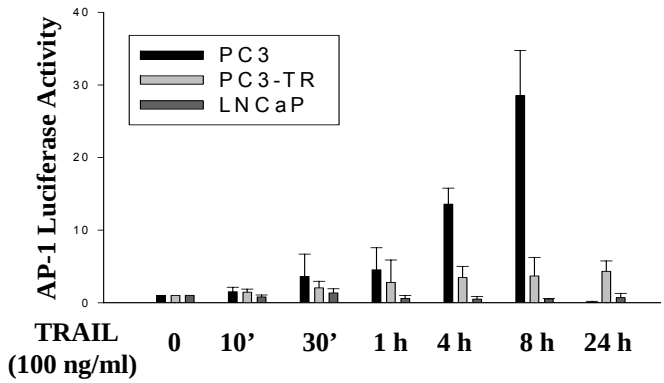


Fig. S3. AP-1 luciferase activity in prostate cancer cells after treatment with TRAIL (100ng/ml) at various time points. After treatment with TRAIL, the AP-1 luciferase activity was significantly increased in the TRAIL-sensitive PC3 cells up to 8 hours of treatment with TRAIL, while there was no significant change of AP-1 activity in the TRAIL-resistant PC3-TR and LNCaP cells. However, the increased AP-1 activity in the PC3 cells was lost after 8 hours, because majority of the PC3 cells had undergone cell death at this stage. The values were normalized with internal control Ranilla activity. Data represents average of at least three independent experiments ± SD.

Fig. S4

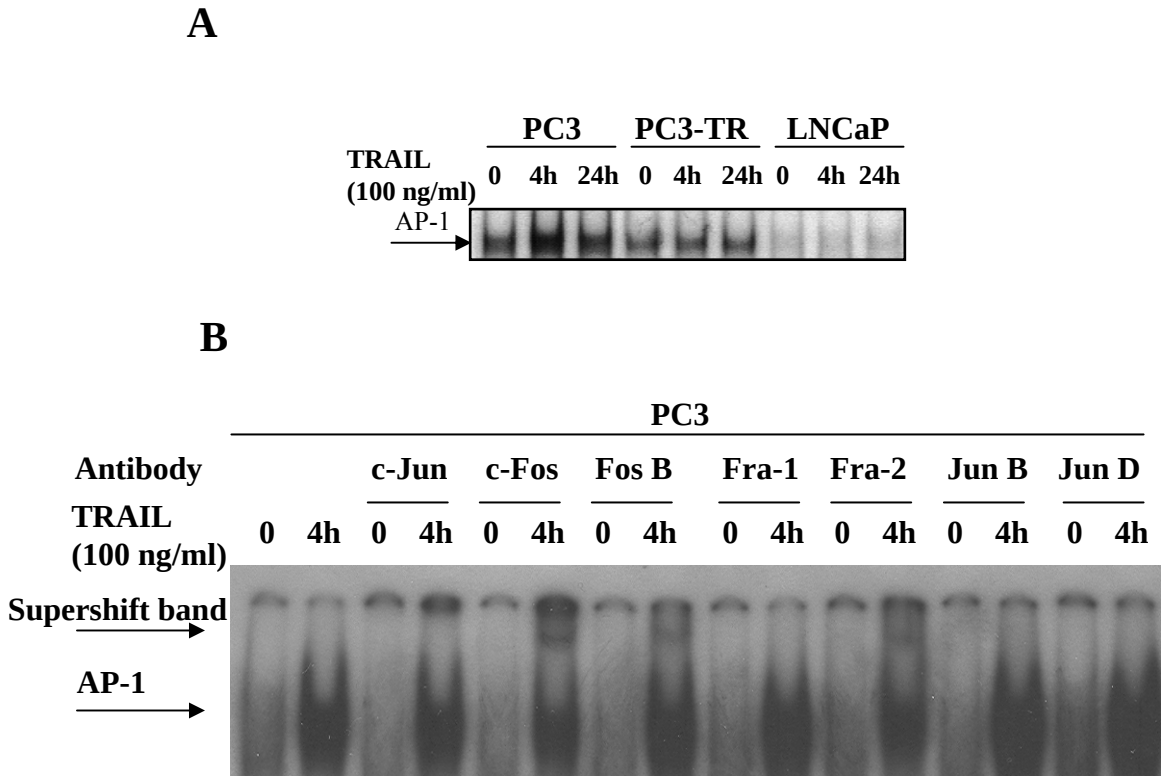


Fig. S4. Electrophoretic mobility shift assay (EMSA) of AP-1 DNA binding activity of TRAIL-sensitive and resistant cancer cells after treatment with TRAIL (100ng/ml). (A) Increased AP-1 DNA binding was detected in the TRAIL-sensitive PC3 cells, particularly after 4 hours of treatment with TRAIL, whereas, no significant change was observed in the AP-1 DNA binding in the TRAIL- resistant PC3-TR and LNCaP cells. (B) Supershift of EMSA for AP-1 DNA binding in the TRAIL-sensitive PC3 cells demonstrated supershift bands for c-Fos and other Fos- related proteins, Fos B and Fra-2. However, no supershift bands were detected for other AP-1 family member proteins, c-Jun, Fra-1, JunB or JunD.

Fig. S5

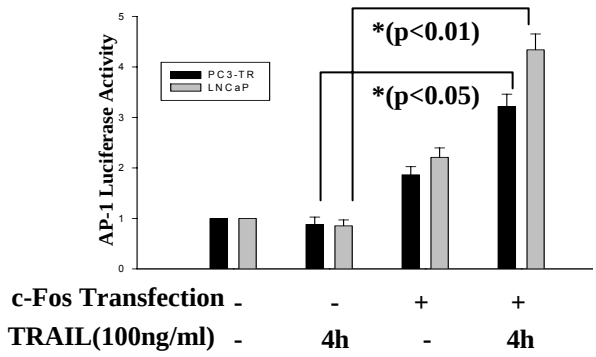


Fig. S5. AP-1 luciferase activity increases with ectopic expression of c-Fos in the TRAIL-resistant PC3-TR and LNCaP cells. AP-1 activity is pronounced with c-Fos ectopic expression, particularly when cells were treated with TRAIL (100 mg/ml). Control samples were transfected with vector pSG5 (-). " ***** " indicates significant differences between control and experimental groups.

Fig. S6

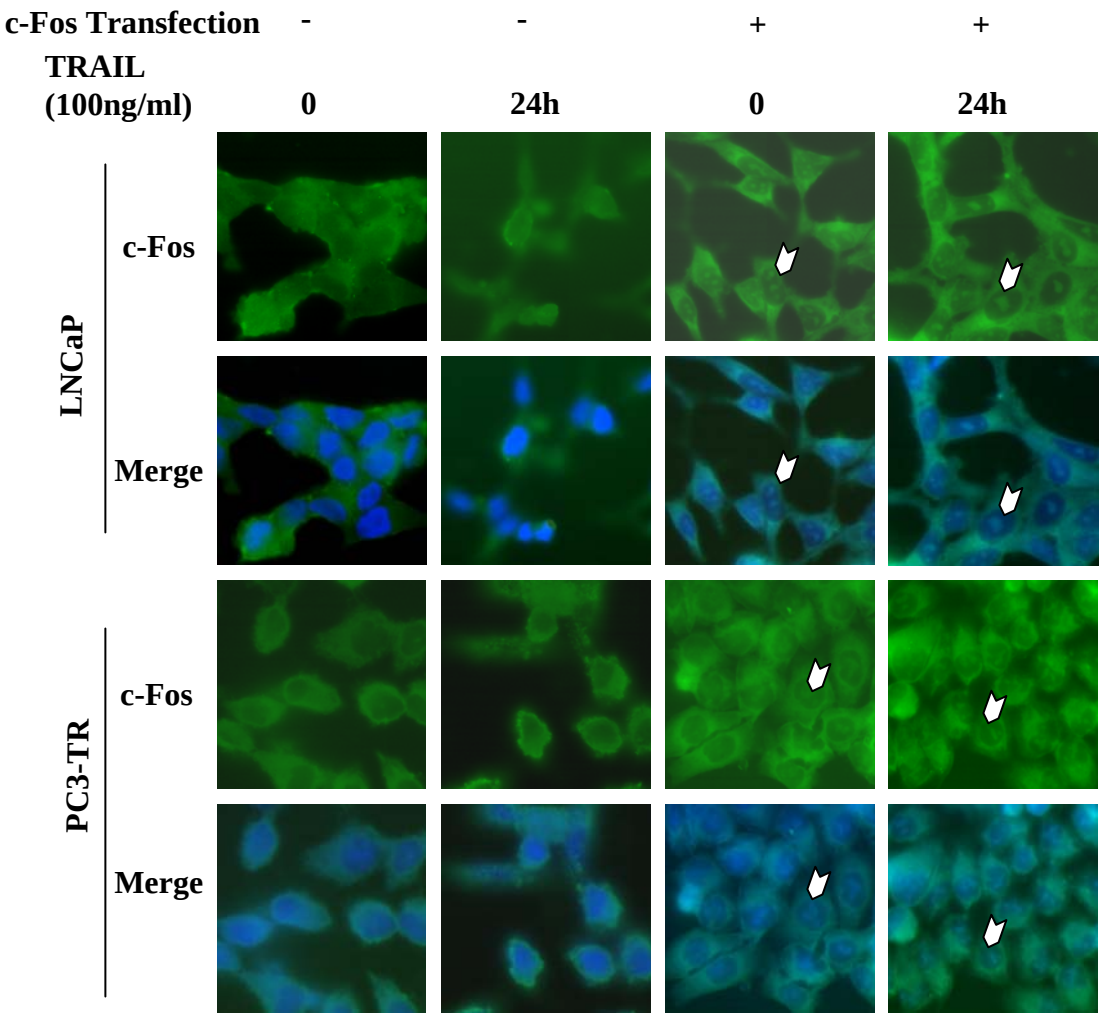


Fig. S6. Ectopic expression of c-Fos leads to increased nuclear c-Fos reactivity in the TRAIL-resistant cells. Nuclear c-Fos level is enhanced when c-Fos is ectopically expressed, regardless of TRAIL treatment. However, c-Fos overexpression alone does not lead to cell death (Fig. 2F), suggesting that nuclear c-Fos expression is necessary but insufficient for TRAIL-induced apoptosis. Arrowheads show examples of nuclear c-Fos staining.

Fig. S7

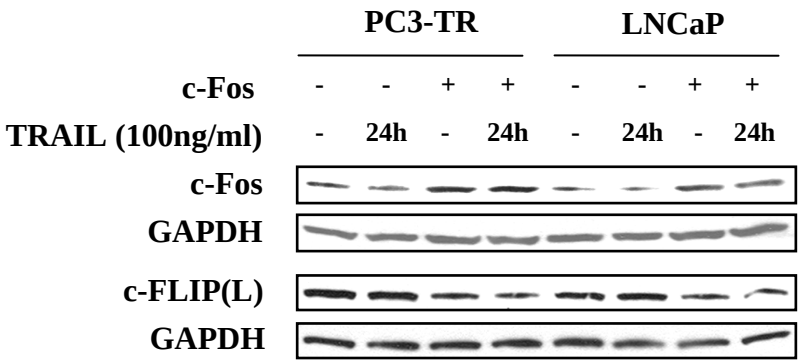


Fig. S7. Ectopic expression of c-Fos reduces c-FLIP(L) protein levels in the TRAIL-resistant PC3-TR and LNCaP cells. TRAIL-resistant PC3-TR and LNCaP cells were treated were transfected with the c-Fos vector and subsequently treated with TRAIL. c-FLIP(L) protein level decreased in the presence of ectopically expressed c-Fos, regardless of treatment with TRAIL. c-Fos (-) refers to empty vector control transfections

Fig. S8

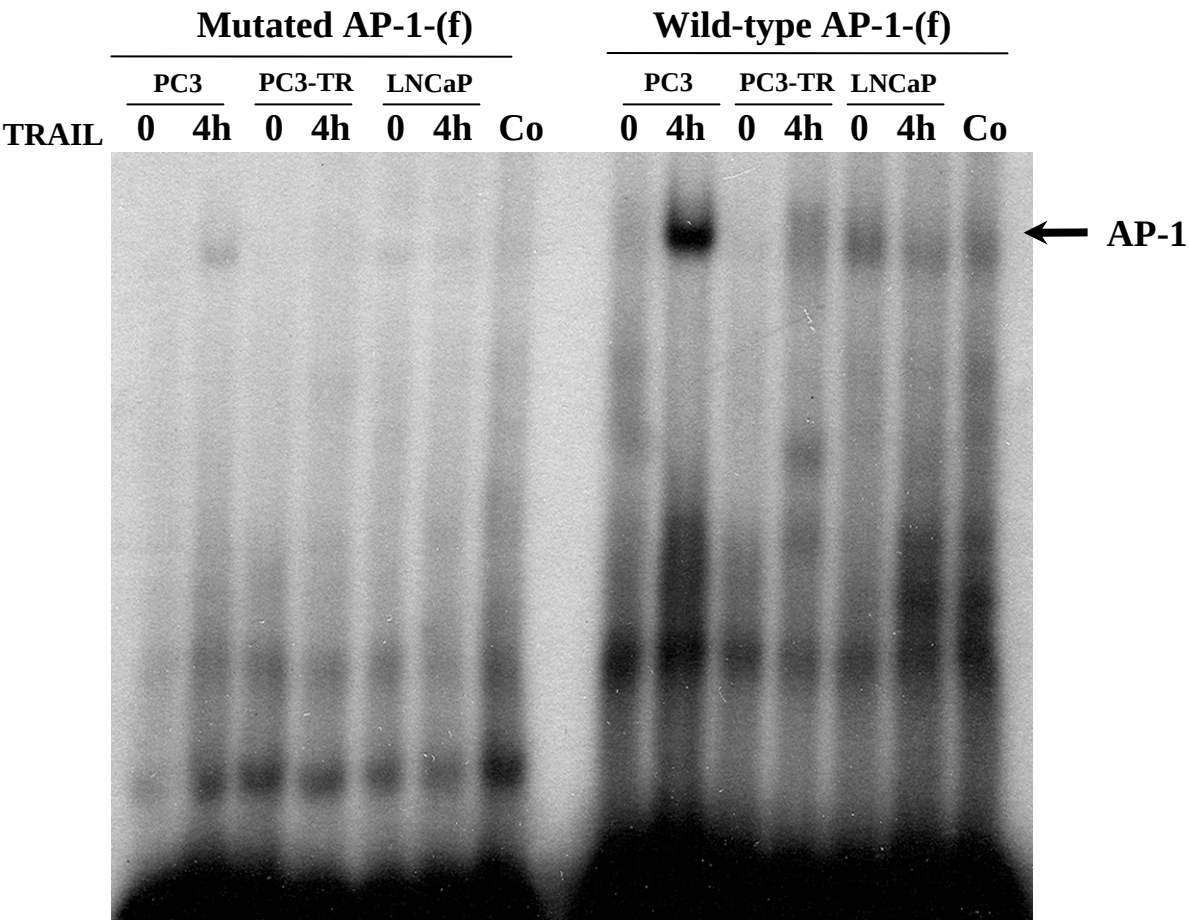


Fig. S8. Binding to c-FLIP(L)'s AP-1-(f) binding site is abolished with mutations. EMSA using a four-tandem oligonucleotide of the c-FLIP(L)'s AP-1-(f) binding site, 5'-at***ca***cttgagg-3', (see Fig. 3E & 3F). In addition, a mutated four-tandem oligo of the c-FLIP(L)'s AP-1-(f) binding site, 5'-at***tg***cttgagg-3', was designed. Binding to the wild-type AP-1-(f) site increases in the TRAIL-sensitive PC3 cells after treatment with TRAIL (100mg/ml) for 4 hours. In contrast, binding to the mutated AP-1-(f) site is abolished in the TRAIL-sensitive PC3 cells. The TRAIL-resistant cells (PC3-TR & LNCaP) do not demonstrate any significant binding to the wild-type or mutated forms of AP-1(f). " **Co** " signifies negative control.

Fig. S9-S10

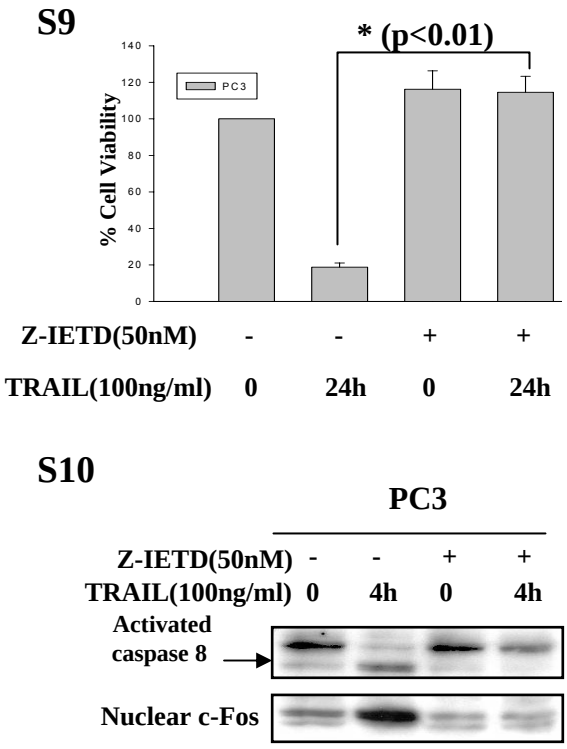


Fig. S9-S10. Nuclear expression of c-Fos is caspase 8 dependent. **Fig. S9** PC3 cells were pretreated with caspase 8 inhibitor Z-IETD (50nM) for one hour, then treated with TRAIL (100ng/ml) for additional 24 hours. In presence of the caspase 8 inhibitor, cell viability of the TRAIL-sensitive PC3 is converted to a TRAIL-resistant phenotype, " ***** " denotes statistically significant difference. **Fig. S10** Activation of caspase 8 is associated with increased c-Fos nuclear level after treatment of the TRAIL-sensitive PC3 cells. Inhibition of caspase 8 by Z-IETD abrogated expression of nuclear c-Fos.

Fig. S11

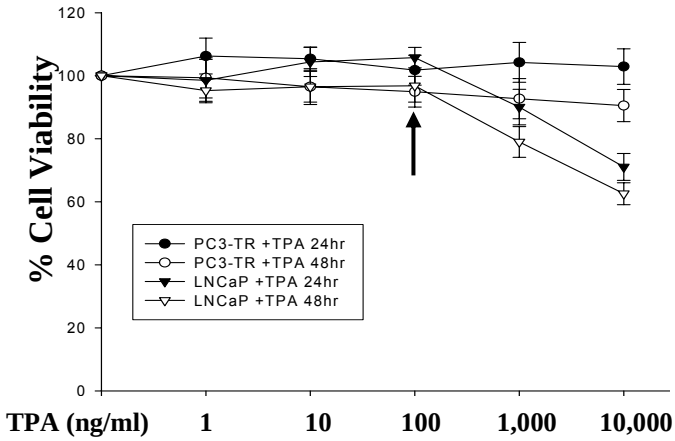


Fig. S11. Determining a non-toxic dose of TPA in TRAIL-resistant PC3-TR and LNCaP cells by cell viability assay. Cells were treated with increasing doses of TPA for 24 or 48 hours. The results demonstrate that higher dose of TPA can induce apoptosis in LNCaP cells but not in PC3-TR cells. Arrow indicates the non-toxic dose of TPA (100ng/ml) used in the following experiments.

MG-132 Sensitizes TRAIL-resistant Prostate Cancer Cells by Activating c-Fos/c-Jun Heterodimers and Repressing c-FLIP(L)

Wenhua Li, Xiaoping Zhang and Aria F. Olumi[1]

Division of Urologic Surgery

Beth Israel Deaconess Medical Center,

Harvard Medical School

Boston, MA 02215

[1]Corresponding Author:
330 Brookline Ave., Rabb 440
Boston, MA 02215
aolumi@bidmc.harvard.edu
(617) 667-4075 (phone)
(617) 975-5570 (fax)

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising anti-cancer agent since it induces apoptosis in cancer cells but not in normal cells. Unfortunately, some cancer cells develop resistance to TRAIL-induced apoptosis. Therefore, it is clinically important to determine the molecular mechanisms that differentiate between TRAIL-sensitive and –resistant tumors. Previously, we have shown that the anti-apoptotic molecule, c-FLIP(L) (cellular-FLICE-inhibitory proteins long isoform), is necessary and sufficient to maintain resistance to TRAIL-induced apoptosis. We have found that c-FLIP(L) is transcriptionally regulated by the AP-1 family member protein, c-Fos. Here we report that MG-132, a small molecule inhibitor of the proteasome, sensitizes TRAIL-resistant prostate cancer cells by inducing c-Fos and repressing c-FLIP(L). c-Fos, which is activated by MG-132, negatively regulated c-FLIP(L) by direct binding to the putative promoter region of the c-FLIP(L) gene. In addition to activating c-Fos, MG-132 activated another AP-1 family member, c-Jun. We show that c-Fos heterodimerizes with c-Jun in order to repress transcription of c-FLIP(L). Therefore, MG-132 sensitizes TRAIL-resistant prostate cancer cells by activating the AP-1 family members, c-Fos and c-Jun, which in turn repress the anti-apoptotic molecule, c-FLIP(L).

INTRODUCTION

Prostate cancer is the second leading cause of cancer death in American men. In 2005, about 232,900 new prostate cancers were diagnosed, and it accounted for 30,350 deaths in the United States. In a majority of cases, early-stage prostate cancer can be treated effectively with surgery or radiotherapy. However, advanced hormone refractory metastatic prostate cancer can be a fatal disease without effective treatment (1).

Cell surface death receptor ligand, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), has attracted attention to cancer therapy not only because of its ability to effectively kill cancer cells, but also because it has little effect on normal cells, therefore, TRAIL has minimal cytotoxicity (2). TRAIL induces apoptosis by binding to DR4 and DR5 — two related death receptors, causing the formation of a death-inducing signaling complex (DISC), which includes the receptors, the adaptor protein FADD, and caspase 8 (3, 4). Auto-activated caspase 8 initiates the apoptotic executing caspase cascade and subsequent programmed cell death (extrinsic cell death pathway). Activation of Bid to its truncated form, tBid, leads to the release of cytochrome c from the mitochondria, which then activates the mitochondrial-mediated pro-apoptotic pathway (intrinsic cell death pathway) (5).

Although many cancers undergo TRAIL-induced apoptosis, some cancer cells are resistant to TRAIL. Moreover, some sensitive cancer cell lines develop resistance when they are treated with TRAIL (6). Cellular sensitivity for TRAIL induced apoptosis can be modulated at several levels. Inducing the expression of DR5 can enhance TRAIL signal and overcome TRAIL resistance in cancer cells (7, 8). Other examples include modulation of the proapoptotic molecules Bax and Bak and the antiapoptotic molecule,

Bcl-2, which can modify TRAIL-induced mitochondrial apoptosis (9). c-FLIP (cellular-FLICE-inhibitory proteins), is another class of important intracellular anti-apoptotic molecules, which can block the apoptotic signaling pathway of TRAIL induced-apoptosis. c-FLIP protein homologues interrupt apoptotic signaling by competing with caspase 8 for binding to the DED domains of FADD and also regulate apoptosis through their interference with the recruitment of caspase 8 to FADD (4, 10, 11). The levels of intracellular c-FLIP, therefore, may determine the sensitivity of cancer cells to apoptosis triggered by TRAIL (6, 12). The c-FLIP family of proteins, c-FLIP(L), c-FLIP(s), and perhaps the newly detected c-FLIP(r) (13), can bind to the DED domains of FADD and caspase 8 and regulate apoptosis through their interference with the recruitment of caspase 8 to FADD.

We have shown in the past that persistent expression of c-FLIP(L) is necessary and sufficient in order to maintain resistance to TRAIL-induced apoptosis in prostate cancer cells (6). Intracellular c-FLIP(L) can be regulated at either the transcriptional, translational or post-translational levels. Expression of c-FLIP(L) has been shown to be modulated by NF-κB (14-16), Akt (17, 18), c-Myc (12), p53 (19), and E3-ubiquitin ligase (20). We have found that transcriptional repression of c-FLIP(L) by the AP-1 family member protein, c-Fos, is critical in modulating resistance and sensitivity of cells in TRAIL-induced apoptosis (Zhang, X. et al. – manuscript submitted).

In order to sensitize TRAIL-resistant cancer cells, proteosome inhibitors have been combined with TRAIL in a variety of different cancer models. For example, the proteasome inhibitor, PS-341, has been shown to help overcome TRAIL-resistance in colon and bladder cancer cells (21-25). Another proteosome inhibitor, MG-132, has a

potent anti-tumor function, and has been shown to sensitize resistant cancer cells to the pro-apoptotic effects of TRAIL (7, 8, 26, 27). In this study, we examined the mechanism that MG-132 sensitizes prostate cancer cells to TRAIL-induced apoptosis. We demonstrate that MG-132 sensitizes TRAIL-resistant prostate cancer cells by up-regulating the AP-1 family proteins, c-Fos and c-Jun, which in turn repress the anti-apoptotic molecule, c-FLIP(L). c-Fos/c-Jun heterodimers bind to the c-FLIP(L) promoter, repress its transcriptional activity, and reduce c-FLIP(L) mRNA and protein levels. These findings suggest that elevated c-Fos and c-Jun can play an important role in determining whether a cell is responsive or resistant to the pro-apoptotic effects of TRAIL.

MATERIALS AND METHODS

Chemicals and antibodies

Recombinant human TRAIL/TNFSF10 was obtained from R&D Systems Inc. (Minneapolis, MN). Proteasome inhibitor MG-132 was obtained from EMD Calbiochem (La Jolla, CA), and dissolved in DMSO at a stock concentration of 2 mM at -20°C. Fresh dilutions in medium were made for each experiment. Antibodies were obtained from the following sources: Horseradish peroxidase-conjugated secondary antibody (goat-anti-mouse, goat-anti-rabbit, goat-anti-rat antibodies), Oct-1 (C-21), c-Fos (D1), Fos B (C-11), Fra-1 (N-17), Fra- 2 (L-15), JunB (N-17), Jun D (329) and c-Fos siRNA were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). c-Jun, phospho-c-Jun (Thr 91) and c-Fos antibodies were obtained from Cell Signaling (Beverly, MA). Monoclonal c-FLIP(L) antibody (Dava II) was obtained from Apotech Corp. (San Diego, CA). Phospho-c-Fos (T232) and GAPDH antibodies were from Abcam, Inc. (Cambridge, MA).

Cell lines and culture conditions

All of the cell culture materials were from Cellgro (Herndon, VA), and plastic ware was obtained from Becton Dickinson Labware (Bedford, MA). PC3 and LNCaP prostate cancer cell lines and HEK 293T cells were obtained from the American Type Culture Collection (Manassas, VA). BPH-1 (benign prostatic hyperplasia cells immortalized with SV-40 large T antigen) cells were kindly provided by Dr. Simon Hayward (Vanderbilt University) (28). PC3-TR was a TRAIL-resistant subline established from parental PC3 cells by TRAIL treatment selection (6). All of the cells were cultured in RPMI 1640 tissue culture medium supplemented with 2 mM L-

glutamine, 10% FBS, and 1% penicillin-streptomycin (each at 50 μg/ml) at 37°C with 5% CO_2.

Cell viability and apoptosis assays

Cell viability was determined by MTS method in accordance with the manufacturer's instructions (Cell TITER 96 Aqueous Assay, Promega, Madison, WI). In brief, 5×10^4 PC3, PC3-TR, BPH-1, HEK 293T cells and 7.5×10^4 LNCaP cells were seeded in 96-well plates and cultured for 48 hours before treatment. Cells were then treated with TRAIL and/or MG-132 for 24 hours. MTS substrates were added and incubated for 2 hours at 37°C. Absorbance was measured at 490 nm using a microtiter plate reader. Viability of control cells of just treated with DMSO was set at 100%, and absorbance of wells with medium and without cells was set at zero.

For apoptosis assays, cells were washed twice with PBS and resuspend in binding buffer (10 mM HEPES, 140 mM NaCl, and 5 mM $CaCl_2$ (pH 7.4) all from Sigma Chemical Co.), and stained with FITC-conjugated annexin V (Roche Diagnostic Co., Indianapolis, IN) and propidium iodide (PI) for 15 min at room temperature. Annexin V fluorescence was determined with a FACScan flow cytometer, and the membrane integrity of the cells was simultaneously assessed by the PI exclusion method. Annexin V binds to those cells that express phosphatidylserine on the outer layer of their membranes, and PI stains the cellular DNA of those cells with a compromised membranes.

Cell extracts and Western blot analysis

Cells were harvested for total cell lysates with RIPA buffer (1% NP-40, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% deoxycholate, and 0.1% SDS) containing a mixture of protease inhibitors (cocktail 1 ×, 1 mM PMSF, 20 mM, 40 mM NaF and 3

mM Na_3VO_4). After sonication for 15 seconds, cell debris was discarded by centrifugation at 12,000 × g for 10 min at 4°C, and the protein concentration was determined by BCA protein assay reagent (Pierce, Rockford, IL). The procedure for the nuclear protein extraction was carried out according to the manufacturer's instructions (NE-PER nuclear and cytoplasmic extraction reagents Kit, (Pierce Biotechnology, Rockford, IL)). Cells were swollen with hypotonic buffer and then disrupted. The cytoplasmic fraction was removed, and the nuclear protein was released from the nuclei by a high-salt buffer. The lysate was boiled for 10 min and frozen at -80°C. Western blot was carried out as previously described (6).

Semi-quantitative reverse transcription-PCR analysis.

Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The RNA yield and purity were evaluated by measuring A_{260}/A_{280} and agarose gel electrophoresis. RT–PCR was performed using a Superscript One-Step RT–PCR kit (Invitrogen Life Technologies, Carlsbad, CA). 0.4 μg of the total RNA was used to RT-PCR of 25 μl reaction system. cDNA synthesis was performed at 50°C for 30 minutes using and the following cycle temperatures and times: denaturation at 94°C for 50 seconds, annealing at 56°C for 50 seconds, and polymerization at 72°C for 2 minutes (total number of cycles = 30), final extension at 72ºC for 10 min. In each reaction, the same amount of GAPDH was used as an internal control. The primers used for PCR were as follows: c-FLIP(L), 5'-GTC TGCTGA AGT CAT CCA TCAG-3' (forward) and 5'-CTT ATG TGT AGG AGA GGA TAA G-3' (reverse); c-Fos, 5'-GAA TAA GAT GGC TGC AGC CAA ATG C-3' (forward) and 5'-AAG GAA GAC GTG TAA GCA GTG CAG C-3' (reverse); GAPDH, 5'-TCC ACC

ACC CTG TTG CTG TA-3' (forward); and 5'-ACC ACA GTC CAT GCC ATC AC-3' (reverse). The PCR products were resolved on 1% agarose gels, stained with ethidium bromide, and then photographed.

Luciferase Assay

c-FLIP(L) promoter luciferase structure was kindly provided by Dr. W.S. El-Deiryin, (University of Pennsylvania, Pennsylvania, PA) (12). Cells were seeded into 24-well plates. When cells reached 50-80% confluence, both AP-1 luciferase reporter (25 ng/well) and Ranilla reporter (5 ng/well) from Stratagene (La Jolla, CA) or c-FLIP(L) promoter and Ranilla reporter were co-transfected into cells. In some other experiments, c-Fos siRNA or full-length human c-Fos cDNA plasmid was transfected into cells for 24 hours before transfection of luciferase and Renilla. Renilla acted here as an internal control for transfection efficiency. After 24 hours of transfection, cells were treated with TRAIL (100 ng/ml) for the indicated time. Thereafter, cells were collected, prepared, and further detected by using Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's protocol. Samples were stored at -20°C until detection. All results represent an average of at least three independent experiments ± SD.

Transfection with c-Fos vector or c-Fos siRNA

Dr. L Shemshedini kindly provided full-length human c-Fos cDNA which was cloned into a pSG5 vector (University of Toledo, OH) (29). Plasmids with or without c-Fos were transfected with Lipofectamine^{TM} 2000 (Invitrogen Life Technologies, Carlsbad, CA). siRNA of c-Fos was then transfected into cells by TransMessenger^{TM} Transfection Reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. After transfection with the c-Fos vector for 24 hours or the c-Fos siRNA for 36-48 hours, the

cells were seeded in 96-well plates for cell viability assays or treated with TRAIL for Western blot assays.

Immuno-coprecipitation and Immunoblotting

Cells were lysed at 4°C for 30 min in RIPA lysis buffer containing protease inhibitors. Lysates were centrifuged at 12,000 × *g* at 4°C for 10 min to remove insoluble materials. The supernatants were then collected and the total protein was determined using the BCA assay (Pierce). Supernatants of equal amounts of protein were incubated at 4°C overnight with either c-Fos antibody or IgG control antibody. Protein A-Sepharose was added and incubated at 4°C for 1-4 hours. The immuno-complexes were washed three times in cold lysis buffer. The bound proteins were eluted from the column in preheated sample buffer (50 mM Tris-HCl pH 6.8, 50 mM dithiothreitol, 1% SDS, 0.005% bromphenol blue, and 10% glycerol) and denatured by boiling for 5 min. The immunoprecipitates and whole lysate proteins were then subjected to 4-12% SDS-PAGE. Immunoblot analysis was performed with the indicated antibodies.

Cell Extracts and Electrophoretic Mobility Shift Assay (EMSA)

Frozen cell pellets were resuspended in 4 volumes of lysis buffer: 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 0.2 mM EGTA, 10% glycerol, 10 mM Na molybdate, 2 mM Na pyrophosphate, 2 mM Na orthovanadate, 0.5 mM spermidine, 0.15 mM spermine, 50 μM TPCK, 25 μM TLCK, 1 μg/ml each of aprotinin, pepstatin A, and leupeptin, 0.5 mM benzamidine, 1 mM DTT, and 0.5 mM PMSF. KCl was added to 400 mM final, and the extracts were incubated at 4°C for 30 min and centrifuged at 10,000 × g for 5 min. The supernatant contained the whole cell extracts. The reactions were made using 3 μl of whole cell extract and 0.1–0.5 ng of ^{32}P-labeled double-stranded specific oligonucleotides

(5,000–25,000 cpm) and run on 5-7% polyacrylamide gels containing 0.5 × Tris glycine EDTA. Gels were dried with Bio-Rad gel dryer (Hercules, CA) and imaged using Kodak BioMax MR Film (Fisher Scientific, Atlanta, GA). General AP-1 gel shift oligonucleotide was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Wild-type oligonucleotides of the c-FLIP(L)'s AP-1-(f) site was designed as 5'-atcacttgagg atcacttgagg atcacttgagg atcacttgagg -3' .

ChIP assay

ChIP assay was performed using the ChIP Assay Kit (Upstate Cell Signaling Solutions, Lake Placid, NY). In brief, PC3-TR cells were cultured in 10cm dishes and treated with TRAIL and/or MG-132 for 4 hours. Cross-linking of DNA and proteins were fixed by adding formaldehyde directly to the culture medium to a final concentration of 1% and incubated for 10 min at 37°C. Cells were collected and washed twice using ice-cold PBS containing protease inhibitors. Harvested cells were resuspended in 200µl of SDS lysis buffer provided by the manufacturer and incubated for 10 min. Cell lysates were sonicated to break the DNA to 200-1,000 bp fragments. Samples were centrifuged at 12,000 × *g* for 10 min at 4°C and the supernatant was harvested. Concentration of each sample was quantitated using BCA protein assay reagent (Pierce Biotechnology). Positive controls were 10% of each DNA sample, which did not include the immunoprecipitation (IP) step. The remainder of the samples was divided equally into two groups. The experimental group was immunoprecipitated with specific c-Fos (D-1) antibody, while the negative control group was immunoprecipitated with general IgG antibody. After eluting protein-DNA from antibody, protein-DNA crosslinking was reversed by heating at 65°C for 4 hours. The isolated genomic DNA was first purified by

phenol/chloroform extraction and ethanol precipitation. Then the DNA was amplified by PCR, using specific primers encompassing the region containing the AP-1-(f) binding site according to the human c-FLIP(L) sequence (GeneBank). The conditions were as follows: primers 5'-CCT GTG ATC CCA GCA CTT TG-3' (forward) and 5'-CAC CAT GCC CGA CTA ATT TT-3' (reverse): denaturation at 94°C for 30 seconds; annealing at 56°C for 45 seconds; polymerization at 72°C for 30 seconds, for 25 cycles. Finally, PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining.

RESULTS

MG-132 sensitizes TRAIL-resistant prostate cancer cells to undergo apoptosis.

Although PC3 cells are sensitive to TRAIL-induced apoptosis, PC3-TR and LNCaP cells are resistant to the pro-apoptotic effects of TRAIL (Fig. 1A and (6)). Combination of TRAIL with MG-132 sensitizes resistant prostate cancer cells, PC3-TR and LNCaP, to undergo apoptosis (Fig. 1B & C). Since TRAIL is more effective against cancer cells than benign immortalized cells (2), we wished to determine whether the effect of TRAIL + MG-132 is specific to cancer cells or whether immortalized but non-tumorigenic cells undergo cell death. Non-tumorigenic and immortalized 293T (human embryonic kidney) and BPH-1 (benign prostatic hyperplasia) cells were treated with MG-132, TRAIL or in combination with MG-132 + TRAIL. We found that neither treatment as single agents nor combination of treatments promoted cell death in the immortalized non-cancerous cell lines (Fig. 1D). This data suggests that MG-132 is capable of sensitizing cancerous cells, but not benign transformed cells, to undergo TRAIL-induced apoptosis.

Combination of TRAIL and MG-132 repress c-FLIP(L) and induces c-Fos.

The anti-apoptotic protein c-FLIP(L) plays an important role in TRAIL sensitivity of cancer cells. We have shown in the past that persistent expression of c-FLIP(L) is necessary and sufficient to maintain resistance to TRAIL-induced apoptosis (6). In addition, we have found that in TRAIL-sensitive cancer cells, the anti-apoptotic molecule, c-FLIP(L), is repressed by the AP-1 family protein, c-Fos, a mechanism that is lacking in TRAIL-resistant cancer cells (Zhang, X. et al -- submitted). Since MG-132 sensitizes

resistant cancer cells to undergo TRAIL-induced apoptosis, we wished to determine whether c-Fos' ability to repress c-FLIP(L) is restored in the presence of MG-132.

c-FLIP(L) protein and mRNA levels are maintained when the resistant PC3-TR cells are treated with either TRAIL or MG-132 alone (Fig. 2A). However, combination of TRAIL + MG-132 led to reduction of the anti-apoptotic molecule, c-FLIP(L), at the mRNA level and protein levels as demonstrated by the semi-quantitative RT-PCR and Western blot analyses (Fig. 2A). The c-FLIP(L) mRNA level was noticeably reduced 12 hours after treatment with MG-132 and TRAIL.

Since we have found that up-regulation of the AP-1 family protein, c-Fos, is necessary for TRAIL-induced apoptosis, we examined whether c-Fos levels are upregulated in the presence of MG-132. We found that c-Fos protein and mRNA levels are increased in the presence of MG-132 alone (Fig. 2B), a condition that does not promote cell death in resistant prostate cancer cells (Fig. 1B and 1D). However, combination of MG-132 and TRAIL sensitizes prostate cancer cells to undergo cell death while promoting c-Fos levels at the mRNA and protein levels (Fig. 2B). Decrease in c-FLIP(L) and increase in c-Fos protein levels are observed in a time-dependent (Fig. 2C) and dose-dependent (data not shown) fashion. Since c-Fos is a well established transcription factor (30, 31), we determined whether there is any significant change in nuclear c-Fos levels in the presence of MG-132. We found that nuclear c-Fos, and more specifically, phosphorylated nuclear c-Fos, was increased when treated with MG-132 or MG-132 + TRAIL (Fig. 2D). This data demonstrates that MG-132 sensitizes resistant prostate cancer cancers to undergo apoptosis (Fig. 1) by repressing expression of c-FLIP(L) and promoting expression of c-Fos. Similar to our previous results, increased

expression of c-Fos in response to MG-132 does not induce cell death, but only primes resistant prostate cancer cells to undergo apoptosis (Figs. 1 and 2).

Combination of TRAIL and MG-132 increases AP-1 activity and decreases c-FLIP(L) promoter activity.

Since combination of MG-132 and TRAIL reduces the expression of c-FLIP(L) and enhances the expression of the AP-1 family member, c-Fos (Fig. 2), we wished to determine whether there is any direct interaction between the transcription factor, AP-1/c-Fos, and the anti-apoptotic molecule, c-FLIP(L). First, we examined the luciferase AP-1 activity in the resistant PC3-TR cells. We found that MG-132 alone or combination of MG-132 + TRAIL significantly enhanced the AP-1 activity in the resistant PC3-TR cells after 24 hours of treatment (Fig. 3A). Enhancement of AP-1 activity was particularly pronounced when MG-132 was combined with TRAIL (Fig. 3A). c-FLIP(L) promoter activity was not significantly changed in the presence of MG-132, however, combination of MG-132 + TRAIL led to significant reduction of the c-FLIP(L) promoter activity (Fig. 3B). This data, again, suggests that the proteosome inhibitor, MG-132 alone, sensitizes resistant prostate cancer cells to undergo apoptosis by enhancing AP-1 activity, which only in the presence of the pro-apoptotic agent, TRAIL, will lead to repression of the anti-apoptotic molecule, c-FLIP(L) (Fig. 2).

Next, we examined whether inhibition of c-Fos by siRNA can affect c-FLIP(L)'s promoter activity. To assure that our siRNA was functioning as expected, AP-1 activity and c-Fos protein levels were assessed in presence or absence of c-Fos siRNA. We found that AP-1 activity and c-Fos protein levels were reduced in the PC3-TR cells in the

presence of c-Fos siRNA (Fig. 3C). In addition, inhibition of c-Fos by siRNA led to increased c-FLIP(L)'s promoter activity (Fig. 3C).

Next, we wished to examine the effect of inhibiting c-Fos by siRNA on c-FLIP(L)'s promoter activity when treated with MG-132 and TRAIL. The luciferase activity in the control groups were normalized (Fig. 3D). Then we examined the luciferase activity when the cells were treated with MG-132, TRAIL or MG-132 + TRAIL. We found that c-FLIP(L) promoter luciferase activity did not differ significantly from the controls when the cells were treated with MG-132 or TRAIL alone. c-FLIP(L) promoter activity decreased in the cells that were treated with MG-132 + TRAIL (Fig. 3D, last panel). However, c-Fos siRNA rescued and promoted c-FLIP(L)'s promoter activity when the cells were treated with MG-132 + TRAIL (Fig. 3D, last panel). In addition, c-Fos siRNA helped maintain the expression of c-FLIP(L) protein (Fig. 3E). To determine whether inhibition of c-Fos by siRNA had any functional role, we examined the cell viability of PC3-TR cells. As previously shown, we found that combination of MG-132 + TRAIL sensitized prostate cancer cells to undergo apoptosis. However, when c-Fos was inhibited by siRNA, PC3-TR cells become more resistant to cell death than controls when treated with MG-132 + TRAIL (Fig. 3 F, last panel).

In contrast, ectopic expression of c-Fos (Fig. 3G) increased AP-1 activity and c-Fos protein level as expected, but also led to reduction of c-FLIP(L) promoter activity (Fig. 3G). Ectopic expression of c-Fos and reduced c-FLIP(L) promoter activity are associated with sensitizing resistant prostate cancer cells to undergo TRAIL-induced apoptosis (data not shown). Therefore, MG-132 enhances the c-Fos activity, reduces c-FLIP(L) promoter activity and sensitizes prostate cancer cells to undergo apoptosis.

MG-132 upregulated AP-1 activity by increasing nuclear translocation of c-Fos/c-Jun and their interaction.

AP-1 family transcription factors are dimeric protein complexes composed of heterodimers between Fos (c-Fos, FosB, Fra-1, and Fra-2), Jun (c-Jun, JunB, and JunD) and ATF family gene products, which convert extracellular signals into changes of specific target gene expression (31, 32). Since we found that the AP-1 activity of PC3-TR cells are increased in response to MG-132 (Fig. 3A), we wished to examine whether any other AP-1 family members, besides c-Fos (Fig. 2), plays a key role in sensitizing cancer cells and regulating promoter activity of c-FLIP(L) during TRAIL-induced apoptosis. We found that MG-132 increased levels of c-Jun protein in PC3-TR cells, while there was no significant change in the protein levels of other AP-1 members (FosB, JunB, JunD, Fra-1, and Fra-2) (Fig. 4A). In particular, nuclear levels of total c-Jun and phospho c-Jun were significantly increased (Fig. 4B).

c-Fos functions as a transcription factor by hetero-dimerizing with c-Jun and other AP-1 family members (31, 32). To determine whether the increased c-Fos and c-Jun nuclear levels after MG-132 treatment is associated with direct interactions between c-Fos and c-Jun, we performed immunoprecipitation experiments between c-Fos and c-Jun. We found that direct interactions between c-Fos and c-Jun were increased in PC3-TR cells when the cells were exposed to MG-132. Similar results were obtained when MG-132 was combined with TRAIL. However, TRAIL alone did not enhance c-Fos/c-Jun interactions (Fig. 4C). Similar results were obtained when c-Jun antibody was used for the immunoprecipitation experiments (Fig. 4C). Therefore, the proteosome inhibitor,

MG-132, enhances c-Fos and c-Jun levels, enhances direct interactions between c-Fos and c-Jun, and presumably promotes heterodimerization and transcriptional activity.

c-Fos and c-Jun bind to the c-FLIP(L) promoter region.

In order to determine whether increased protein levels of c-Fos and c-Jun in response to MG-132 is associated with increased DNA binding, we performed EMSA and EMSA supershift assays. We found that AP-1 DNA binding is increased in the presence of either MG-132, TRAIL or MG-132 + TRAIL. However, we observed supershift bands for c-Fos and c-Jun particularly when the cells were treated with MG-132, demonstrating the specificity of binding of these AP-1 family member proteins in response to MG-132 (Fig. 5A).

Since c-Fos and c-Jun DNA binding is increased in response to treatment of cells with MG-132, and c-Fos represses the anti-apoptotic molecule, c-FLIP(L), we wished to determine if c-Fos and c-Jun specifically bind to the c-FLIP(L) putative promoter region. Previously, we examined fourteen potential AP-1 binding sites upstream and within the first intron of c-FLIP(L)'s coding region (Fig 5B). We found binding of c-Fos only to the AP-1-(f) site (see Fig. 5B) in c-FLIP(L)'s putative promoter region (Zhang, X. et al. – submitted). We have found that in prostate cancer cells which are sensitive to TRAIL-induced apoptosis, mutations or deletions to the AP-1-(f) site abrogates binding of c-Fos, increases c-FLIP(L) promoter activity and converts the phenotype of TRAIL-sensitive prostate cancer cells to become TRAIL-resistant. Therefore, in our current model, with TRAIL-resistant prostate cancer cells which are sensitized by MG-132 we wished to determine whether there is increased binding of c-Fos and/or c-Jun at c-FLIP(L)'s AP-1-(f) site. We performed chromatin immunoprecipitation (ChIP) experiments to determine

direct binding of c-Fos and c-Jun at the AP-1-(f) site. There was no significant binding of either c-Fos or c-Jun to c-FLIP(L)'s AP-1-(f) site without treatment or with TRAIL treatment alone. However, in the presence of MG-132 both c-Fos and c-Jun demonstrated enhanced binding to c-FLIP(L)'s AP-1-(f) site (Fig. 5C). This data demonstrates that MG-132 sensitizes resistant prostate cancer cells to pro-apoptotic effects of TRAIL by enhancing c-Fos and c-Jun interactions and transcriptionally repressing the expression of c-FLIP(L) by binding to c-FLIP(L)'s AP-1-(f) site.

DISCUSSION

TRAIL has great potential as an anti-tumor agent in that it can selectively induce apoptosis in cancer cells, yet spare most normal cells. Although many cancer cells are sensitive to pro-apoptotic effects of TRAIL, some develop resistance to TRAIL-induced apoptosis. Many groups have been investigating the synergistic effects of different drugs in combination with TRAIL in order to overcome the resistance developed by cancer cells (8, 21, 33-39). In the present study, we demonstrated that TRAIL combined with the proteasome inhibitor, MG-132, could effectively sensitize TRAIL-resistant prostate cancer cells to undergo apoptosis. Moreover, this combined treatment did not induce death in nonmalignant cell (BPH-1 and HEK 293T) (Fig.1). MG-132 sensitizes TRAIL-resistant prostate cancer cells by upregulating the AP-1 family proteins, c-Fos and c-Jun, which in turn repress the anti-apoptotic molecule c-FLIP(L). As for the other well studied c-FLIP isoform, c-FLIP(s), we have not found c-FLIP(s) to be expressed in our prostate cancer cells. Therefore, the effects of MG-132 on c-FLIP(s) was not examined in our study.

Proteasome inhibitors are attractive cancer therapeutic agents in that they have been involved in regulation of apoptosis-related proteins, (e.g. TRAF2, BAX, IAP, p53 proteins) (23-25, 40). PS-341 has been approved by the FDA for treatment of patients with multiple myeloma, and many clinical trials are ongoing to examine the efficacy of PS-341 for treatment of other malignancies (41, 42). MG-132 is another small molecule proteasome inhibitor, and numerous reports have demonstrated that MG-132 inhibits NF-κB activation through stabilization of the inhibitor of the κB (IκB)/ NF-κB complex, as well as prevention of nuclear translocation of the NF-κB (43, 44). Additionally, MG-132

induces MAP kinase expression and activates c-Jun N-terminal kinase (JNK) (26, 27). Some reports have shown that MG-132 sensitizes TRAIL-resistant cancer cells by up-regulating death receptor DR5 expression and Bik accumulation (7, 8, 26, 27).

In the current study we determined whether TRAIL-resistant prostate cancer cells, which are sensitized by MG-132 have changes in the AP-1/c-Fos and c-FLIP(L) signalling pathway. In the presence of MG-132, we found that inhibition of c-Fos by siRNA led to upregulation of c-FLIP(L) promoter activity, and conversely, ectopic expression of c-Fos reduced c-FLIIP(L) promoter activity (Fig. 3). After priming the resistant prostate cancer cells by MG-132 to undergo apoptosis, we showed that the AP-1 family members, c-Fos and c-Jun, directly bind to the c-FLIP(L)-AP-1(f) site (Fig. 5) after treatment with TRAIL.

In TRAIL-sensitive prostate cancer cells, we have found that the AP-1 family member proteins only bind to the AP-1-(f) site of the c-FLIP(L) promoter region, and none of the other putative AP-1 binding sites in the putative promoter region of c-FLIP(L). Deletions and mutations at the c-FLIP(L)-AP-1(f) site abrogates binding of c-Fos to the c-FLIP(L) promoter and maintains expression of c-FLIP(L) promoter activity (Zhang, X et al. – submitted). The current study demonstrates that treatment of resistant prostate cancer cells with MG-132 potentiates binding of c-Fos and c-Jun proteins to the c-FLIP(L)-AP-1-(f) site (Fig. 5B & C). Although, binding of c-Fos/c-Jun to c-FLIP(L)'s putative promoter region after treatment with MG-132 may be necessary, it is not sufficient to reduce c-FLIP(L)'s m-RNA and protein levels (Fig. 2). Therefore, addition of TRAIL to MG-132 induces other factors to repress c-FLIP(L) levels and potentiate cell death.

The AP-1 transcription factor is composed of protein dimers between the Jun, Fos, and ATF family members. The predominant forms of AP-1 in most cells are Fos/Jun heterodimers, which have a high affinity for binding to an AP-1 site. The regulation of these transcription factors is critical in determining the response to various physiological and environmental stimuli (31, 32). In addition to c-Fos, we found that c-Jun was another AP-1 family protein that was activated by MG-132. c-Jun protein levels increased in the TRAIL-resistant cancer cells after treatment with MG-132. More specifically, MG-132 promoted expression of nuclear c-Jun protein and hetero-dimerization with c-Fos and binding to the c-FLIP(L) promoter (Fig. 4). These results suggest that c-Fos/c-Jun heterodimers may act concomitantly to down-regulate c-FLIP(L) expression and sensitize resistant cancer cells to undergo TRAIL-induced apoptosis.

Activation of AP-1 family members by other TNF family signalling pathways, besides TRAIL, has been implicated in the past. For example, the TNF receptor member, Receptor Activator of NF-κB Ligand (RANKL), is a key regulator of bone homeostasis. RANKL induces expression of c-Fos, an important step in proper bone development. In order to maintain proper balance in bone development, c-Fos activates its own inhibitor, interferon-beta, to reduce RANKL signalling. Thus, an auto-regulatory mechanism involving c-Fos, the TNF receptor family member, RANKL, and interferon-beta play a crucial role in proper bone development (45). In the present study we identified a similar auto-regulatory mechanism that involves c-Fos/c-Jun heterodimerization in resistant cancer cells that were sensitized to TRAIL-induced apoptosis.

We postulate that post-translational modifications of AP-1 family member proteins, particularly c-Fos and c-Jun, play an important role in determining whether cancer cells are sensitive or resistant to TRAIL-induced apoptosis. Cellular localization and activation of c-Fos and c-Jun can depend on their phosphorylation, protein stability and other chaperone proteins. Recent work has suggested that phosphorylation of c-Fos, which is an important determinant of its activity and expression, is tightly regulated by a variety of kinases such as mitogen-activated protein kinase (MAPK) (20, 46), Fos-regulating kinase (FRK) (47), ribosomal S6 kinase 2 (RSK2) (48), casein kinase II (CKII) (49), PI3K-dependent kinase 1 (PDK1) (50). Protein stability of c-Fos, another regulator of its physiologic function, has been shown to be dependent on its C-terminal PEST3 domain which modulates c-Fos' proteosome mediated degradation (51). Associated proteins in the form of chaperone proteins or hetero-dimers can also regulate c-Fos' structure and function. Therefore, we believe that c-Fos and c-Jun's post-translational modifications can significantly affect its ability to regulate c-FLIP(L) gene expression and TRAIL-induced apoptosis, and it's an area under investigation in our laboratory.

Some limitations of our study are that MG-132 is a general proteosome inhibitor, and can affect many different molecular pathways in cancer cells. Noting this limitation, we focused our attention on the effect of MG-132 on AP-1 related protein, c-Fos. Since our prior work has suggested that c-Fos, and not other AP-1 protein family members, is an important modulator of c-FLIP(L) protein, we primarily focused our attention on the effects of c-Fos and c-FLIP(L). However, our present results suggest that sensitization of TRAIL-resistant cancer cells by MG-132 lead to increased levels of c-Jun, as well as to c-Fos – a finding not seen in TRAIL-sensitive cells. In particular, we showed that DNA

binding of c-Jun to potential AP-1 sites after treatment with TRAIL may be more pronounced than binding of c-Fos to potential AP-1 sites (Fig. 5). Our future studies will determine whether c-Fos and c-Jun have an equal or disproportionate effect on transcriptional regulation of c-FLIP(L) and modulation of TRAIL-induced apoptosis in cancer cells that are sensitized by the proteosome inhibitor, MG-132.

In summary, we demonstrate that MG-132 primes and sensitizes TRAIL-resistant prostate cancer cells to undergo apoptosis by activating the AP-1 family member proteins, c-Fos and c-Jun (Fig. 6A). Combination of MG-132 with TRAIL in TRAIL-resistant prostate cancer cells promotes cell death by increased heterodimerization of c-Fos/c-Jun and direct repression of the anti-apoptotic molecule, c-FLIP(L) (Fig. 6B). Therefore, we report a new regulatory pathway which MG-132 sensitizes cancer cells for apoptosis, and combination of TRAIL with proteosome inhibitors may be an effective strategy for treating TRAIL-refractory tumors.

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FIGURE LEGENDS

Fig. 1. Cell viability and apoptosis assays after treatment with TRAIL and/or MG-132. Data represent an average of at least three independent experiments ± SD.

(A) Sensitivity of prostate cancer cells to TRAIL. Cell viability was evaluated by MTS assay after treatment with TRAIL for 24 hours with increasing concentrations of TRAIL. Viability of untreated cells was set at 100%.

(B) TRAIL-resistant prostate cancer cells PC3-TR or LNCaP cells were treated with MG-132, TRAIL or TRAIL (100ng/ml) + MG-132 (1μM) for 24 hours. Cell viability was evaluated by MTS.

(C) FACS analysis for apoptosis after treatment with MG-132 (1μM), TRAIL (100ng/ml) or combination of the two for 24 hours. Apoptosis was assessed by FITC-conjugated annexin V and propidium iodide (PI) staining for 15 minutes at room temperature. The percentage of apoptotic cells was determined by annexin V stained positive cells.

(D) Cell viability after exposure of nonmalignant benign prostatic hyperplasia (BPH-1) or HEK 293T cells to MG-132 (1μM), TRAIL (100ng/ml) or combination of the two did not induce cell death after 24 hours of treatment.

Fig. 2. TRAIL combined with MG-132 represses c-FLIP(L) and induces c-Fos.

(A) c-FLIP(L) and c-Fos (B) expression by Western blot (upper panel) and semi-quantitative reverse transcription-PCR analysis (lower panel in A). PC3-TR cells were treated with MG-132 (1μM), TRAIL (100ng/ml) or MG-132 combined with TRAIL. Western blot results represent 24 hours after treatment. GAPDH is used as loading control.

(C) Protein expression of c-FLIP(L) and c-Fos. PC3-TR cells were treated with MG-132 (1μM) + TRAIL (100ng/ml) for different times (1, 4, 12, and 24 hours).

(D) Western blot analysis of nuclear c-Fos and phosphorylated c-Fos after treatment with MG-132 (1μM), TRAIL (100ng/ml) or MG-132 combined with TRAIL for 24 hours. Oct-1 is used as loading control of nuclear extracts.

Fig. 3. MG-132 combined with TRAIL increases the AP-1 activity and decreases the c-FLIP(L) promoter activity. Data are means ± SD from four independent experiments. * $P< 0.05$ and ** $P< 0.01$.

(A) AP-1 reporter luciferase activities and (B) c-FLIP(L) promoter luciferase activities in PC3-TR cells after treatment with MG-132 (1μM), TRAIL (100ng/ml) or MG-132 combined with TRAIL for 4 or 24 hours . Luciferase activity of control samples without treatment were set at 1 and fold increase or fold decrease are represented, accordingly.

(C) PC3-TR cells were transfected with c-Fos siRNA for 48 hours, and then assessed for AP-1 reporter luciferase activities or c-FLIP(L) promoter luciferase activities. Western blot demonstrates successful reduction of c-Fos after siRNA-c-Fos treatment.

(D) c-FLIP(L) promoter luciferase activities. PC3-TR cells were transfected with c-Fos siRNA for 48 hours, and then treated with MG-132 (1μM), TRAIL (100ng/ml) or MG-132 combined with TRAIL for 24 hours.

(E) c-Fos and c-FLIP(L) protein levels of PC3-TR cells were assayed by Western blot after transfected with c-Fos siRNA for 48 hours, and then treated with MG-132 (1μM), TRAIL (100ng/ml) or MG-132 combined with TRAIL for 24 hours.

(F) Cell viability of PC3-TR cells transfected with c-Fos siRNA or non-specific siRNA for 48 hours and then treated with MG-132 (1μM), TRAIL (100ng/ml) or MG-132 combined with TRAIL for 24 hours. Cell viability was evaluated by MTS assay. Viability of untreated cells was set at 100%.

(G) AP-1 reporter and c-FLIP(L) promoter luciferase activities analysis. Ectopic expression of c-Fos for 48 hours in PC3-TR cells, and then transfected with AP-1 reporter or c-FLIP(L) promoter plasmids and Ranilla for another 24 hours.

Fig. 4 MG-132 upregulates AP-1 activity by increasing nuclear c-Fos and c-Jun and their heterodimerization.

(A) Western blot analysis of AP-1 member proteins (FosB, c-Jun, JunB, JunD, Fra-1, and Fra-2). PC3-TR cells were treated with MG-132 (1μM), TRAIL (100ng/ml) or MG-132 combined with TRAIL for 24 hours.

(B) Western blot analysis of nuclear protein for total c-Jun and phosphorylated c-Jun in PC3-TR cells.

(C) Immunoprecipitation assay between c-Fos and c-Jun protein. PC3-TR cells were treated with MG-132 (1μM), TRAIL (100ng/ml) or MG-132 combined with TRAIL for 4 hours. Equal quantity whole-cells lysates were immunoprecipitated using c-Fos or c-Jun antibody. The resulting immune complex was subjected to Western blot analysis. Input was 2% of the total from each sample of IP.

Fig. 5. AP-1 binding to c-FLIP(L) promoter was analyzed by EMSA and ChIP assay.

(A) EMSA and EMSA supershift assay for c-Fos and c-Jun binding to DNA.

(B) AP-1 binding sites of the putative regulatory region of c-FLIP(L) before the ATG start-codon.

(C) AP-1 binding to “f” site of c-FLIP(L) putative promoter region was analyzed by ChIP assay. Negative controls are samples using non-specific IgG. Positive controls are whole cell lysates without the immunoprecipitation step, and experimental samples include the ChIP assay using c-Fos or c-Jun antibodies.

Fig. 6. Model for MG-132 priming and sensitization of TRAIL-resistant cancer cells. Cells are primed after exposure to MG-132 by upregulation of AP-1 (c-Fos/c-Jun) but do not undergo cell death (A). However, combination of MG-132 and TRAIL primes cancer cells and promotes apoptosis of TRAIL-resistant prostate cancer cells (B).

Fig. 1

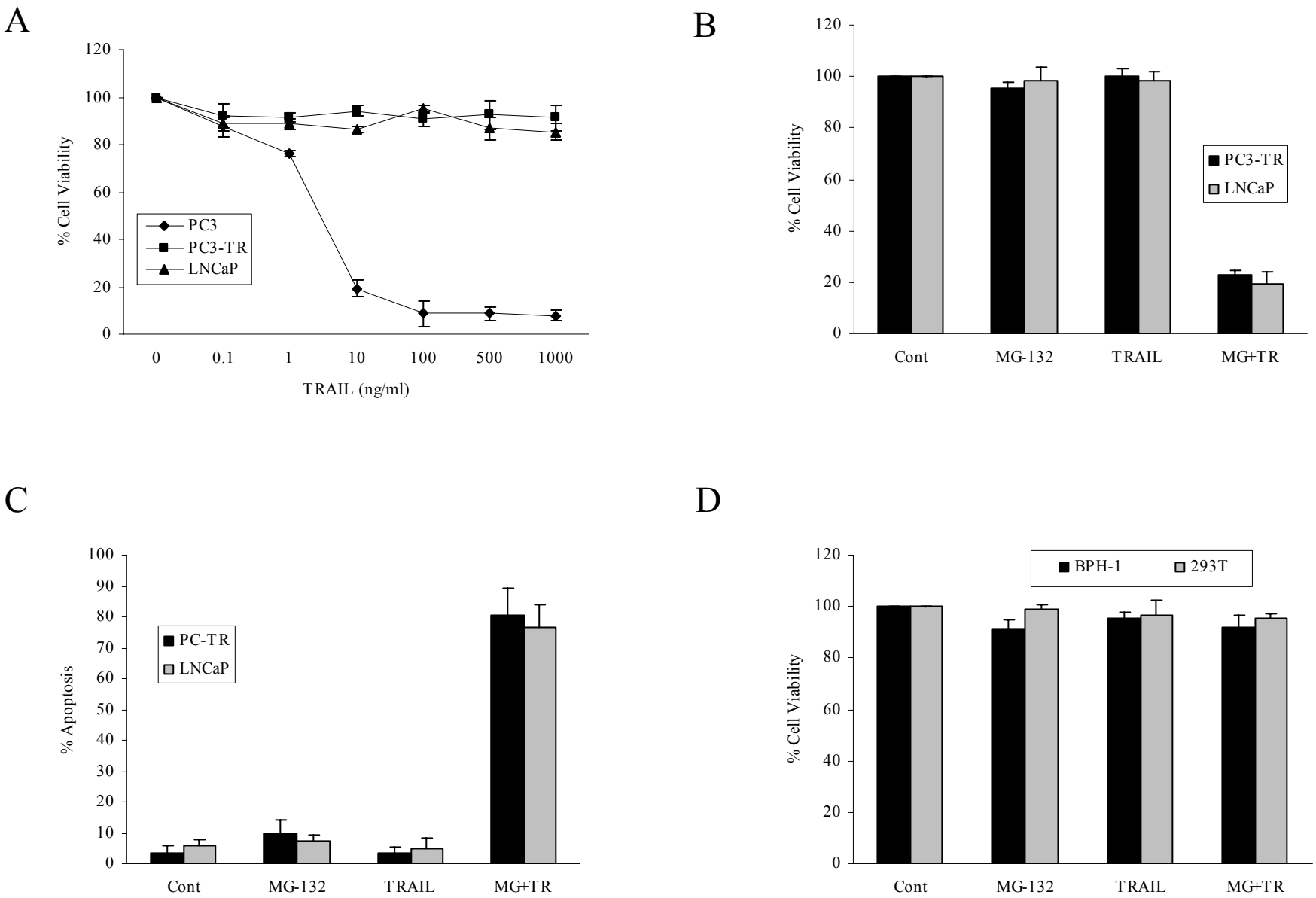

A
% Cell Viability
120
100
80
60
40
20
0
PC3
PC3-TR
LNCaP
0
0.1
1
10
100
500
1000
TRAIL (ng/ml)
B
% Cell Viability
120
100
80
60
40
20
0
PC3-TR
LNCaP
Cont
MG-132
TRAIL
MG+TR
C
% Apoptosis
100
90
80
70
60
50
40
30
20
10
0
PC-TR
LNCaP
Cont
MG-132
TRAIL
MG+TR
D
% Cell Viability
120
100
80
60
40
20
0
BPH-1
293T
Cont
MG-132
TRAIL
MG+TR

Fig. 2

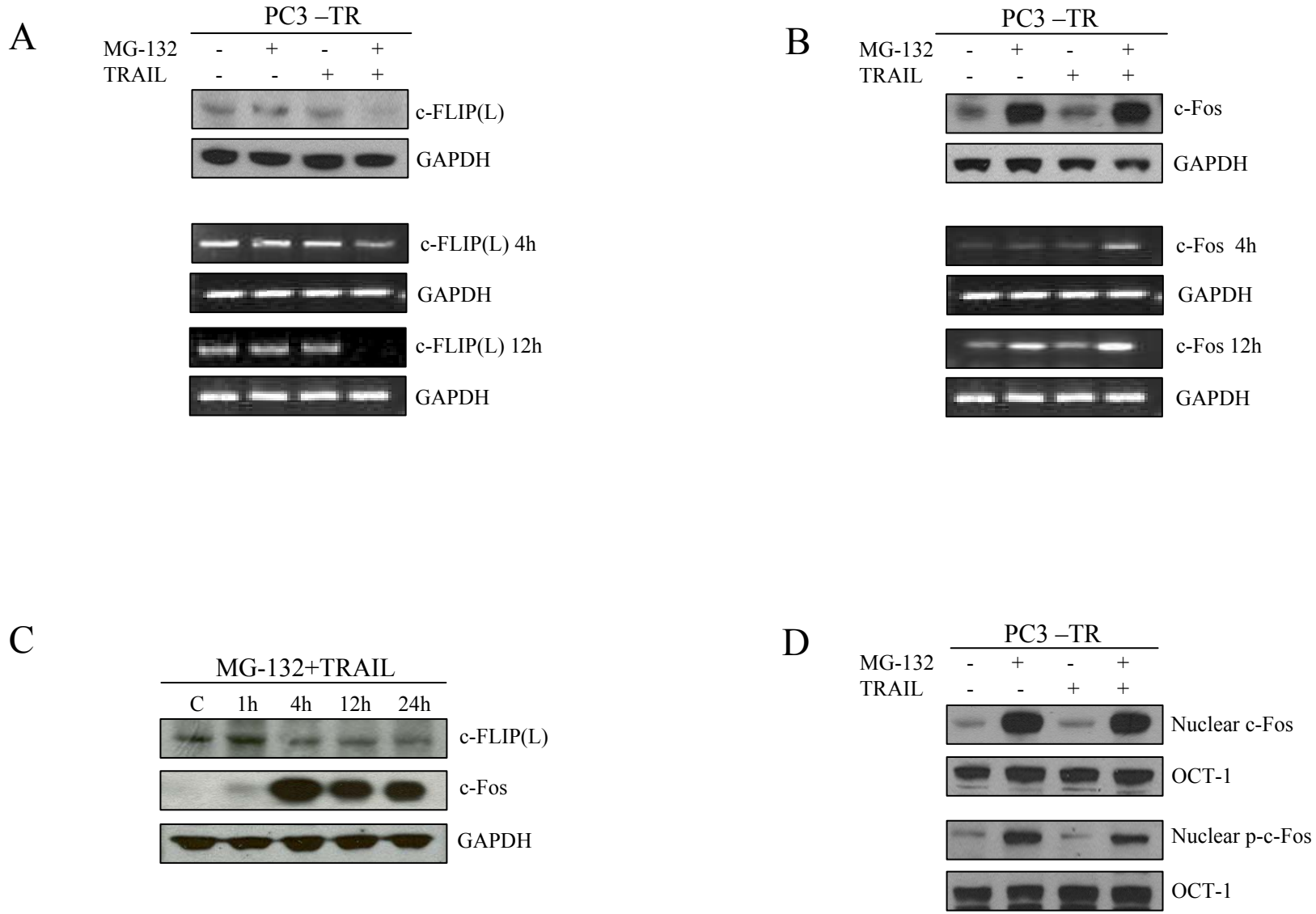

A
PC3 –TR
MG-132 - + - +
TRAIL - - + +
c-FLIP(L)
GAPDH
c-FLIP(L) 4h
GAPDH
c-FLIP(L) 12h
GAPDH
B
PC3 –TR
MG-132 - + - +
TRAIL - - + +
c-Fos
GAPDH
c-Fos 4h
GAPDH
c-Fos 12h
GAPDH
C
MG-132+TRAIL
C 1h 4h 12h 24h
c-FLIP(L)
c-Fos
GAPDH
D
PC3 –TR
MG-132 - + - +
TRAIL - - + +
Nuclear c-Fos
OCT-1
Nuclear p-c-Fos
OCT-1

Fig. 3

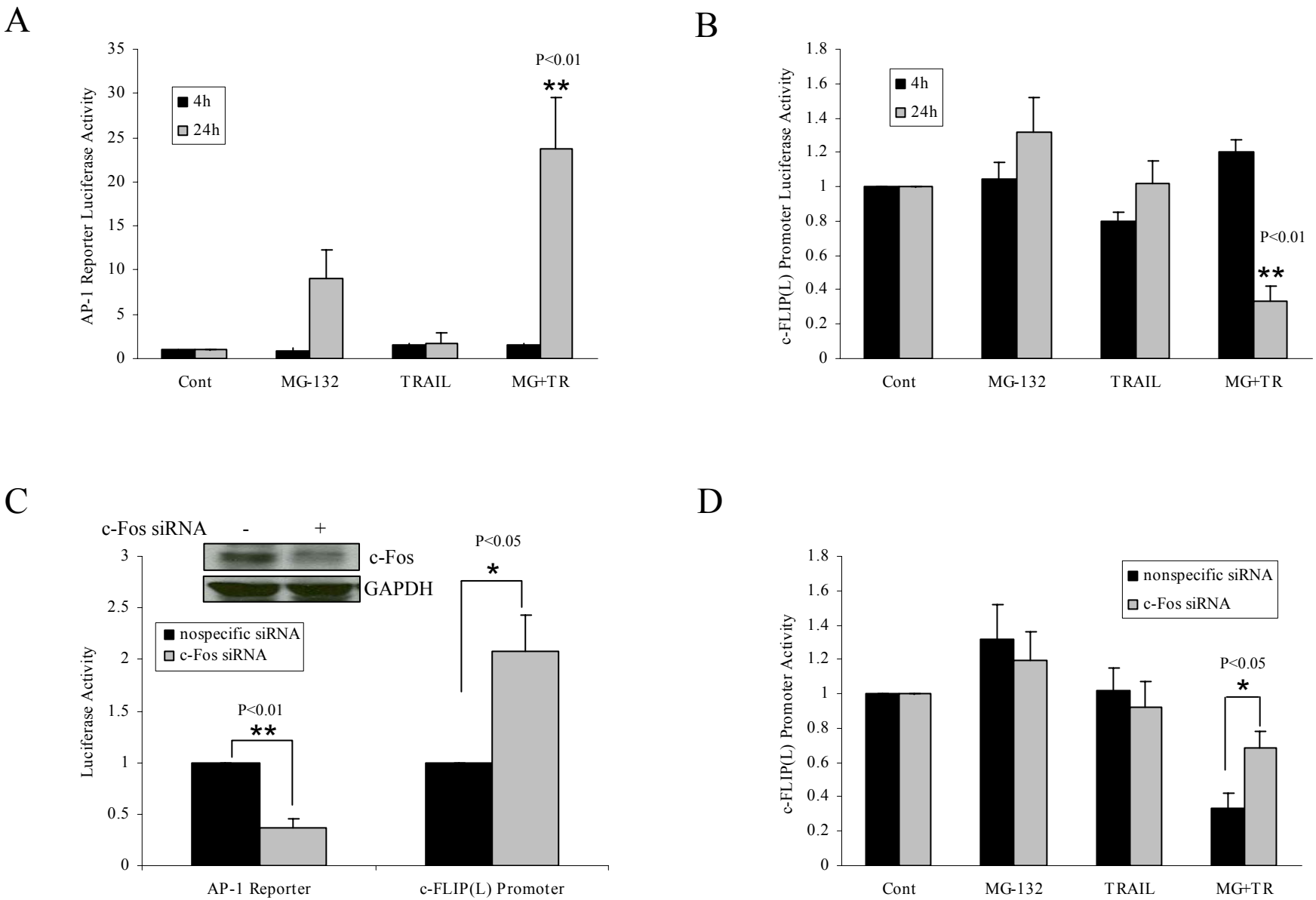
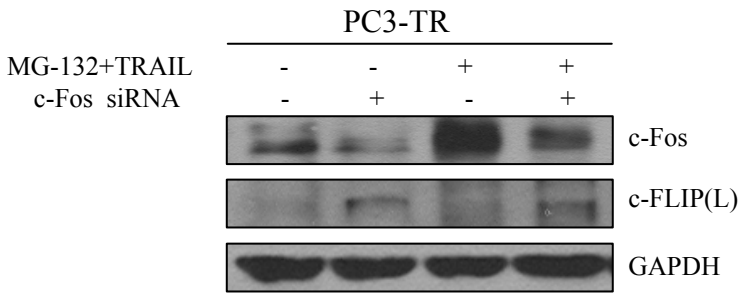
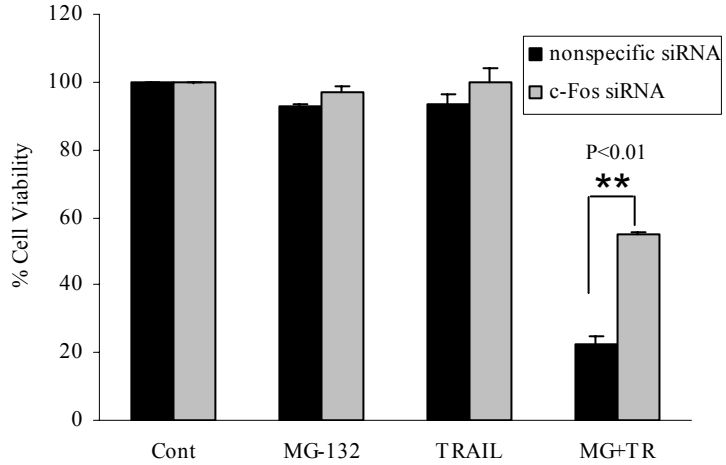

A
AP-1 Reporter Luciferase Activity
4h
24h
P<0.01
**
Cont
MG-132
TRAIL
MG+TR
B
c-FLIP(L) Promoter Luciferase Activity
4h
24h
P<0.01
**
Cont
MG-132
TRAIL
MG+TR
C
c-Fos siRNA - +
c-Fos
GAPDH
nospecific siRNA
c-Fos siRNA
P<0.01
**
P<0.05
*
Luciferase Activity
AP-1 Reporter
c-FLIP(L) Promoter
D
c-FLIP(L) Promoter Activity
nonspecific siRNA
c-Fos siRNA
P<0.05
*
Cont
MG-132
TRAIL
MG+TR

Fig. 3

E


PC3-TR
MG-132+TRAIL - - + +
c-Fos siRNA - + - +
c-Fos
c-FLIP(L)
GAPDH

F


% Cell Viability
120
100
80
60
40
20
0
nonspecific siRNA
c-Fos siRNA
P<0.01
**
Cont
MG-132
TRAIL
MG+TR

G

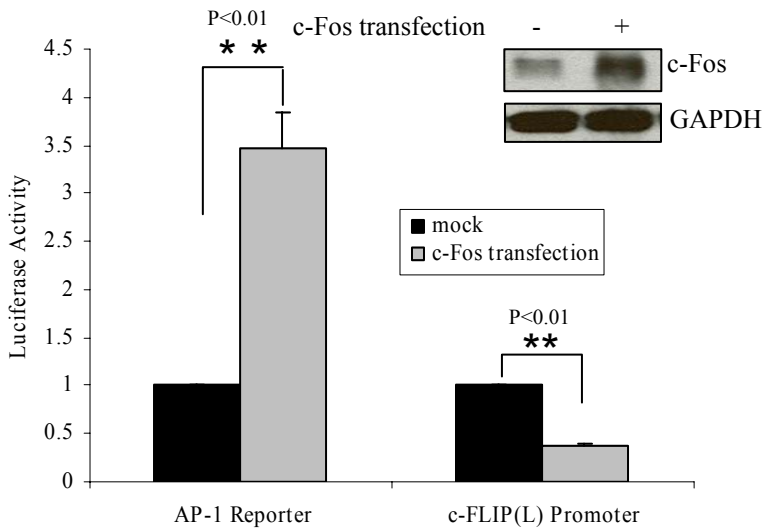

P<0.01
* *
c-Fos transfection
- +
c-Fos
GAPDH
Luciferase Activity
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
mock
c-Fos transfection
P<0.01
**
AP-1 Reporter
c-FLIP(L) Promoter

Fig. 4

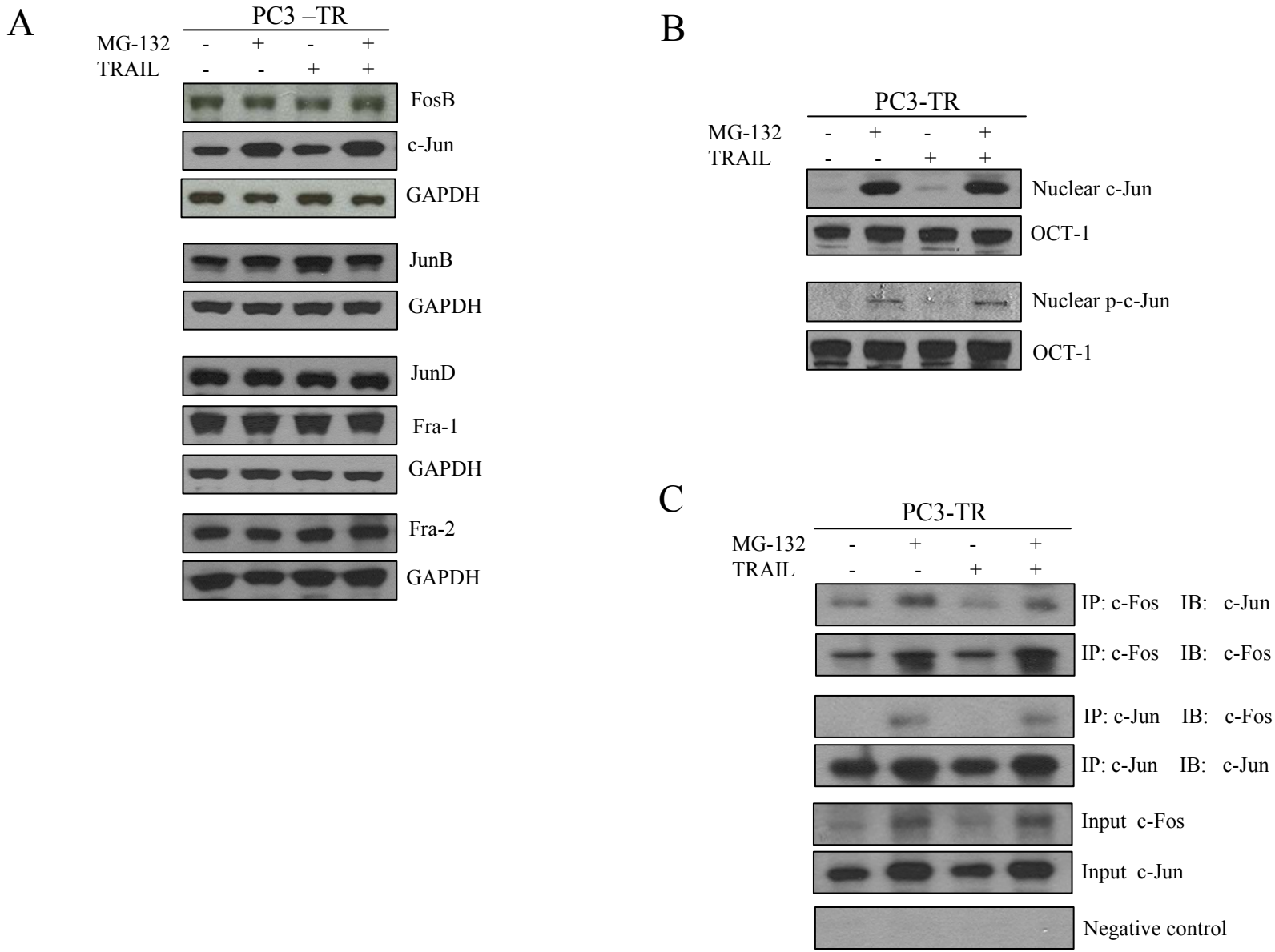
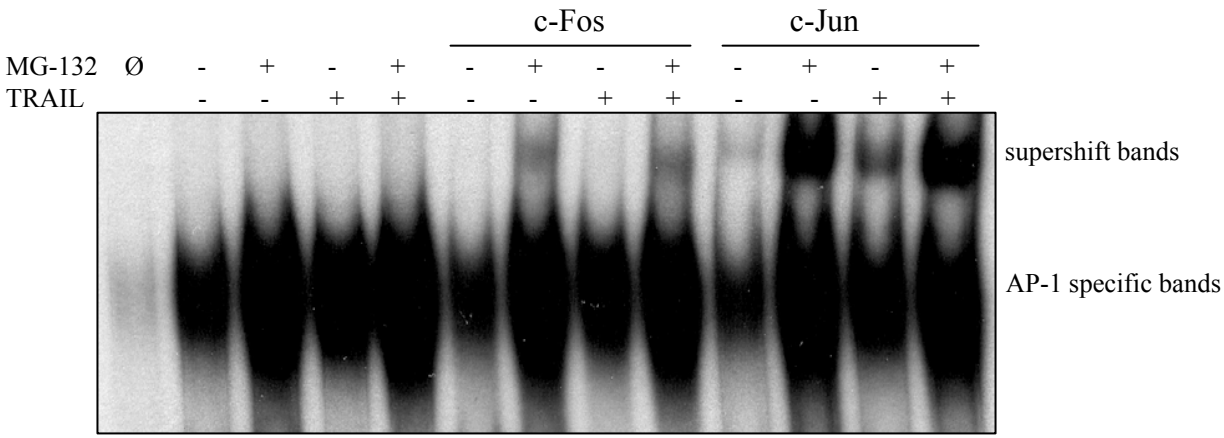
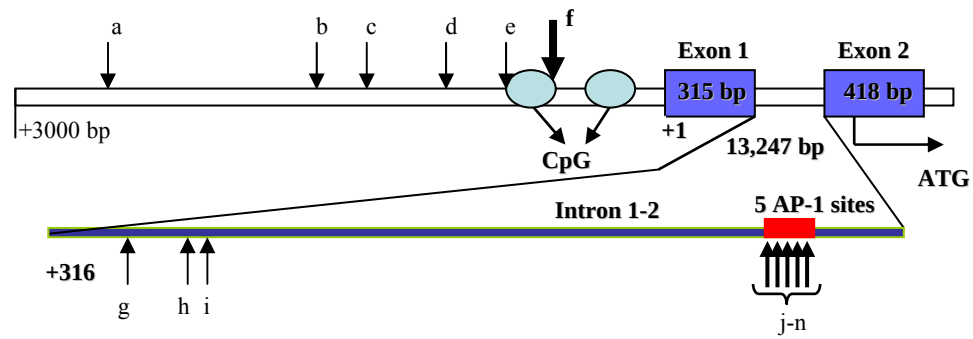

A
PC3 –TR
MG-132 - + - +
TRAIL - - + +
FosB
c-Jun
GAPDH
JunB
GAPDH
JunD
Fra-1
GAPDH
Fra-2
GAPDH
B
PC3-TR
MG-132 - + - +
TRAIL - - + +
Nuclear c-Jun
OCT-1
Nuclear p-c-Jun
OCT-1
C
PC3-TR
MG-132 - + - +
TRAIL - - + +
IP: c-Fos IB: c-Jun
IP: c-Fos IB: c-Fos
IP: c-Jun IB: c-Fos
IP: c-Jun IB: c-Jun
Input c-Fos
Input c-Jun
Negative control

Fig. 5

A


c-Fos
c-Jun
MG-132 Ø - + - + - + - + - + - +
TRAIL - - + + - - + + - - + +
supershift bands
AP-1 specific bands

B


a
b
c
d
e
f
Exon 1
Exon 2
315 bp
418 bp
+3000 bp
+1
13,247 bp
CpG
ATG
Intron 1-2
5 AP-1 sites
+316
g
h i
j-n

C

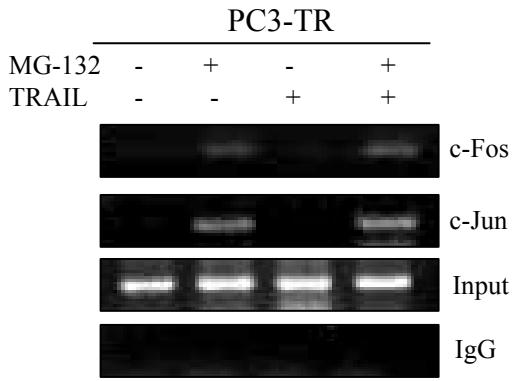
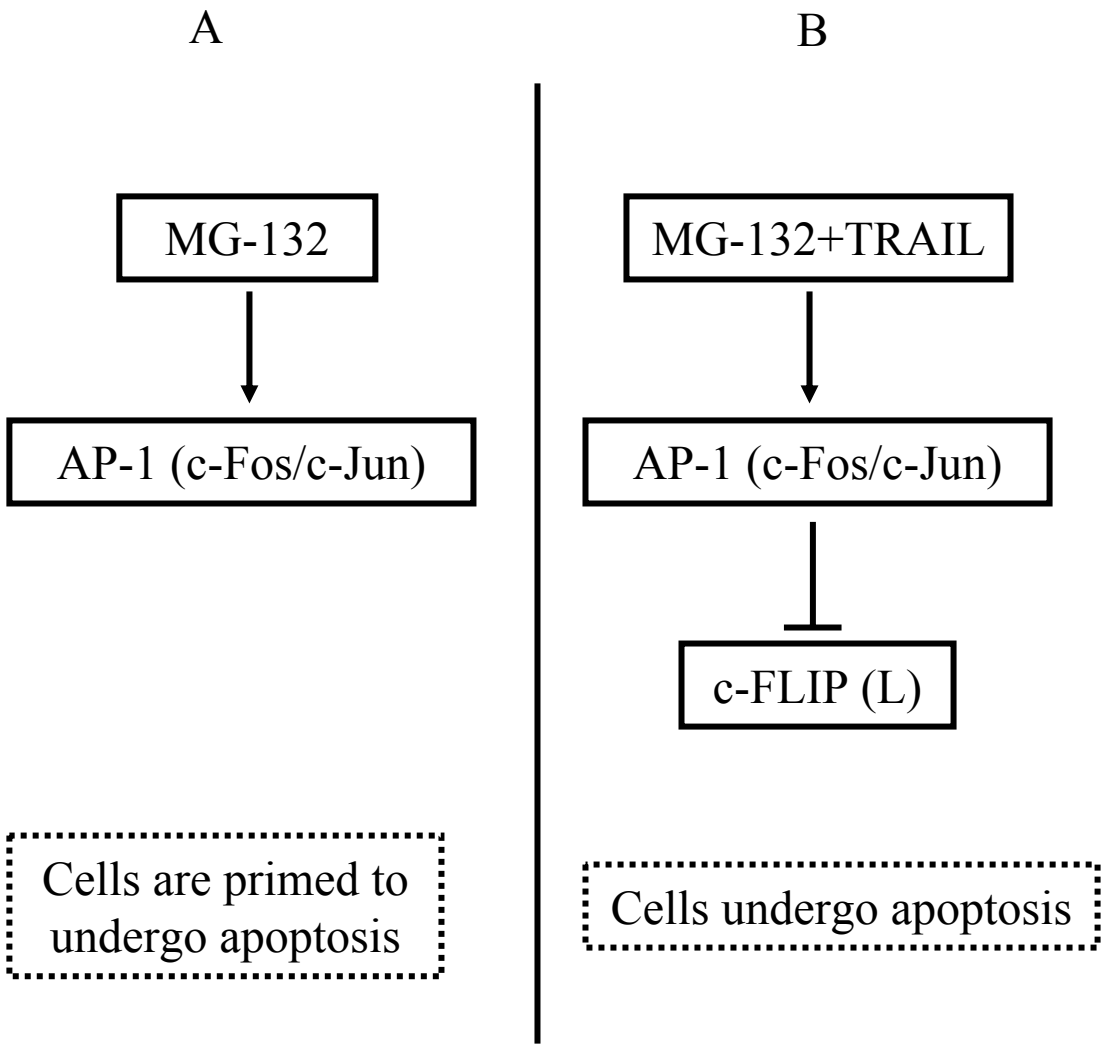

PC3-TR
MG-132 - + - +
TRAIL - - + +
c-Fos
c-Jun
Input
IgG

Fig. 6


A
B
MG-132
AP-1 (c-Fos/c-Jun)
Cells are primed to undergo apoptosis
MG-132+TRAIL
AP-1 (c-Fos/c-Jun)
c-FLIP (L)
Cells undergo apoptosis