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# Changes in FADD levels, distribution, and phosphorylation in TNF$\alpha$-induced apoptosis in hepatocytes is caspase-3, caspase-8 and BID dependent

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## Abstract

FADD/MORT1 (The adaptor protein of Fas Associate Death Domain/Mediator of Receptor Induced Toxicity) is essential for signal transduction of death receptor signaling. We have previously shown that FADD is significantly up-regulated in TNF$\alpha$/ActD induced apoptosis. Over-expression of FADD also induces death of lung cancer cells and primary hepatocytes. We hypothesize that the increase in detectable FADD levels require the proximal steps in apoptotic signaling and speculated that FADD would be redistributed in cells destined to undergo apoptosis. We show that monomeric non-phosphorylated FADD is up-regulated in hepatocytes treated with TNF$\alpha$/ActD and that it accumulates in the cytoplasm. Nuclear phosphorylated FADD decreases with TNF$\alpha$/ActD treatment. Dimeric FADD in the cytoplasm remains constant with TNF$\alpha$/ActD. The change in FADD levels and distribution was dependent on caspase-3, caspase-8 activity and the presence of BID. Thus, changes in FADD levels and distribution are downstream of caspase activation and mitochondria changes that are initiated by the formation of the DISC complex. Changes in FADD levels and distribution may represent a novel feed-forward mechanism to propagate apoptosis signaling in hepatocytes.

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**Keywords**

FADD; TNF$\alpha$; Hepatocytes; Apoptosis; Caspse-3; Caspase-8; BID; Nuclear/cytoplasm translocation; Phosphorylation; Dimerization

## Introduction

Death ligands, such as Tumor Necrosis Factor $\alpha$ (TNF$\alpha$) are thought to cause the widespread apoptosis seen in some forms of fulminant hepatic failure [1, 2]. Death receptors on the cell surface of hepatocytes respond to death ligands to initiate the apoptotic cascade. Upon TNF$\alpha$ binding the TNF Receptor 1 (TNFR1) trimerizes and the death inducing signaling complex (DISC) forms. In TNF induce apoptosis the DISC is comprised of TNF Receptor Associated Death Domain Protein (TRADD) protein, Fas-associated death domain protein (FADD) and pro-caspase-8 [3]. The formation of the DISC causes catalytic cleavage of pro-caspase-8 to its cleaved activated form which in turn causes cleavage of the Bcl-2 like protein BID to generate tBID. tBID translocates to the mitochondrial membrane and, along with BAX, forms a pore to cause release of cytochrome c [4]. Cytochrome c then binds with Apaf-1 and caspase-9 to form the apoptosome which leads to further downstream apoptotic signaling including caspase-3 activation.

FADD is an essential component of the DISC for all death receptors [5–7]. Over-expression of FADD causes activation of the apoptotic death signaling pathway even in the absence of a death ligand [7]. We and others have demonstrated that FADD levels increase in cells exposed to TNF and a sensitizing agent such as Actinomycin D (Act D) [7, 8]. FADD can also undergo post translational modifications, including phosphorylation [9, 10] and homotypic interaction [11–14]. Furthermore, FADD can be found in both the nucleus [10, 15] and cytosol [7, 8]. FADD can be phosphorylated on sites Ser194 (human) and Ser191 (mouse) [16] and Screaton et al. [10] suggest that pFADD is able to shuttle between the nucleus and cytoplasm during apoptosis. FADD forms aggregates in vitro and when transfected in mammalian cells forms microscopically visible large filamentous structures termed death effector filaments (DEF) [17]. Artificial FADD dimers produced by chemical inducers of dimerization have been shown to be sufficient to trigger apoptosis in Jurkat-TAg cells [12]. It has also been suggested that FADD dimerization could be functionally involved in apoptosis signaling [13]. Natural FADD is self associated through a conserved RXDLL motif in the death effector domain (DED) as demonstrated using fluorescence resonance energy transfer (FRET) in MCF7-Fas cells [13]. Sandu et al. [14] further found that over-expressed FADD uses the DED to self associate into an arrangement that is necessary to stabilize FADD at the intracellular domain of an activated Death Receptor (DR) in FADD deficient Jurkat I2.1 cells and 293T cells. Thus, changes in not only the levels of FADD but also the forms and distribution of FADD might be expected as cells proceed toward apoptosis.

Here we sought to define the changes in the posttranslational modification of FADD and FADD distribution as FADD levels increased in cell destined to undergo apoptosis. We demonstrate that FADD exists in hepatocytes in dimeric and monomeric forms and both

can be detected in phosphorylated and non-phosphorylated forms. Apoptosis signaling activated by TNF$\alpha$/ActD up-regulated only monomer cytoplasmic FADD which is not phosphorylated. Levels of nuclear FADD which exists in a monomeric, phosphorylated form reduced with TNF$\alpha$/ActD treatment. We found that these changes were dependent on caspase-3 and caspase-8 activation and the presence of BID. These changes in FADD levels and distribution could represent a novel feed-forward mechanism to promote apoptosis cell death.

## Materials and methods

### Materials

Hepatocyte culture medium, antibiotics and serums used were as previously described [7]. Mouse recombinant TNF$\alpha$ was obtained from R&D Systems (Minneapolis, MN). Actinomycin D was purchased from Sigma (A9415). Caspase-8 inhibitor (z-IETD-fmk, # 550380) and caspase-3 inhibitor (z-DMEM-fmk) were from BD Biosciences Pharmingen (San Diego, California,) and prepared according to manufactures instruction. $\beta$-Actin (ab8226) and $\beta$-tubulin (ab11313) were obtained from Abcam (Cambridge, MA). Lamin B receptor was purchase from Abcam (ab45848, Cambridge, UK). The transfection reagent used was Lipofectimine2000 (Invitrogen, Burlington, ON). FADD antibodies were acquired as described in Table 1. Anti-c-myc antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents are from Sigma.

### Preparation of primary hepatocytes and cell culture

Primary hepatocytes were isolated and purified from either male C57BL/6 mice (Charles River Laboratories, Wilmington, MA) or BID−/− mice [18] by a collagenase perfusion method as described previously [7, 8]. Highly purified hepatocytes (~98% purity) of ~95% viability by trypan blue exclusion) were suspended in Williams' E medium supplemented with 10% calf serum, 1 uM insulin, 2 mM Lglutamine, 15 mM HEPES, pH 7.4, 100 units/ml penicillin, and 100 (μg/ml streptomycin. The cells were plated on collagen-coated tissue culture plates at a density of $2 \times 10^5$ cells/ml/12 well, or $5 \times 10^6$ cells/5 ml/10-cm dish for Western blotting assays. Cells were allowed to adhere overnight at 37°C in 5% $CO_2$ prior to describe treatment.

Apoptosis was induced by incubating the hepatocytes with culture medium containing 2000 units/ml TNF$\alpha$ and 200 ng/ml ActD for specified monolayers. For whole cell lysates (WL), cells were washed with cold phosphate-buffered saline (PBS) and resuspended in 5-fold volume of cell lysis buffer with protease inhibitors (Sigma) (0.5 mM Phenylmethylsulfonyl fluoride; 5 μg/ml Aprotinin; 5 μg/ml Pepstatin; and 10 μg/ml Leupeptin). After three cycles of freeze-thaw, cell debris was removed by centrifugation at 13,000g at 4°C for 20 min. The 13,000$g$ supernatant is termed as whole cell lysate (WCL). Protein concentration was determined using BCA assay (Pierce, Rockford, Ill.) and WCL were processed for Western Blot.

### Cell viability assay

Cell viability was determined by the crystal violet method as described previously [4, 7]. In brief, cells were stained with 0.5% crystal violet in 30% ethanol and 3% formaldehyde for 5 min at room temperature followed with six times wash with tap water. Plates were leave to dry in room temperature. After drying, cells were lysed with 10% sodium dodecyl sulfate (SDS) solution, and dye uptake was measured at 550 nM using a 96-well microplate reader. Cell viability was calculated from relative dye intensity of the mean for four parallel samples and presented as percentages relative to untreated samples. Cell viability is presented with the representative Western blotting results from the same hepatocytes harvest.

### Cell fractionation and western blot

Cytoplasmic and nuclear extracts were prepared as described [19]. In briefly, for cell fractionation procedures, protease inhibitors (Sigma) were added to all the buffers in the following concentrations: pefablock, 0.2 mg/ml; aprotinin, 0.01 mg/ml; pepstatin, 0.01 mg/ml; and leupeptin, 0.01 mg/ml. Cells grown in 10-$cm^2$ dish were rinsed two times with cold PBS then were harvested by scraping into 1.0 ml ice cold PBS. The PBS suspensions were spin down at 2,000 rpm for 3 min. Collect the pellet and re-suspend it in Buffer-A (Hepes, 0.1M; $MgCl_2$, $1.5 \times 10^{-3}$ M; KCl, 0.01M; Nonidet P-40, 0.5%) and incubated on ice for 10 min. Nuclei were pelleted by centrifugation at 5,000*g* for 5 min at 4°C. The supernatant fraction from Buffer-A was collected and classified as cytoplasm. The pellet left from Buffer-A was re-suspended in Buffer-B (Hepes, 0.1M; $MgCl_2$, $1.5 \times 10^{-3}$M; KCl, 0.01M) and spin down at 5,000*g* for 5 min at 4°C for 3 times. The pellet was collected as nucleus. This nucleus pellets were lysed by CD-buffer (Hepes, 0.02M; $MgCl_2$, $1.5 \times 10^{-3}$ M, KCl, 1.6M; EDTA, $2 \times 10^{-4}$ M; Glycerol, 10%) and incubated on ice for 1–2 h. The supernatant fraction was collected by centrifugation at 15,000*g* for 15 min and classified as nucleus fraction. Protein concentration was determined in all cell fractions using a Pierce BCA protein assay reagent as per manufacturer's instructions.

Analyzing of FADD protein monomer-dimer status was accomplished comparing samples separated on 12% SDS-PAGE. Samples (50 μg) were prepared in both denatured and mild-denatured sample buffer as described previously [20]. All the western blots for cell fraction studies used mild-denature conditions. Immunoblot for tubulin (Upstate Biotechnology, NY, USA) was used to confirm nuclear isolation integrity. Results were confirmed with commercially available fractionated nuclear extracts were also used to confirm subcellular fractionation (4C Biotech, Belgium; data not shown).

### Immunostaining and confocal microscopy

Primary hepatocytes were seeded at a density of $2.5 \times 10^5$ cells/well in commercially available collagen-1 coated 22mm coverslips (BD Biocscience, San Jose, CA). Following an overnight incubation at 37°C, 5% $CO_2$, cells were treated as described. Following treatments, monolayers were fixed with 2% Paraformaldehye, followed by wash of 2× PBS to minimize autofluorescence created by the paraformaldehyde. Cell monolayers were permeabilized with 0.2% Triton X-100 for 15 min, followed by a 1h block in 2% BSA–PBS. Samples were incubated with the different FADD primary antibodies as described in Table 1. Following primary antibody incubation, monolayer were washed 3× with 0.5%BSA-PBS,

secondary antibodies as detailed in Table 1 for 1h. Secondary antibody was removed with three washes of 0.5%BSA-PBS and the BSA was removed with three additional washes with PBS alone. The fixed cells were mounted and preserved with water soluble mounting medium [7]. Primary antibody deleted samples used as negative controls for staining. DRAQ5 (Molecular Probes, Eugene, OR) or DAPI (Sigma) was added where indicated to the samples to enable visualization of the nuclei. Fluorescent images were captured with an Olympus Fluoview 1000 confocal microscope (Malvern, NY), fitted with an Olympus CCD digital camera.

## Immunoblotting Analysis

Protein samples (40 μg) were separated on 12% SDS-PAGE and transferred onto a nitrocellulose membrane. Loading of equal protein amounts was assessed by staining of nitrocellulose membranes with 0.1% Ponceau S (Sigma) in 5% acetic acid. Nonspecific binding was blocked with Tris-Buffered Saline with 0.1% Tween-20 (TBS-T, pH 7.4) containing 5% nonfat milk for 1 h of incubation with agitation at room temperature. The FADD antibodies were used as described in Table 1, the other antibodies were used as follows: anti-caspase-8 (1:1000), anti-$\beta$-actin (1:5000) and anti-$\beta$ tubulin (1:1000). After primary antibodies incubation, the membranes were washed three times with TBS-T and incubated with horseradish peroxidase-conjugated goat secondary antibodies (see Table 1) for 1 h at room temperature. Following five washes with TBS-T, the membranes were incubated with Supersignal TM (Pierce, Rockford, IL) according to the manufacturer's instructions. Chemiluminescence was visualized on Kodak film (Eastman Kodak).

## Cell line culture conditions and transfections

Human embryonic kidney (HEK 293T; ATCC) were grown in Dulbecco's Modified Medium (DMEM) containing 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine and 4.5 g/l glucose. Cell cultures were allowed to grow to 50–70% confluence prior to transfection. Cells were washed with Opti-MEM and transfected Lipofectamine2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. The DNA and lipofectamine mixture were incubated with cells in Opti-MEM medium (Invitrogen Life Technologies, Gaithersburg, MD), which was replaced by complete medium (DMEM+10% serum) after 4 h of transfection. Cells were maintained at 37°C and 5% $CO_2$, 95% air until collected. Transfection efficiency was routinely measured by GFP and was determined to be between 40% and 70% (data not shown).

Mouse FADD cDNA (wild type c-myc-tagged mouse FADD(myc-WT), phosphorylation site (Serine191) mutant c-myc-tagged mouse FADD(myc-SA) were over-expressed in 293T cells and protein levels and form were compared to rat hepatocytes treated with TNF$\alpha$/ActD for 6 h (+ve) for BD-FADD antibody. Whole cell lysates were analyzed by western blot and probed as follows: polyclone myc antibody (myc), polyclone mouse specific pFADD, monoclonal BV-FADD antibody, monoclonal BD-FADD antibody (see Table 1).

## Preparation of DNA constructs

pcDNA3 vector containing the full length of myc-tagged FADD was kindly provided by Dr. Dai-Wu Seol from the University of Pittsburgh School of Medicine. Plasmid DNA was

prepared by using Qiagen Maxiprep kits (Valencia, CA, USA). Site-specific mutagenesis at serine191 to arginine was introduced by PCR with the QuikChange kit (Stratagene, La Jolla, CA, USA) by following the manufacturer's instructions and using pcDNA3-myc-FADD as templates with 12 cycle PCR with the polymerase, Pfu Ultra Hotstart, was performed using the forward primer agagtgagaatatg**g**ccccagtactaagg (bolded nucleotide changed from "t" in wild type) and its antiparallel compliment. The amplimer was then digested with Dpn I, a restriction enzyme that will only digest the methylated, parental, nonmutated strand. The DNA was then transformed into bacteria XL10 Gold (stratagene). Plasmids were purified (Qiagen) from several bacterial colonies and mutation confirmed by DNA sequencing.

## Results

### Detectable FADD increase in TNF/ActD treated mouse hepatocytes is not dependent on transcription or translation

Previous studies have identified the up-regulation of FADD in rat hepatocytes treated with TNF$\alpha$/ActD [7, 8]. The increased levels of the detectable protein were not due to increased copies of FADD mRNA. The mechanistic studies reported here required the use of mouse hepatocytes. Here, we found the same protein (Fig. 1a) and mRNA (Fig. 1b) response in mouse hepatocytes. TNF$\alpha$/ActD induces mouse hepatocyte apoptosis similar to that reported in rat hepatocytes (Fig. 1c). The detectable FADD protein levels recognized by specific antibody were dramatically increased in mouse hepatocytes 6 h following TNF$\alpha$/ActD treatment (Fig. 1a), whereas FADD mRNA levels (Fig. 1b) were unaltered. TNF treatment alone increased FADD mRNA levels without increasing protein levels. In contrast to our previous observation in rat hepatocytes, ActD alone did not increase detectable FADD protein levels or cell death in mouse hepatocytes. The protein translational inhibitor cyclohexamide (CHX), in combination with TNF$\alpha$/ActD, failed to block the increased detection of FADD (Fig. 1a). In fact, CHX alone induced increases in detection of FADD protein levels. Thus, TNF$\alpha$/ActD ± CHX caused an increase in the detectable amount of FADD. This appears to occur through a post-translational mechanism in mouse hepatocytes following the exposure to the pro-apoptotic stimulus TNF$\alpha$/ActD.

### TNF/ActD induce monomeric non-phosphorylated FADD up-regulation

The antibody we utilized to assess FADD levels (BD-FADD antibody) recognizes a monomeric form of FADD. However, FADD is known to undergo posttranslational modification including phosphorylation [9, 10] and homotypic interaction [11–14]. The specificity of two additional anti-FADD antibodies was assessed by western blotting lysates from 293T cells transfected with plasmid for myc-tagged and non-myc-tagged FADD. A phosphorylation site mutant (Serine191A) was used to assess detection of the phosphorylated form of FADD, while both denaturing and mild-denaturing conditions were carried out to assess the detection of dimeric FADD. The specificity and source of FADD antibodies is summarized in Table 1 and confirmed by western blots (Fig. 2).

As shown in Fig. 2a, mouse specific FADD antibody from BioVision (BV-FADD) recognized both 30 kD and 60 kD forms of FADD in the wild-type non-myc tagged tansfected cells. An anti-mouse FADD antibody (Fig. 2b) directed against the

phosphorylated form of FADD (pFADD) detected strong bands in the cells transfected with the wild type myc-tagged (~35 kD) and non-myc-tagged forms of FADD(~30 kD) and only a faint band in the cells transfected with the phosphorylation mutant forms of FADD (~35 kD). Like the BV-FADD antibody the anti-pFADD antibody detected both 30 kD and 60 kD forms in the cells transfected with the wild type non-myc-tagged form of FADD. It is possible that the addition of the myc-tag interfered with the detection of dimmer FADD by BV-FADD and pFADD antibodies. This would explain the detection of bands at the lower molecular weight range (~35 kD) in the myc-tagged FADD transfected cells with BD-FADD antibody (Fig. 2c). The mouse specific FADD antibodies (BV-FADD, pFADD) were not able to detect FADD in rat hepatocytes. A possible explanation could be that these two antibodies were generated with c-terminal of FADD which has a significant sequence difference between rat and mouse. $\beta$-Actin levels were used to assess protein loading (Fig. 2d) and Myc expression was assessed to detect presence of the over-expressed protein (Fig. 2e).

## Cytoplasmic FADD up-regulation is coupled with nuclear pFADD down-regulation

Phosphorylated FADD has been found to be localized in the nucleus in several different cell lines [10, 15, 21, 22]. We hypothesized that the phosphorylation and dimerization status of FADD would determine its subcelluar location in hepatocytes in apoptotic signaling. Cytoplasmic and nuclear fractions of TNF$\alpha$/ActD treated hepatocytes were prepared and probed with BD-FADD, pFADD, and BV-FADD antibodies. Monomeric non-pFADD only localized to the cytoplasm and was dramatically increased in TNF$\alpha$/ActD stimulation in a time dependent manner (Fig. 3a). However, monomeric pFADD localized exclusively in the nucleus and this nuclear pFADD decreased in a time dependent fashion following TNF$\alpha$/ActD stimulation (Fig. 3a). Dimeric FADD was found localized predominately in the cytoplasm and remained constant with the TNF$\alpha$/ActD stimulation (Fig. 3b). Trace amounts of phosphorylated and dimeric non-pFADD were also present in the nuclear fraction of non-stressed hepatocytes but disappeared upon treatment with TNF$\alpha$/ActD (Fig. 3b). The changing subcellular location of the various forms of FADD with pro-apoptotic stimuli may play an important role in the localization, phosphorylation and dimerization status of FADD in apoptotic signaling. However, more direct evidence will be needed to prove the possible translocation leads to the redistribution of various forms of FADD.

## Detectable FADD induced by TNF/Act D is caspase-3, caspse-8 and BID dependent

Hepatocytes undergo Type II apoptosis [23, 24], in which the mitochondria is essential for efficient apoptotic cell death. Upstream apoptotic signals such as Caspase-8 activation and BID cleavage are required to elicit opening of the permeability transition pore and release of pro-apoptotic signaling from mitochondria. Upon TNF$\alpha$ binding to TNFR1, TRADD, FADD and pro-caspase-8 are recruited to the DISC, leading to caspse-8 cleavage and activation [25, 26]. Although FADD is part of the DISC it is unclear if DISC assembly is required for the increase in FADD levels and for redistribution in the cell. Both a pan-caspase (z-vad-fmk, 50 uM) as well as a caspase-8 specific inhibitor (z-ietd-fmk, 50 uM) (Fig. 4a) and a caspse-3 specific inhibitor (DEVD-fmk, 20 uM) (Fig. 4b) prevented monomeric FADD up-regulation following stimulation with TNF$\alpha$/ActD. Block caspase-8 activation and pan caspases activation not only abolished monomeric FADD upregulation

but also rescued hepatocytes from TNF$\alpha$/ActD induced cell death (Fig. 4c). Because BID cleavage is thought to be required for mitochondria dysfunction, we also evaluated whether BID is necessary in FADD up-regulation. In BID −/− mice, there was no up-regulation of monomeric FADD from baseline following TNF$\alpha$/ActD treatment (Fig. 4a).

Confocal imaging was used to determine the location of FADD and pFADD in hepatocytes. Consistent with our western blot analysis, immunofluorescence staining using BD-FADD or anti-pFADD antibody revealed that monomeric non-pFADD was up-regulated in the cytoplasm with TNF$\alpha$/ActD at 6 h. However, the nuclear pFADD disappeared with TNF$\alpha$/ActD treatment. When the caspase-8 inhibitor was administered to TNF$\alpha$/ActD-treated hepatocytes, no increase in cytoplasmic FADD was observed and nuclear pFADD remained unchanged (Fig. 4d). FADD up-regulation and redistribution also did not occur in the BID−/− cells following TNF$\alpha$/ActD treatment (Fig. 4e). In parallel to the cell imaging experiment, cell fraction and western blotting was performed under denaturing and non-denaturing conditions. As shown in Fig. 5, inhibition of caspase-8 prevented the accumulation of monomeric non-pFADD in the cytoplasm and preserved nuclear pFADD levels. Of note, levels of dimeric FADD in the cytoplasm were not altered by TNF$\alpha$/ActD treatment. However, addition of the caspase-8 inhibitor in the setting of TNF$\alpha$/ActD treatment caused dimeric non-pFADD to appear in the nucleus. Taken together these observations indicate that caspase-8 activation is required for the changes in FADD levels and distribution in response to TNF$\alpha$/ActD. Caspase-3 activation is necessary for the increase in monomeric non-pFADD. The appearance of dimeric FADD in the nucleus following TNF$\alpha$/ActD treatment in the setting of caspase-8 inhibition suggests that a caspase-8 independent pathway may also regulate FADD.

## Discussion

Previous observations show that FADD levels increase in cells destined for apoptosis [7, 8] suggesting that cells may be programmed to increase the abundance of FADD to promote apoptosis in cells committed to die. However, FADD can exist in both phosphorylated [9, 10] and non-phosphorylated forms [7, 8] and also undergo homotypic interaction [11–14]. Furthermore, FADD can be found in both the nucleus [10, 15, 21, 27] and cytosol [7, 8]. Experiments were undertaken to understand how the changes in total FADD correlated with FADD localization and post-translational modification. Our studies show that level of cytosolic non-phosphorylated, monomeric FADD increases in a caspase-3, caspase-8 and BID dependent manner in hepatocytes exposed to TNF$\alpha$/ActD. This was associated with a decline in nuclear pFADD. Thus, the change in FADD localization and abundance occur as the result of the activation of proximal signaling events in apoptosis.

One of the best known functions of FADD is its role in apoptosis signaling when recruited to the DISC as an adaptor protein. In TNF$\alpha$ induced apoptosis in hepatocytes, TNFR1 and TRADD recruit FADD and pro-caspase-8 to the DISC. Caspase-8 is further activated by oligomerization of recruited procaspase monomers [28]. Active caspase-8 initiates the apoptotic cascades via cleavage of BID [29]. It is well accepted that FADD is required for caspase-8 activation and is upstream of BID cleavage. However, the processing of p10 and p20 active forms of caspase-8 are also detected after 6 h in TNFa/ActD induced hepatocytes

[30]. This suggests that caspase-8 is also processed at a step downstream of mitochondria in TNF-a-treated hepatocytes, as occurs in other type II cells [31–33]. We observed that not only caspase-8 but also caspase-3 cleavage is required for the increase in cytoplasmic FADD levels. This suggests a feed forward loop initiated to enhance apoptosis that requires both proximal and downstream caspses. Over expression of FADD alone leads to cell death in hepatocytes [7]. This observation supports the notion that FADD levels are a determinant of the efficiency of apoptosis. Other feed forward mechanisms have been proposed. Ding et al. proposed that BID could retro-regulate caspase-8 activation by controlling reactive oxygen species (ROS) production induced by TNF$\alpha$ or anti-Fas antibody in hepatocytes [34]. ROS contributed to cell death and caspase-8 activation by promoting FLICE-inhibitory protein degradation and mitochondrial release of cytochrome c [34]. These findings put together make it very plausible that this feed forward amplification loop is present and an important part of up-regulating apoptosis.

The significance of the loss of nuclear pFADD with TNF$\alpha$/ActD stimulation is uncertain. Screaton et al. [10] first showed that pFADD was localized only in the nucleus and that this was due to phosphorylation. Furthermore, FADD was shown to shuttle between the nucleus and cytoplasm in several human cancer cell lines [15]. FADD has been shown to regulate the cell cycle which is related to phosphorylation of Ser194(S191 in murine FADD) [35–37]. Nuclear localization of FADD and the elevation of the pFADD is correlated with NF-kB activation and lung adenocarcinoma cell cycle control, therefore leading to increased cell survival [21, 22]. FADD phosphorylation at Ser194 was also found to be critical for increasing the sensitivity of tumor cells to Taxol either alone or in combination with other chemotherapeutic compounds [38, 39]. We found monomeric pFADD to be predominantly localized in the nucleus in hepatocytes. This nuclear form of FADD declined following TNF$\alpha$/ActD. The 60 kD pFADD, presumed to be dimeric is localized to the cytoplasm and changed little prior to apoptosis. Whether the loss of nuclear pFADD is due to dephosphorylation and transport from the nucleus is not known. It is tempting to speculate that one source of the increased cytoplasmic FADD is from the nucleus. However, we still lack of direct evidence to support the cytoplasmic and nuclear translocation and dephosphorylation of pFADD in TNF$\alpha$/ActD induced hepatocytes apoptosis.

Although dimerization of FADD has been demonstrated in several different cell lines using FADD over-expression systems [12–14], the function of dimeric FADD has not been definitively established. FADD dimers have been reported to trigger apoptosis [12]. FADD dimerization via DED has also been found to be important for FADD and TNFR1 interaction [14]. However, there have been no reports on how FADD dimerization is involved in the TNF induced apoptosis signaling pathway or FADD localization. Our results showing that monomeric FADD is the predominant form upregulated during apoptosis suggests that this form also contributes to cell death signaling. Our experiments did not determine which form of FADD participates in the initial DISC formulation that occurs prior to the changes in the changes in FADD levels or distribution.

Interestingly, in the setting of caspase-8 inhibition, following TNF$\alpha$/ActD treatment we found that dimeric FADD accumulated in the nucleus. This change in FADD distribution in

the setting of increases in total FADD suggest that nuclear-cytoplasmic shuttling could be part of response and that caspase-8 independent mechanism are involved.

Accumulating evidence suggests that Casein Kinase I$\alpha$ (CKI$\alpha$) may be involved in the regulation of death receptor induced apoptosis. CKI$\alpha$ [36, 40] can phosphorylate FADD. Meanwhile, phosphorylation of BID by Casein Kinases I and II regulates its cleavage by caspase-8. Although not studied here, it is interesting to speculate that the regulation of CKI might be one mechanism by which FADD distribution and levels are regulated. Taken together our current and previous work point to a response whereby detectable FADD levels increase in the cytosol through mechanism that involves a redistribution of FADD and a reduction in FADD degradation. How activation of caspase-3, caspase-8 and BID then lead to these changes are not known.

In summary, the identification and characterization of different forms of FADD and their translocation during apoptotic signaling indicates a complex response that is unique from the current linear cascade proposed for apoptosis signaling. The initial engagement of proximal apoptotic signaling sets into motion a response that includes the accumulation of monomeric FADD in the cytoplasm with a loss of nuclear pFADD. It seems likely that the accumulation of this cytoplasmic non-pFADD in TNF$\alpha$/ActD treated cells could then contribute to the efficient propagation of cell death signaling.

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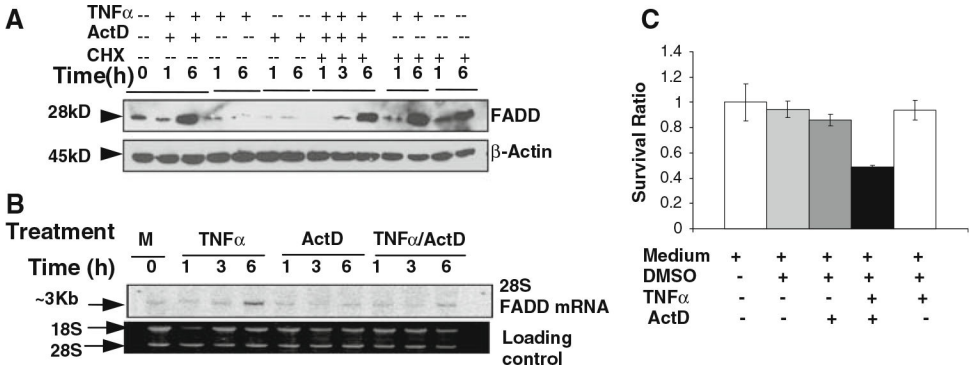


**Fig. 1.**

FADD is up-regulated in a post-translational manner with TNF$\alpha$/ActD stimulation. (a) Primary cultured mouse hepatocytes were treated with TNF$\alpha$ (2000 units/ml) with or without ActD (0.2 μg/ml) or CHX (50 μg/ml). FADD protein was probed with BD-FADD antibody (Table 1) with $\beta$-Actin used as a loading control. **(b)** Total mRNA was extracted from hepatocytes treated with TNF$\alpha$, ActD, or TNF$\alpha$/ActD treated cells. The Northern blot was probed with mouse FADD cDNA. Blot representative of three independent experiments. **(c)** Primary mouse hepatocyte cultures were exposed to TNF$\alpha$±ActD and 16 h later cell viability was assessed by crystal violet staining. DMSO (1% in cell culture medium) was tested for the dissolve reagent toxicity as a media control

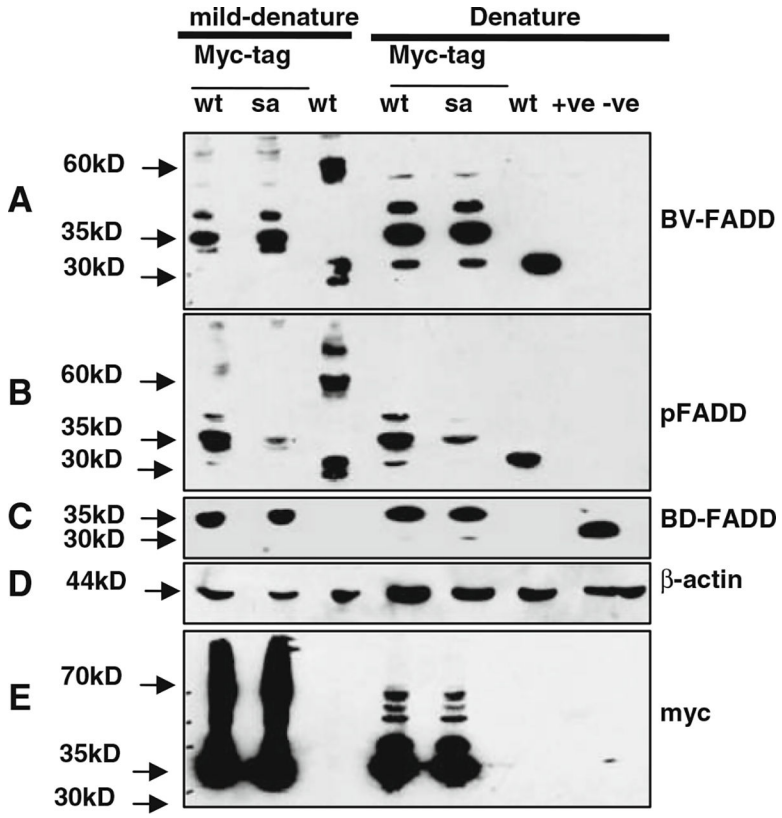


**Fig. 2.**

Three different mouse FADD constructs were overexpressed in 293T cells, including wild type (wt) with or without myc-tag and a myc-tagged serine191 mutant (sa). Proteins were separated by gel electrophoresis and western blots were performed under denaturing and mild denaturing conditions. Cell lysate from rat hepatocytes exposed to TNF/ActD for 6h was used as a positive control (+ve). Cell lysate from 293T mock transfects was used as negative control (−ve). **(a)** BV-FADD antibody detected wild type(wt) 60 kD and 30 kD FADD forms under nondenaturing conditions without the myc tag. Under denaturing conditions only 30 kD monomeric wt FADD without Myc tag was detectable. **(b)** The anti-pFADD (serine 191) antibody detected phosphorylated myc- and non-myc-tagged forms of wt FADD but not the sa mutant. **(c)** The antibody designated as BD-FADD (rat specific, BD Bioscience) detected 30 kD FADD including both wt and the phosphorylation mutant. It recognized FADD in the positive control from rat hepatocytes. **(d)** $\beta$-actin was probed to assess protein loading. **(e)** Myc was probed to show equal transfection

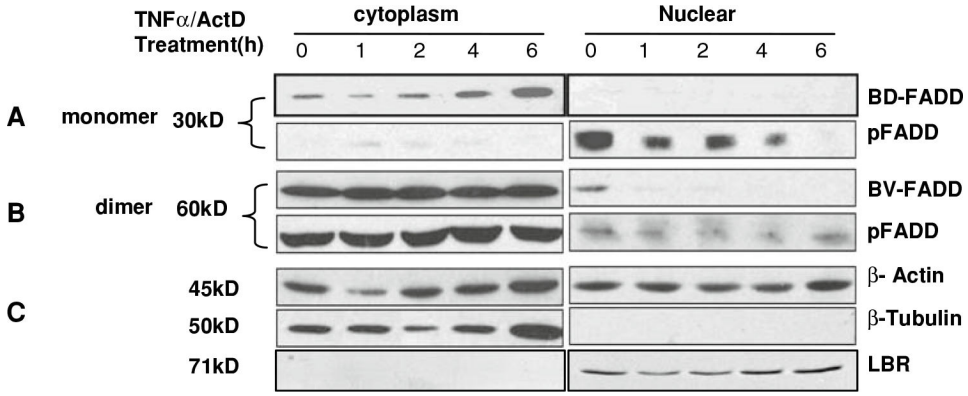


**Fig. 3.**

Cellular distribution of FADD in hepatocytes following TNF$\alpha$/ActD treatment over-time. Hepatocytes were treated with TNF$\alpha$/ActD and cells isolated at the time points shown. Cytoplasm and nuclear fractions were separated under denaturing and mild denaturing conditions. **(a)** Denaturing conditions to detect the changes in total FADD and pFADD. **(b)** Mild denaturing conditions to assess the level of dimeric FADD and pFADD. **(c)** $\beta$-actin levels to assess loading and $\beta$-tubulin as cytoplasm marker and lamin B receptor (LBR) as nuclear marker to establish purity of the nuclear preparation. Blots are representative of three experiments that yielded similar results

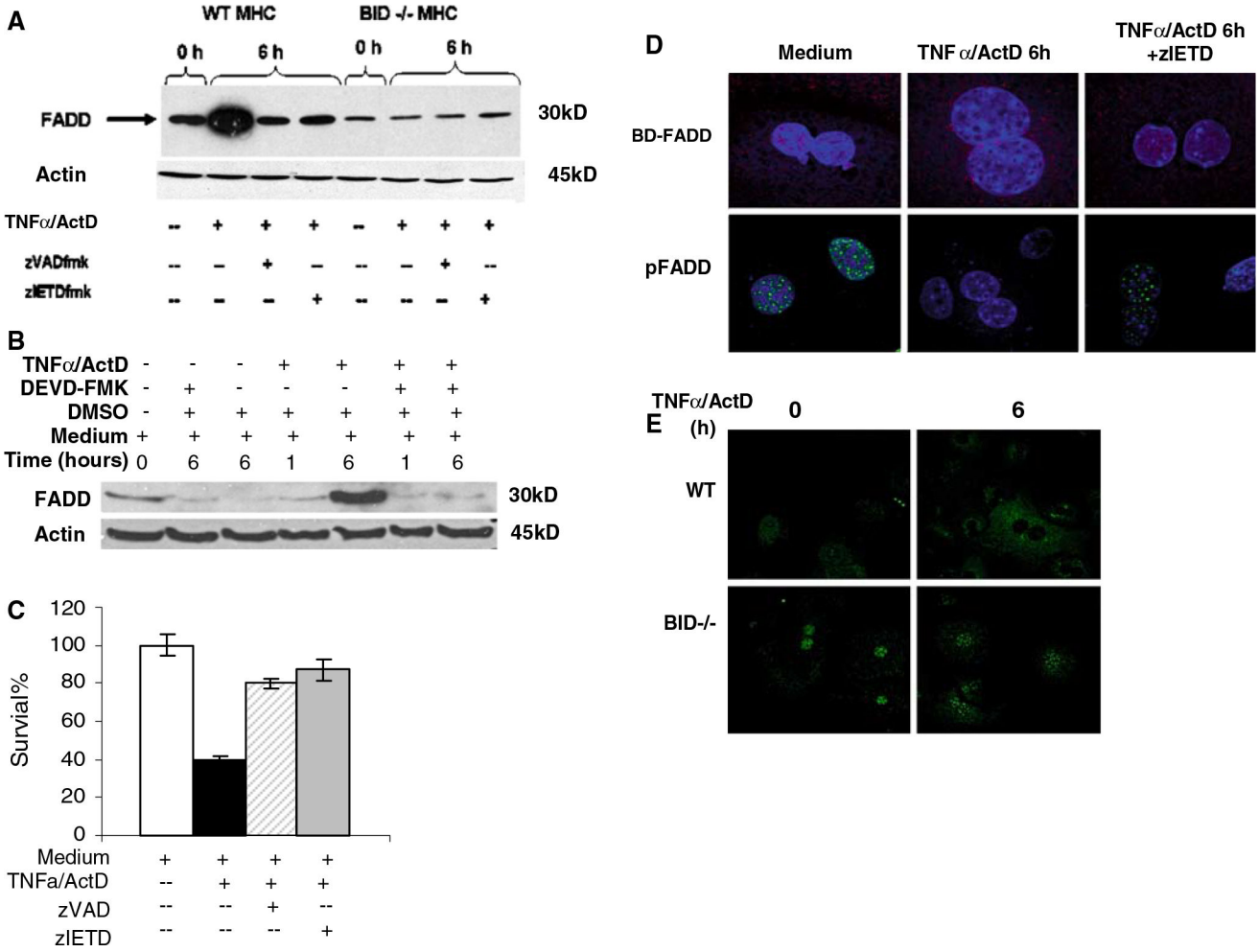


**Fig. 4.**

FADD subcellular localization is dependent on caspase-8 cleavage and BID. **(a)** Hepatocytes from wild type or BID knock-out mice were treated with or without TNF$\alpha$/ActD in combination with or without the pan-Caspase inhibitor z-VAD (50 μM) or Caspase-8 inhibitor z-IETD (50 μM). FADD protein levels were detected by Western Blot analysis with BD-FADD antibody. **(b)** Hepatocytes were treated with TNF$\alpha$/ActD in combination with or without Caspse-3 inhibitor z-DEVD-fmk (20 μM) blocked the monomer non-pFADD up-regulation. DMSO(1% in cell culture medium) was tested for control. **(c)** Primary mouse hepatocyte cultures were exposed to TNF$\alpha$/ActD with or without caspase-8 inhibitor z-IETD (50 μM) or pan-Caspase inhibitor z-VAD (50 μM) and 12 h later cell viability was assessed by crystal violet staining. **(d)** Inhibition of caspase 8 cleavage causes retention of non-phosphorylated FADD in nucleus and prevents down-regulation of nuclear pFADD with TNF$\alpha$/ActD treatment. Confocal microscopy of FADD (red) and pFADD (green) localization was performed with TNF$\alpha$/ActD± caspase-8 inhibition. Nuclei were stained with DRAQ5 dye. Magnification is 100×. (e) Non-phosphorylated FADD in BID−/− hepatocytes resides in the nucleus and does not respond to TNF$\alpha$/ActD stress. Confocal microscopy of FADD (green) from WT or BID−/− mouse hepatocytes treated with TNF$\alpha$/

ActD for 0 or 6 h. Magnification is 60×. Results are representative of at least three separate experiments that yielded similar results

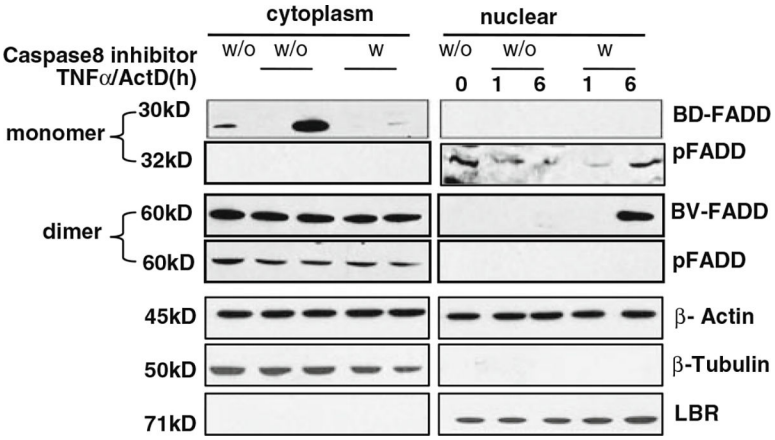


**Fig. 5.**

Inhibition of Caspase-8 abrogates FADD up-regulation in the cytoplasm and loss of in monomeric pFADD in the nucleus in associate with increase in non-phosphorylated dimeric FADD in the nucleus. Mouse hepatocytes were treated with TNF$\alpha$/ActD in combination with or without a caspase-8 inhibitor for 0 h, 1 h, 6 h. Nuclear and cytoplasm fractions were subjected to electrophoresis under mild denaturing conditions and subjected to western blot analysis. Antibodies specific for different FADD forms were used to probe the membrane. Blots are representative of three experiments yielding similar results. $\beta$-actin levels to assess loading and $\beta$-tubulin as cytoplasm marker and lamin B receptor as nuclear marker to establish purity of the nuclear preparation. Blots are representative of three experiments that yielded similar results

**Table 1**

FADD antibody summary

| Name | Antigen | Protocol | Specificity for FADD forms | 1° Antibody Source (dilution and duration) | 2° Antibody Source/Fluorophor (Dilution) |
|---|---|---|---|---|---|
| BD-FADD | FADD | IF | Only monomeric non-phosphorylated FADD | Monoclone mouse @ rat FADD BD Pharmingen™ (558202)1:1000 1h RT | Goat anti-mouse Alex Cy3(1:1000) |
| | FADD | WB | Only monomeric non-phosphorylated FADD | Monoclone mouse @ rat FADD BD Pharmingen™ (558202)1:200 o/n 4°C | Goat anit-mouse (Amersham Biosciences) (1:10000) |
| BV-FADD | FADD | WB | Dimeric FADD in hepatocytes; both dimeric and monomeric FADD in over express system | Monoclone rat@mouse Biovision (3343–100)1:1000 1h RT | Goat anti-rat (Amersham Biosciences) (1:10000) |
| pFADD | pFADD | IF | Monomeric phosphorylated FADD | Polyclone rabbit@mouse Cell-Signaling Technology #2785 1:200 o/n 4°C | Goat anti-rabbit Alexa 488, Invitrogen(1:500) |
| | pFADD | WB | Both dimeric and monomeric phosphorylated FADD | Polyclone rabbit@mouse Cell-Signaling Technology #2785 1:1000 overnight, 4°C | Goat anti-rabbit (Amersham Biosciences) (1:10000) |