

# Nuclear and cytoplasmic shuttling of TRADD induces apoptosis via different mechanisms

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The adapter protein tumor necrosis factor receptor (TNFR)1-associated death domain (TRADD) plays an essential role in recruiting signaling molecules to the TNFR1 receptor complex at the cell membrane. Here we show that TRADD contains a nuclear export and import sequence that allow shuttling between the nucleus and the cytoplasm. In the absence of export, TRADD is found within nuclear structures that are associated with promyelocytic leukemia protein (PML) nuclear bodies. In these structures, the TRADD death domain (TRADD-DD) can activate an apoptosis pathway that is mechanistically distinct from its

action at the membrane-bound TNFR1 complex. Apoptosis by nuclear TRADD-DD is promyelocytic leukemia protein dependent, involves p53, and is inhibited by Bcl-xL but not by caspase inhibitors or dominant negative FADD (FADD-DN). Conversely, apoptosis induced by TRADD in the cytoplasm is resistant to Bcl-xL, but sensitive to caspase inhibitors and FADD-DN. These data indicate that nucleocytoplasmic shuttling of TRADD leads to the activation of distinct apoptosis mechanisms that connect the death receptor apparatus to nuclear events.

## Introduction

Tumor necrosis factor (TNF)\*- $\alpha$  regulates inflammation, survival, proliferation, and apoptosis by activating the membrane-bound receptors, TNF receptors (TNFRs)1 and 2 (for a review of TNF family signaling see Ashkenazi and Dixit, 1998; Locksley et al., 2001). The adapter protein TNFR1-associated death domain (TRADD) is an essential component of the TNFR1 complex (Hsu et al., 1995), and functions by recruiting other members of the complex to the receptor. Among these are TRAF-2, which binds the NH<sub>2</sub>-terminal domain portion of TRADD (Hsu et al., 1996b), and Fas-

associated death domain protein (FADD) and receptor-interacting protein (RIP), which binds to its COOH-terminal death domain (DD) (Hsu et al., 1996a, 1996b).

FADD contains a death effector domain (DED) that binds the DED of caspase-8. Recruitment of this caspase through TRADD and FADD results in caspase activation and subsequent apoptosis (Chinnaiyan et al., 1996; Hsu et al., 1996b). RIP is required for activation of NF- $\kappa$ B, which results in the transcription of antiapoptotic genes, whereas TRAF-2 is required for initiation of the JNK signaling pathway (Yeh et al., 1997; Kelliher et al., 1998). In addition, TRAF-2 may recruit inhibitor of apoptosis proteins (IAPs) to the complex, resulting in inhibition of apoptosis (Shu et al., 1996). RIP is also thought to recruit RAIDD, which has a caspase recruitment domain (CARD) that binds caspase-2, and may thus initiate apoptosis through activation of this caspase (Duan and Dixit, 1997). A product generated by caspase-8 cleavage of RIP seems to stabilize the TRADD-FADD interaction, resulting in further caspase-8 activation (Lin et al., 1999).

Thus, a delicate balance is maintained between pro- and antiapoptotic signals that depend on TRADD binding to TNFR1 at the membrane-bound death-inducing signaling complex (DISC). The result of receptor activation (cell survival or death) is dependent on the context of its activation. In some cases, inhibition of caspases fails to block, and may

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\*Abbreviations used in this paper: AA, amino acid(s); CARD, caspase recruitment domain; CRM, chromosomal region maintenance protein; DAPK, death-associated protein kinase; DD, death domain; DED, death effector domain; FADD, Fas-associated DD protein; DISC, death-inducing signaling complex; FADD-DN, dominant negative FADD; GFP, green fluorescent protein; HIPK, homeodomain-interacting protein kinase; IAP, inhibitor of apoptosis protein; LMB, leptomycin B; NES, nuclear export sequence; MEF, mouse embryo fibroblast; NLS, nuclear localization signal; PML, promyelocytic leukemia protein; RIP, receptor-interacting protein; TNF, tumor necrosis factor; TNFR, TNF receptor; TRADD, TNFR1-associated DD protein; TRADD-DD, TRADD death domain; YFP, yellow fluorescent protein.

Key words: apoptosis; TRADD; leptomycin B; PML; p53

even increase, TNF-induced cell death. However, necrotic rather than apoptotic death occurs in some of these processes (Vercammen et al., 1998; Jones et al., 2000; Luschen et al., 2000; Denecker et al., 2001). In some cells, p53 is required for TNFR1-dependent apoptosis (Cai et al., 1997; Ameyar et al., 1999; Rokhlin et al., 2000), but it is unclear why a nuclear transcription factor should be required for apoptosis when complex formation at the receptor can directly activate caspases.

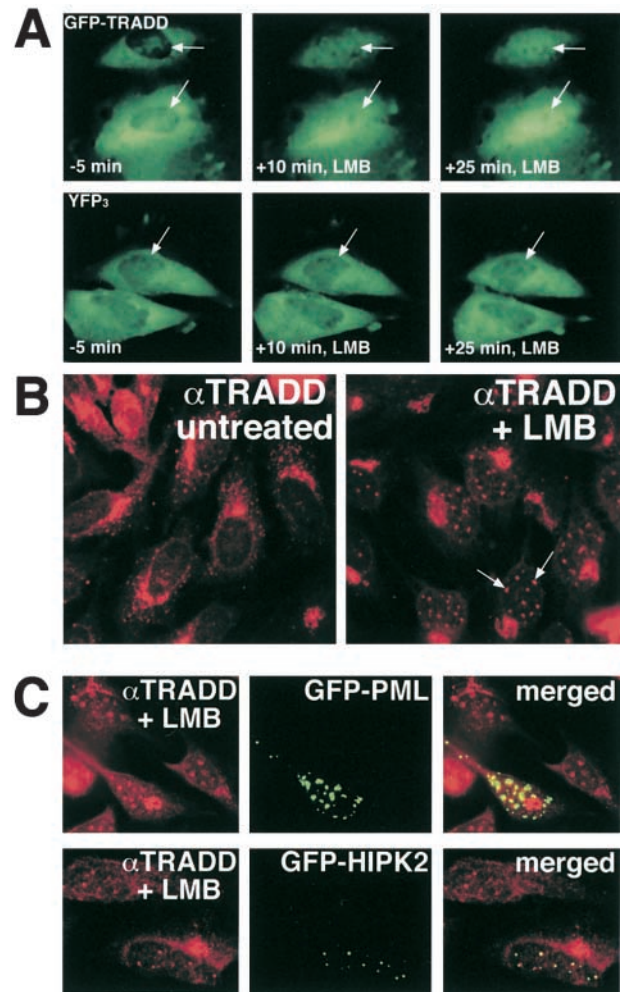
The promyelocytic leukemia protein (PML) is a tumor suppressor (Rego et al., 2001) found in discrete bodies in the nucleus known as PML oncogenic domains, or PML nuclear bodies. PML-null cells are resistant to TNF $\alpha$  and the PML-RAR $\alpha$  fusion protein, which is delocalized from nuclear bodies to nonfunctional nuclear microspeckles in acute promyelocytic leukemia patients, and antagonizes Fas ligand- and TNF $\alpha$ -induced death (Wang et al., 1998). These data suggest that functional PML (and PML nuclear bodies) is required for death receptor-induced apoptosis. This raises the question of how nuclear PML might be involved in apoptosis that is initiated at the cell membrane by cytoplasmic proteins such as TRADD, FADD, etc. In the case of Fas, this link may involve Daxx, which has been reported to bind Fas in some conditions (Chang et al., 1998; Ko et al., 2001) and be in PML nuclear bodies in other situations (Torii et al., 1999; Zhong et al., 2000b). However, a protein that is at the TNFR1 DISC and can mediate apoptosis from PML nuclear bodies has not been identified.

Here we report that TRADD contains both nuclear export and import sequences, allowing it to shuttle through the nucleus. Upon inhibition of nuclear export with leptomycin B (LMB), TRADD accumulates in nuclear structures that are associated with PML nuclear bodies. A fragment of TRADD-DD that is localized exclusively to these structures induces death through a mechanism that involves p53 and PML. These data suggest that shuttling of TRADD between the cytoplasm and the nucleus activates different apoptotic mechanisms and provides a link between components of the TNF receptor signaling machinery and the nucleus.

## Results

### TRADD translocation to the nucleus

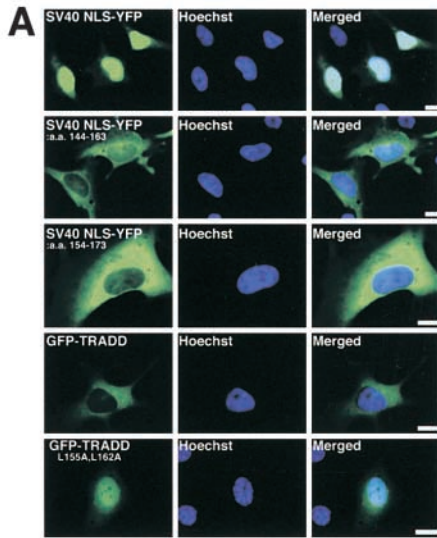
To study the subcellular localization of TRADD in living cells, we transfected HeLa cells with a plasmid encoding a green fluorescent protein (GFP)-TRADD fusion protein. GFP-TRADD was excluded from the nucleus and had a perinuclear or a diffuse cytoplasmic distribution (Fig. 1 A, top left) similar to what has been described previously in fixed cells (Jones et al., 1999). At higher expression levels, GFP-TRADD accumulated in what appeared to be the previously observed death effector filaments (Siegel et al., 1998; Guet and Vito, 2000; Cottin et al., 2001). Expression of a GFP-TRADD construct with an exogenous SV40-nuclear localization signal (NLS) fused to the NH<sub>2</sub> terminus of the GFP (NLS-GFP-TRADD) failed to change the cytoplasmic distribution, suggesting that GFP-TRADD might be actively exported from the nucleus. LMB binds to and inhibits the nuclear export receptor chromosomal region maintenance protein (CRM)1, and has been widely used to



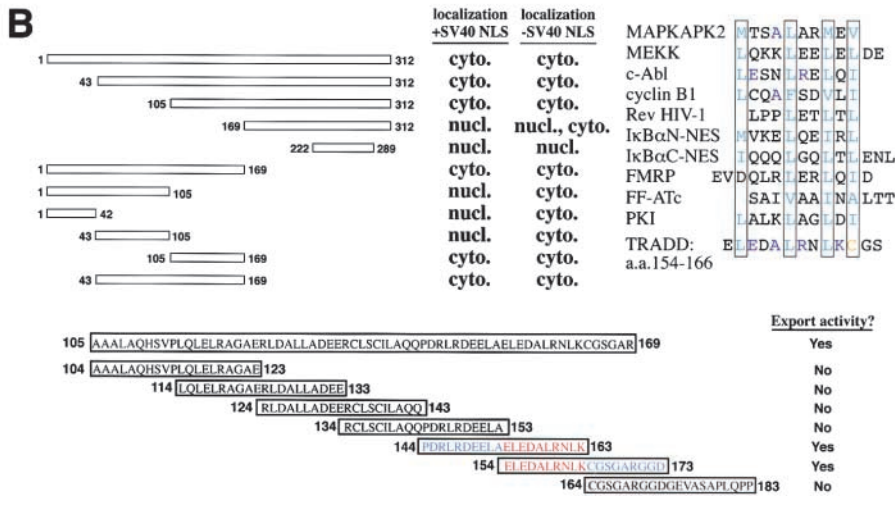
**Figure 1. Nuclear translocation of TRADD.** (A) Time-lapse fluorescence microscopy of HeLa cells expressing GFP-TRADD or YFP<sub>3</sub>. (Left) Cells 5 min before treatment; (Center and right) The same cells 10 and 25 min after LMB treatment. (B) Immunofluorescence staining of endogenous TRADD in HeLa cells. (Left) Untreated cells; (Right) Cells treated for 8 h with LMB. (Arrows) Examples of nuclear TRADD localized in dots after LMB treatment. (C) Colocalization of endogenous TRADD (left) with GFP-PML (top middle) or GFP-HIPK2 (bottom middle) 6 h after LMB treatment. (Right) A merged image shows colocalization of the two proteins (yellow).

test whether proteins are actively exported from the nucleus (Henderson and Eleftheriou, 2000). Cells expressing either GFP-TRADD or triple yellow fluorescent protein (YFP<sub>3</sub>) proteins were followed by time-lapse video microscopy before and after LMB treatment. Although both proteins were initially cytoplasmic, LMB treatment led to rapid accumulation of GFP-TRADD, but not YFP<sub>3</sub>, within the nucleus (Fig. 1 A). These data suggest that GFP-TRADD moves between the cytoplasm and the nucleus.

To test whether the endogenous protein behaved like the GFP-tagged molecule, we stained cells with an anti-TRADD monoclonal antibody. TRADD has previously been shown to localize to the medial- or cis-Golgi, as well as to the cytoplasm (Jones et al., 1999). Consistent with these previous observations, immunostaining of untreated cells revealed that endogenous TRADD had a cytosolic localization and a punctate perinuclear Golgi distribution (Fig. 1 B) that



**Figure 2. Mapping of an NES in TRADD.** (A) Fluorescence microscopy of cells expressing GFP or YFP fusion proteins (left). (Middle) Cell nuclei stained with Hoechst dye; (Right) The respective merged images. YFP fusion proteins in the top three panels contain an exogenous SV40 NLS. Bars, 10 mM. (B) Schematic diagram of NH<sub>2</sub>- and COOH-terminal deletions of TRADD expressed as COOH-terminal GFP fusion proteins (left). The table indicates whether fusion proteins were primarily nuclear or cytoplasmic in the presence or absence of an exogenous SV40-NLS fused to the NH<sub>2</sub> terminus of GFP. (Bottom) Overlapping 20-aa segments of TRADD. The ability of these sequences to export GFP or NLS-GFP is indicated to the right. The TRADD NES sequence is shown in alignment with other known NES sequences (right).



dispersed to a diffuse cytosolic pattern upon treatment with Brefeldin A (unpublished data). LMB treatment resulted in the accumulation of endogenous TRADD in the nucleus, where it formed dot-like structures in many cells (Fig. 1 B).

We investigated whether endogenous TRADD would colocalize with PML, which is found in subnuclear domains known as PML oncogenic domains or PML-nuclear bodies (for review see Maul et al., 2000; Zhong et al., 2000a). Upon expression of GFP-PML in LMB-treated cells, a clear correlation could be detected between the nuclear TRADD structures and GFP-PML. Where present in the same nuclei, the two proteins appeared to be partially colocalized or in structures juxtaposed to each other (Fig. 1 C, top).

A second protein that localizes to nuclear dots is HIPK2, which has been reported to bind to TRADD (Li et al., 2000). A related protein, HIPK3, binds both FAS and FADD and colocalizes with PML (Rochat-Steiner et al., 2000). HIPK2 colocalizes with PML and activates p53-

dependent apoptosis in nuclear bodies (D’Orazi et al., 2002). Like GFP-PML, nuclear dots containing HIPK2-GFP colocalized with, or were adjacent to, nuclear dots containing endogenous TRADD in LMB-treated cells (Fig. 1 C, bottom).

**TRADD contains a nuclear export sequence**

Rapid nuclear accumulation of endogenous TRADD and GFP-TRADD in LMB treated cells suggests that TRADD contains a nuclear export sequence (NES) that is dependent on CRM1, or binds to another protein that contains a CRM1-dependent NES. To map this region, combinations of NH<sub>2</sub>- and COOH-terminal deletions of TRADD were created as COOH-terminal fusion proteins to GFP in the presence or absence of an exogenous SV40-NLS to target the molecule to the nucleus (Fig. 2 B, left). A fragment containing amino acids (aa) 104–168 was sufficient to overcome the exogenous SV40-NLS and target fusion constructs to

the cytoplasm. Conversely, constructs that lacked this region were primarily localized to the nucleus when an NLS was present. To more precisely map the NES, we expressed overlapping 20-aa segments of this region as COOH-terminal YFP fusion proteins (Fig. 2 B, bottom). Proteins containing aa 144–163 or 154–173 were excluded from the nucleus, indicating that the overlapping aa 154–163 could function as an NES. YFP fusion proteins of these two fragments were absent from the nucleus even when an SV40-NLS was fused to the NH<sub>2</sub> terminus of the YFP (Fig. 2 A), and relocated to the nucleus in LMB-treated cells (unpublished data). The solved solution structure of TRADD indicates that these 10 aa are part of an exposed alpha helix (Tsao et al., 2000). This sequence displays homology to other NESs (Henderson and Eleftheriou, 2000), with three of four leucines being conserved (Fig. 2 B, right). The combination of L155A and L162A mutations in the wild-type GFP–TRADD abolished nuclear export, and resulted in nuclear accumulation (Fig. 2 A). These data indicated that aa 154–163 function as a CRM1-dependent NES.

### TRADD contains a nuclear import sequence

The accumulation of nuclear GFP–TRADD in LMB-treated cells and in the L155A/L162A mutant suggests that TRADD has a nuclear import signal. Consistent with this hypothesis, we found that a YFP fusion protein containing the core helices of the DD (aa 222–289) of TRADD (YFP–TRADD-DD) was exclusively nuclear and localized in dots (Fig. 3 A). This localization pattern in nuclear dots appears to be specific for the TRADD-DD, as expression of the DDs of FADD and death-associated protein kinase (DAPK) in similar YFP fusion proteins resulted in diffuse localization patterns similar to YFP alone.

To further define the sequence important for nuclear localization, we made NH<sub>2</sub>- and COOH-terminal truncations of the YFP–TRADD-DD construct. A construct containing TRADD aa 222–245 was sufficient to retain YFP in the nucleus in a pattern similar to YFP fused with an SV40-NLS (Fig. 3 A). This region contains a stretch of several basic aa, and loosely matches the consensus for a bipartite NLS. To verify that the putative NLS region was responsible for nuclear import, we fused the TRADD-DD to a YFP<sub>3</sub> construct. YFP<sub>3</sub> is normally excluded from the nucleus because it is too large to diffuse through nuclear pores (Fig. 3 B). The resulting construct (YFP<sub>3</sub>–TRADD-DD) was imported into the nucleus where it accumulated in sub-nuclear dots (Fig. 3 B). Individual mutations in four basic residues in this region (K229M, R231L, K232M, and R242A) increased the cytoplasmic distribution of the protein (unpublished data), and a triple point mutant (K229M/R231L/K232M) resulted in a protein that was completely excluded from the nucleus (Fig. 3 B), indicating that this region is a functional NLS.

The previously used anti-TRADD antibody failed to recognize YFP-or GFP–TRADD-DD. Therefore, we used this antibody to determine whether the nuclear DD colocalized with endogenous TRADD in LMB-treated cells. A strong colocalization was observed between GFP–TRADD-DD and endogenous TRADD in treated cells (Fig. 3 C), suggesting that the nuclear structures are identical.

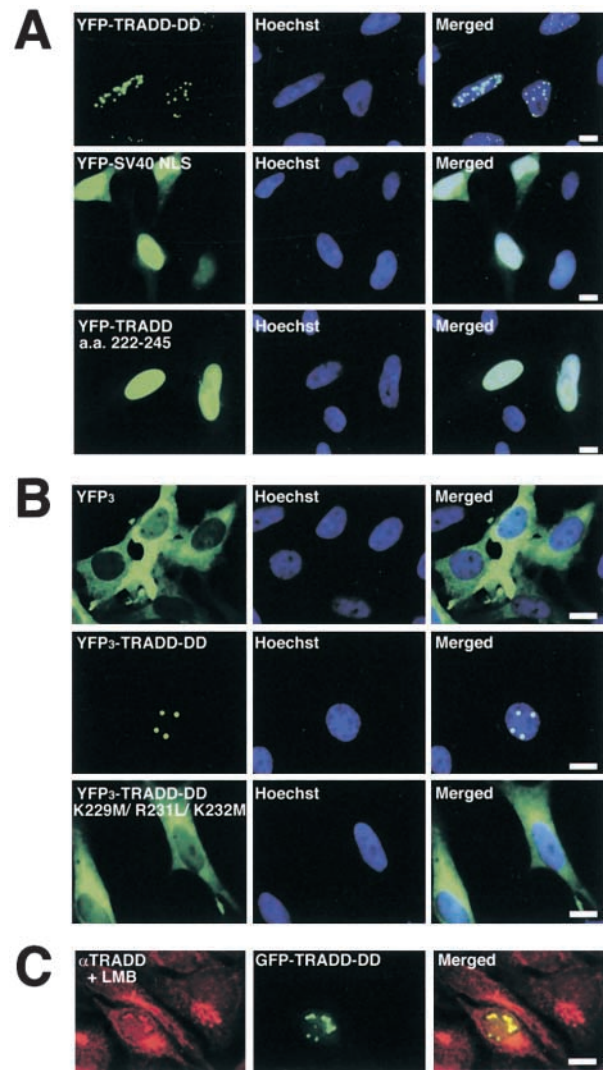


Figure 3. **Mapping of a nuclear localization sequence in TRADD.** (A and B) Fluorescence microscopy of cells expressing YFP (A) or YFP<sub>3</sub> (B) fusion proteins (left) as labeled. (Middle) Cell nuclei stained with Hoechst dye; (Right) The respective merged images. (C) The merged image (right) shows colocalization of endogenous TRADD (left, red) and GFP–TRADD-DD (middle, green) after 6 h LMB treatment. Bars, 10 μm.

### A COOH-terminal element of TRADD regulates the kinetics of nuclear import

A recent report suggested that the COOH-terminal portion of the TRADD-DD (aa 245–312) may associate with acidic keratins 14 and 18, localizing TRADD to cytoplasmic filaments and decreasing sensitivity to TNF (Inada et al., 2001). We noticed that some TRADD constructs lacking the NES accumulated in the nucleus in some cells, and in cytoplasmic filaments in other cells. It is presently unclear whether these cytoplasmic filaments occur under physiological conditions, as we have observed them only at higher expression levels. Nuclear accumulation of TRADD occurred at slower rates for constructs where these filaments were present, and appeared to be dependent on the COOH-terminal 23 aa. Therefore, we compared the cellular localization of TRADD-DD constructs that differ only in the COOH terminus. As shown in Fig. 4, YFP constructs con-

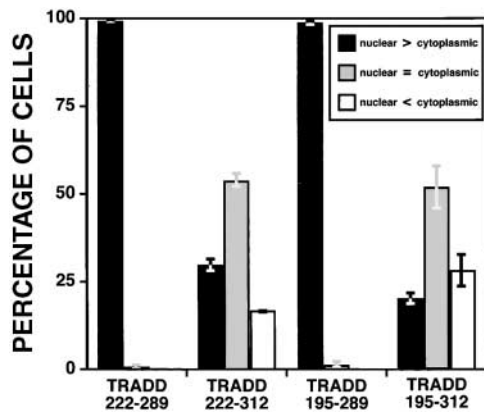


Figure 4. **The COOH-terminal aas of TRADD regulate the nuclear import signal.** The histogram shows the localization of DD constructs with and without 23 COOH-terminal aa 24 h after transfection. Cells were classified into three categories of YFP-TRADD localization: primarily nuclear ( $N > C$ ), primarily cytoplasmic ( $N < C$ ), or equally present in the cytoplasm and nucleus ( $N = C$ ).

taining aa 196–289 or 222–289 were exclusively nuclear, whereas constructs containing aa 196–312 or 222–312 had significant cytoplasmic distribution at 24 h after transfection. These data suggest that the COOH-terminal aa contain an element that regulates the kinetics of nuclear accumulation of TRADD, perhaps by inhibiting the NLS, functioning as another NES, or binding to cytoplasmic proteins such as cytokeratins. Accordingly, for experiments to address the function of TRADD-DD in the nucleus, we used the minimal YFP-tagged DD construct (YFP-TRADD-DD) consisting of aa 222–289, which localizes exclusively to the nucleus in PML-associated structures.

### Nuclear YFP-TRADD-DD induces apoptosis

Time-lapse microscopy indicated that YFP-TRADD-DD-expressing cells died displaying the typical hallmarks of apoptosis, such as membrane blebbing and condensed nuclei (Fig. 5 A; Video 1, available at <http://www.jcb.org/cgi/content/full/jcb.200204039/DC1>). Quantitative cell survival analysis of HeLa cells expressing YFP-TRADD-DD indicated that <25% of these cells remained alive 24 h after microinjection (Fig. 5 B). As controls, we injected plasmids that expressed either YFP alone, YFP fused to an SV40-NLS, or the YFP-tagged DDs of FADD or DAPK. None of these molecules were significantly toxic (Fig. 5 B). These data show that the exclusively nuclear, isolated DD of TRADD is sufficient to induce apoptosis. Truncation of YFP-TRADD-DD to aa 222–275 or 222–266 resulted in concomitant decreases in cell death, whereas the construct containing only aa 222–255 was comparable to YFP-NLS (unpublished data). These data indicate that the DD structure is required for apoptosis.

As previously described, constructs that contained the COOH-terminal 23 aa of TRADD were delayed in their nuclear localization. In addition, the longer constructs were delayed in their ability to induce death (unpublished data), suggesting that YFP-TRADD-DD localization to the nucleus was important for its ability to kill cells. To test this hypothesis, we mislocalized the tagged minimal DD to the plasma membrane using the myristoylation sequence from Src. Relocation of YFP-TRADD-DD to the cell membrane

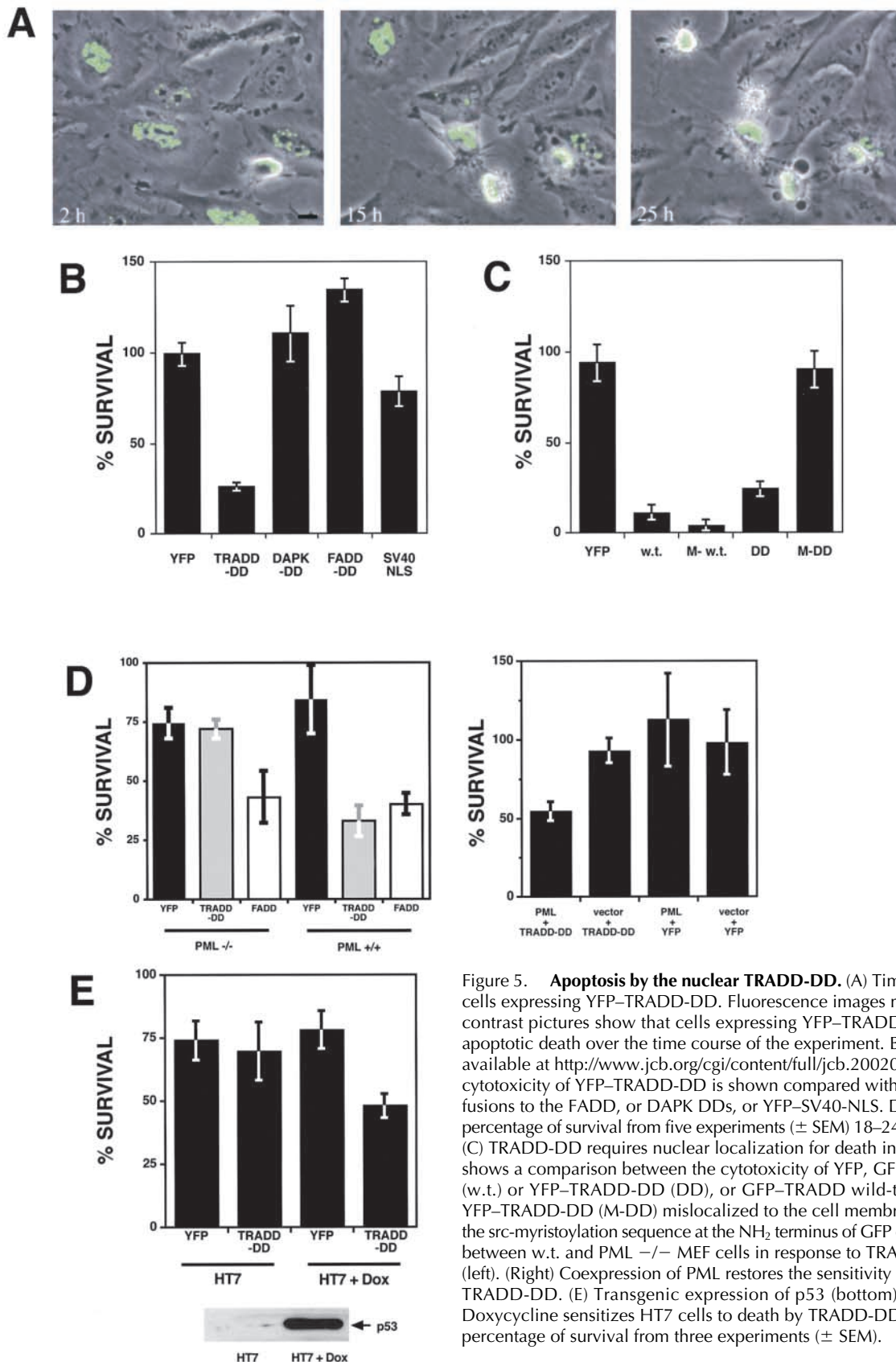
prevented cell death, whereas nuclear YFP-TRADD-DD was effective in killing cells (Fig. 5 C). In contrast, the full-length wild-type GFP-TRADD, which had similarly been modified at the NH<sub>2</sub> terminus with the Src myristoylation sequence (M-w.t.), retained the ability to induce apoptosis (Fig. 5 C). The ability of the membrane-localized full-length TRADD to induce apoptosis is expected because this molecule should still be able recruit FADD as at the TNFR1 DISC. These data suggest that the mechanism of apoptosis induced by the nuclear TRADD-DD molecule may be different from its mode of action at the TNF receptor complex.

### YFP-TRADD-DD requires PML to induce death

We investigated whether PML was required for YFP-TRADD-DD-induced death in mouse embryo fibroblast (MEF) cells. As shown in Fig. 5 D (left), about half of the wild-type MEF cells died in response to YFP-TRADD-DD at 24 h, when compared with YFP-injected control cells. However, MEF cells derived from PML knockout mice were completely resistant to YFP-TRADD-DD when compared with control cells, indicating that PML is required for the death signaling by nuclear YFP-TRADD-DD (Fig. 5 D, left). As a control, an expression plasmid for wild-type FADD was injected into PML-deficient and wild-type MEFs. There was no difference in the sensitivity of PML knockout cells to FADD compared with wild-type cells. This suggests that FADD-dependent caspase activation pathways are intact in PML  $-/-$  MEFs, and that the PML-dependent signal generated by TRADD-DD is likely independent of FADD recruitment. To verify the requirement for PML, we coexpressed PML with TRADD-DD or YFP in PML  $-/-$  MEFs. Injection of TRADD-DD or PML alone in conjunction with YFP or control vector failed to kill cells at this time point (Fig. 5 D, right). However, ~1/2 of the cells injected with both TRADD-DD and PML died after 24 h. These data suggest that PML plays an essential role in a nuclear apoptotic pathway induced by TRADD.

### Expression of p53 in p53-null cells sensitizes them to YFP-TRADD-DD death

p53 is essential for TNF-dependent apoptosis in some cell types (Cai et al., 1997; Ameyar et al., 1999; Rokhlin et al., 2000). p53 has also been shown to localize in PML nuclear bodies, where PML acts a transcriptional coactivator of p53 and regulates its activity (Fogal et al., 2000; Guo et al., 2000). In addition, HIPK2, which binds to TRADD (Li et al., 2000) and colocalizes in nuclear dots (Fig. 1), can activate p53 (D'Orazi et al., 2002). To test whether p53 was involved in death induced by nuclear TRADD-DD, we performed microinjection experiments in HT7 cells, which are a derivative of the p53-null cell line H1299. These cells contain a p53 transgene regulated by a tetracycline-inducible promoter, and express p53 in the presence, but not in the absence, of doxycycline (Koumenis et al., 2001) (Fig. 5 E, bottom). As shown in Fig. 5 E, H7 cells failed to undergo TRADD-DD-induced apoptosis in the absence of doxycycline. However, doxycycline-induced expression of p53 sensitized these cells to death by the TRADD-DD, suggesting that p53 plays an important role in apoptosis induced by nuclear TRADD-DD.



**Figure 5. Apoptosis by the nuclear TRADD-DD.** (A) Time-lapse microscopy of cells expressing YFP-TRADD-DD. Fluorescence images merged with the phase contrast pictures show that cells expressing YFP-TRADD-DD (green) die an apoptotic death over the time course of the experiment. Bar, 10  $\mu$ m (Video 1, available at <http://www.jcb.org/cgi/content/full/jcb.200204039/DC1>).

(B) The cytotoxicity of YFP-TRADD-DD is shown compared with that of YFP, YFP fusions to the FADD, or DAPK DDs, or YFP-SV40-NLS. Data shown is the percentage of survival from five experiments ( $\pm$  SEM) 18–24 h after microinjection.

(C) TRADD-DD requires nuclear localization for death induction. The figure shows a comparison between the cytotoxicity of YFP, GFP-TRADD wild-type (w.t.) or YFP-TRADD-DD (DD), or GFP-TRADD wild-type (M-w.t.) or YFP-TRADD-DD (M-DD) mislocalized to the cell membrane by an addition of the src-myristoylation sequence at the NH<sub>2</sub> terminus of GFP or YFP.

(D) Comparison between w.t. and PML<sup>-/-</sup> MEF cells in response to TRADD-DD and FADD (left). (Right) Coexpression of PML restores the sensitivity of PML<sup>-/-</sup> MEFs to TRADD-DD.

(E) Transgenic expression of p53 (bottom) in the presence of Doxycycline sensitizes HT7 cells to death by TRADD-DD. Data shown is the percentage of survival from three experiments ( $\pm$  SEM).

**TRADD induces apoptosis by different mechanisms in the nucleus and cytoplasm**

The previous data suggest that nuclear TRADD-DD can induce apoptosis via a mechanism that is different from the

current model, by which TRADD induces apoptosis at the TNFR1. Therefore, we compared the effects of various inhibitors on death induction by the exclusively nuclear YFP-TRADD-DD and the nuclear-excluded wild-type

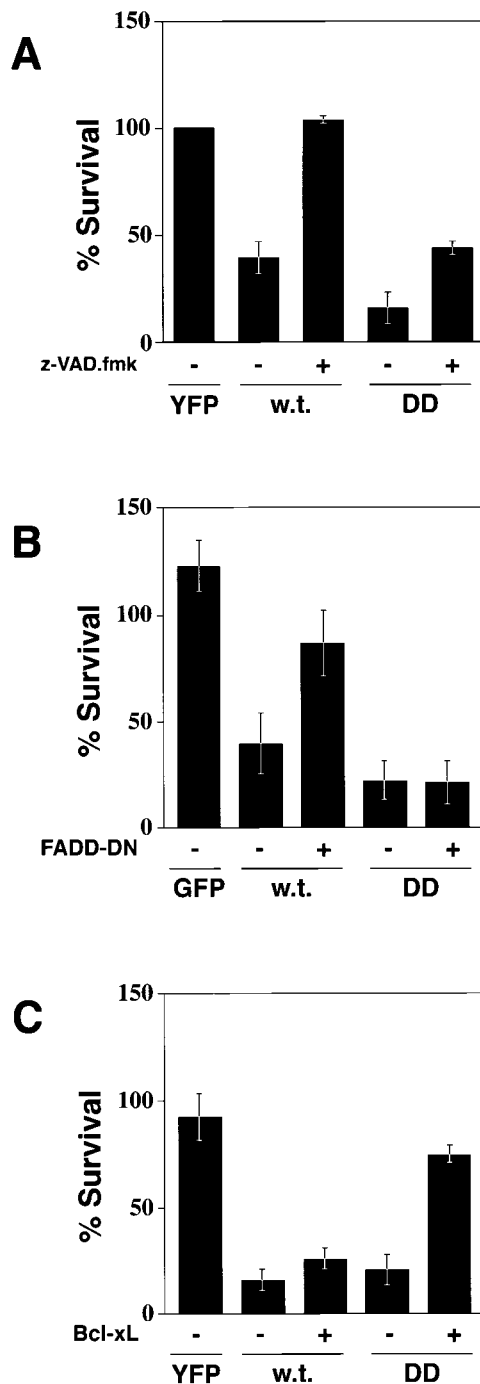


Figure 6. **Mechanistic differences between TRADD w.t. and TRADD-DD death.** (A–C) Survival assays showing comparisons between cells microinjected with YFP–TRADD-DD (DD) or GFP–TRADD (w.t.) 18–24 h after initial expression and quantitation (2 h). Data shown is the percentage of survival  $\pm$  SEM from between five and ten experiments. (A) Survival comparison is shown in the presence of 150  $\mu$ M z-VAD.fmk or DMSO alone and normalized to YFP or GFP. (B and C) Survival comparison after coinjection of (B) dsRED–FADD-DN; (C) Bcl-xL; or control expression plasmids with YFP–TRADD-DD (DD) or GFP–TRADD (w.t.).

GFP–TRADD molecule. The broad-spectrum caspase inhibitor z-VAD (150  $\mu$ M) inhibited cell killing by GFP–TRADD, but had only a modest effect on YFP–TRADD-DD (Fig. 6 A). The baculoviral p35 protein also caused only a modest inhibition when coexpressed with YFP–

TRADD (unpublished data). A FADD-DN molecule lacking the DED has been widely used to inhibit apoptosis induced by death receptors, including TNFR1. Consistent with the previously established role for TRADD (Hsu et al., 1996b), coexpression of a dsRED version of the FADD-DN molecule prevented death induced by GFP–TRADD (Fig. 6 B). However, this molecule failed to inhibit death induced by the nuclear DD construct YFP–TRADD-DD (Fig. 6 B). Conversely, microinjection of Bcl-xL with YFP–TRADD-DD resulted in an almost complete inhibition of YFP–TRADD-DD–induced death, but failed to block apoptosis in GFP–TRADD–expressing cells (Fig. 6 C). These results indicate that when in the nucleus, the TRADD-DD activates an apoptosis pathway that is mechanistically distinct from that activated by TRADD in the cytoplasm. In addition, lack of inhibition by dsRED–FADD-DN on YFP–TRADD-DD implies that the isolated DD does not cause death by upregulating machinery in death receptor pathways upstream of FADD (i.e., FAS, FASL, TNFR1, TNF $\alpha$ , etc.).

## Discussion

In summary, we have shown that TRADD contains both NES and NLS elements capable of nuclear export and import, and causing the protein to shuttle between the cytoplasm and the nucleus. The export activity is sensitive to LMB, implicating a CRM1-dependent export, and treatment with LMB causes nuclear accumulation of endogenous TRADD in dot-like structures that are associated with PML nuclear bodies. The core portion of the TRADD-DD localizes exclusively to such structures even in the absence of LMB, where it can induce apoptosis through a mechanism that is different from TRADD's previously defined role at the cell membrane. This mechanism requires PML and p53, and may involve the mitochondria because it is inhibited by Bcl-xL.

Detailed structure–function analyses have been performed previously with the TRADD-DD (Hsu et al., 1995; Park and Baichwal, 1996). However, these studies utilized a larger construct (aa 196–312) than our minimal DD construct (aa 222–289), which lacks the inhibitory COOH-terminal aas that delay nuclear localization and death. Based on these previous studies, our YFP–TRADD-DD protein should be unable to self associate, bind to TNFR1, or activate NF- $\kappa$ B, consistent with our hypothesis that TRADD acts by a different mechanism in the nucleus than at the TNFR1 DISC.

Previous studies suggest that TRADD can use alternate mechanisms to induce apoptosis in addition to the well-known pathway involving FADD and caspase-8 recruitment. For example, a significant percentage of FADD-deficient cells die upon TRADD overexpression (Yeh et al., 1998). This incomplete rescue implies that TRADD is able to kill cells independent of FADD. Moreover, several studies show that caspase inhibition often fails to prevent cell death associated with TNF $\alpha$  treatment (Vercaemmen et al., 1998; Jones et al., 2000; Luschen et al., 2000; Denecker et al., 2001), suggesting a possible role for TRADD in caspase-independent death.

Previous studies also link TNF to apoptotic signaling in the nucleus involving PML and p53. Consistent with our results, nuclear PML structures are essential for TNF $\alpha$ -medi-

ated death (Wang et al., 1998), and PML has been linked to both caspase-dependent and -independent death pathways (Wang et al., 1998; Quignon et al., 1998). As YFP-TRADD-DD is localized in PML-associated structures where it can induce caspase-independent death, we hypothesize that nuclear shuttling of TRADD may provide a link between nuclear PML and TNF $\alpha$  signaling. p53 is also localized in PML-associated nuclear dots in some instances, and is required for TNF signaling in some contexts (Cai et al., 1997; Ameyar et al., 1999; Rokhlin et al., 2000). Therefore, nuclear shuttling of TRADD may link the TNF receptor with p53 through HIPK2 and/or PML, both of which regulate p53-dependent transcription and apoptosis (Fogal et al., 2000; Guo et al., 2000; Wang et al., 2001; D'Orazi et al., 2002). We have not been able to demonstrate nuclear translocation of TRADD upon TNF $\alpha$  treatment. This may be due in part to technical difficulties in studying such responses in cells that are rapidly undergoing apoptosis. However, we cannot exclude the possibility that nuclear TRADD functions in another pathway outside of TNF signaling.

Death induced by nuclear TRADD-DD is inhibited by Bcl-xL, suggesting that mitochondrial dysfunction occurs in response to nuclear TRADD. Antiapoptotic members of the Bcl-2 family can block some caspase-independent, as well as caspase-dependent, death (Monney et al., 1998; Okuno et al., 1998), consistent with the ability of Bcl-xL to block the caspase-independent death seen here. Bax and other p53 target genes lead to mitochondrial dysfunction (Miyashita and Reed, 1995; Green and Reed, 1998; Oda et al., 2000a, 2000b; Nakano and Vousden, 2001), and activation of the mitochondrial pathway is required for the p53-dependent TNF toxicity seen in MCF-7 cells (Ameyar-Zazoua et al., 2002).

In conclusion, our data suggest that rather than functioning solely in the cytoplasm, TRADD can shuttle through the nucleus where it can activate a different apoptotic pathway involving PML and p53. Therefore, the subcellular distribution of TRADD may modulate its activity and behavior, adding an additional level of complexity to TRADD signaling.

## Materials and methods

### Plasmid construction

The YFP-FADD-DN and YFP-DAPK-DD kinase expression plasmids have been previously described (Morgan et al., 2001). The ds-RED-FADD-DN expression construct was made by removing the YFP coding sequence at the NH<sub>2</sub> terminus of YFP-FADD-DN and replacing it with ds-RED (CLONTECH Laboratories, Inc.). Expression vectors for p35 and BCL-xL were obtained from Invitrogen.

Human TRADD sequences encoding a core DD fragment (aa 222–289) and full-length sequences (aa 1–312) of TRADD were subcloned inframe into the COOH-terminal multiple cloning site of the pEGFP-C2 and pEYFP-C1 vectors (CLONTECH Laboratories, Inc.), respectively, to give GFP-TRADD and YFP-TRADD-DD. Restriction fragments for subcloning of these sequences were obtained from previously constructed cloning vectors generated by blunt-end ligation of PCR products (from a brain cDNA library) into p-Blunt (Invitrogen). A segment of DNA encoding YFP and a small, 15-aa linker was cloned into pEYFP-C1 upstream of the original YFP start codon to give a YFP<sub>2</sub> expression plasmid. The YFP<sub>3</sub> construct was created by subcloning a third YFP sequence downstream of the first two in a strategy that preserved the multiple cloning site from the vector. The DD fragment of TRADD (aa 222–297) was fused downstream of the

YFPs in the YFP<sub>3</sub> construct to give YFP<sub>3</sub>-TRADD-DD. All point mutants were generated by site-directed mutagenesis using the Quick-Change kit (Stratagene).

NLS-GFP-TRADD and NLS-YFP were created by the insertion of annealed DNA oligomers encoding aas (MLYPKKKRGVEDQYK) from the SV40-NLS upstream of the GFP or YFP start codons. This sequence was also inserted COOH-terminal to YFP to make a COOH-terminal YFP-NLS. Similarly, oligomers encoding the Src myristoylation sequence (MGSSKSKPKDPSQR) were used to make M-GFP-TRADD. Annealed oligomers spanning 20-aa segments of TRADD were cloned inframe downstream of YFP or NLS-YFP in pEYFP-c1 to create the constructs used in mapping the NES. GFP fusion proteins with larger segments of TRADD were created from GFP-TRADD and NLS-GFP-TRADD, giving the specified aa in the appropriate reading frames. DNA sequencing was carried out to verify all constructs made.

### Microinjection experiments and death assays

Plasmid DNA was injected into the nucleus of the cell using an Eppendorf microinjector. Injections were performed with the denoted plasmid at a concentration of 250 ng/mL, with the exception of MEF injections, which were performed at 125 ng/mL. To quantitate cell death, healthy living fluorescent cells were counted 1–2 h after microinjection, and the percent survival was determined after overnight incubation. Quantitative data shown in the histograms represent the mean percent survival ( $\pm$  SEM) from 3 or 15 separate experiments, with typically 50–200 cells per sample.

### Cell culture and transfection

All cells were maintained in DME in the presence of 10% fetal bovine serum. Additionally, HT7 cells were supplemented with 100  $\mu$ g/mL Geneticin to maintain the p53 transgene. Where indicated, cells were incubated in the presence of the caspase inhibitor z-VAD-fmk (150  $\mu$ M; Calbiochem) or the corresponding volume of DMSO as a control. Expression of all constructs was by transfection or microinjection. In mapping experiments, nuclei of living cells were stained with Hoechst 33258 at a final concentration of 10  $\mu$ g/mL. Where used, LMB (Sigma-Aldrich) was added to the media at a final concentration of 10  $\mu$ g/mL. Transfections were performed with FuGENE-6 reagent (Roche) according to the manufacturers' protocols, at a concentration of 2  $\mu$ g plasmid DNA/3.5-cm dish.

### Immunofluorescence

For immunofluorescence experiments, cells were grown on glass coverslips, and where indicated, transfected or treated with LMB before permeabilizing/fixing in a 1:1 mixture of methanol/acetone at  $-20^{\circ}$ C for 10 min. Cells were then air dried and blocked in 1% fetal bovine serum in PBS for 10 min. Cells were then stained with a monoclonal anti-TRADD antibody (BD Transduction Laboratories) for 45 min and rinsed three times with PBS for 5 min. For detection, cells were incubated with a rhodamine-conjugated goat anti-mouse immunoglobulin G antibody (Jackson ImmunoResearch Laboratories) for 30 min followed by rinsing three times with PBS for 5 min. Images were obtained with a Hamamatsu digital camera using the Openlab 3.0 software (Improvision).

### Online supplemental material

2 h after microinjection of HeLa cells with YFP-TRADD-DD, time-lapse video microscopy was used to create Video 1 (available at <http://www.jcb.org/cgi/content/full/jcb.200204039/DC1>), which represents apoptosis by the nuclear TRADD-DD and shows apoptotic death of HeLa cells expressing YFP-TRADD-DD (green). Injections were performed 2 h before the start of the video. Images were captured at 30-min intervals for 24 h. Cells were maintained in an environmental chamber at  $37^{\circ}$ C and with 5% CO<sub>2</sub>. Fluorescence images and phase contrast images were obtained at half-hour intervals over 24 h with a Hamamatsu digital camera using the Openlab 3.0 software (Improvision). Images were merged using Openlab 3.0 and exported in Quicktime format.

We thank Constantinos Koumenis (Wake Forest University School of Medicine, Winston-Salem, NC) for the HT7 cells.

This work was supported by grants from the North Carolina Biotechnology Center, American Heart Association, Mid-Atlantic Affiliate, and the National Institutes of Health (NS42662). Initial experiments were supported by the Huntsman Cancer Foundation at the University of Utah during the tenure of a predoctoral fellowship from the American Heart Association, Western States Affiliate (M.J. Morgan).

Submitted: 8 April 2002  
Accepted: 19 April 2002



## References

- Amezar, M., V. Shatrov, C. Bouquet, C. Capoulade, Z. Cai, R. Stancou, C. Badie, H. Haddada, and S. Chouaib. 1999. Adenovirus-mediated transfer of wild-type p53 gene sensitizes TNF resistant MCF7 derivatives to the cytotoxic effect of this cytokine: relationship with c-myc and Rb. *Oncogene*. 18:5464–5472.
- Amezar-Zazoua, M., N. Larochette, G. Dorothee, E. Daugas, H. Haddada, V. Gouloumet, D. Metivier, R. Stancou, F. Mami-Chouaib, G. Kroemer, and S. Chouaib. 2002. Wild-type p53 induced sensitization of mutant p53 TNF-resistant cells: role of caspase-8 and mitochondria. *Cancer Gene Ther.* 9:219–227.
- Ashkenazi, A., and V.M. Dixit. 1998. Death receptors: signaling and modulation. *Science*. 281:1305–1308.
- Cai, Z., C. Capoulade, C. Moyret-Lalle, M. Amor-Gueret, J. Feunteun, A.K. Larsen, B.B. Paillerets, and S. Chouaib. 1997. Resistance of MCF7 human breast carcinoma cells to TNF-induced cell death is associated with loss of p53 function. *Oncogene*. 15:2817–2826.
- Chang, H.Y., H. Nishitoh, X. Yang, H. Ichijo, and D. Baltimore. 1998. Activation of apoptosis signal-regulating kinase 1 (ASK1) by the adaptor protein Daxx. *Science*. 281:1860–1863.
- Chinnaiyan, A.M., C.G. Tepper, M.F. Seldin, K. O'Rourke, F.C. Kischkel, S. Hellbardt, P.H. Kramer, M.E. Peter, and V.M. Dixit. 1996. FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. *J. Biol. Chem.* 271:4961–4965.
- Cottin, V., A.A. Van Linden, and D.W. Riches. 2001. Phosphorylation of the tumor necrosis factor receptor CD120a (p55) recruits Bcl-2 and protects against apoptosis. *J. Biol. Chem.* 276:17252–17260.
- Denecker, G., D. Vercammen, W. Declercq, and P. Vandennebe. 2001. Apoptotic and necrotic cell death induced by death domain receptors. *Cell. Mol. Life Sci.* 58:356–370.
- D'Orazi, G., B. Cecchinelli, T. Bruno, I. Manni, Y. Higashimoto, S. Saito, M. Gostissa, S. Coen, A. Marchetti, G. Del Sal, G. Piaggio, M. Fanciulli, E. Appella, and S. Sodd. 2002. Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser 46 and mediates apoptosis. *Nat. Cell Biol.* 4:11–19.
- Duan, H., and V.M. Dixit. 1997. RAIDD is a new “death” adaptor molecule. *Nature*. 385:86–89.
- Fogal, V., M. Gostissa, P. Sandy, P. Zacchi, T. Sternsdorf, K. Jensen, P.P. Pandolfi, H. Will, C. Schneider, and G. Del Sal. 2000. Regulation of p53 activity in nuclear bodies by a specific PML isoform. *EMBO J.* 19:6185–6195.
- Green, D.R., and J.C. Reed. 1998. Mitochondria and apoptosis. *Science* 281:1309–1312.
- Guiet, C., and P. Vito. 2000. Caspase recruitment domain (CARD)-dependent cytoplasmic filaments mediate bcl10-induced NF- $\kappa$ B activation. *J. Cell Biol.* 148:1131–1140.
- Guo, A., P. Salomoni, J. Luo, A. Shih, S. Zhong, W. Gu, and P.P. Pandolfi. 2000. The function of PML in p53-dependent apoptosis. *Nat. Cell Biol.* 2:730–736.
- Henderson, B.R., and A. Eleftheriou. 2000. A comparison of the activity, sequence specificity, and CRM1-dependence of different nuclear export signals. *Exp. Cell Res.* 256:213–224.
- Hsu, H., J. Xiong, and D.V. Goeddel. 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell*. 81:495–504.
- Hsu, H., J. Huang, H. B. Shu, V. Baichwal, and D. V. Goeddel. 1996a. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. *Immunity*. 4:387–396.
- Hsu, H., H.B. Shu, M.G. Pan, and D.V. Goeddel. 1996b. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell*. 84:299–308.
- Inada, H., I. Izawa, M. Nishizawa, E. Fujita, T. Kiyono, T. Takahashi, T. Momoi, and M. Inagaki. 2001. Keratin attenuates tumor necrosis factor-induced cytotoxicity through association with TRADD. *J. Cell Biol.* 155:415–426.
- Jones, B.E., C.R. Lo, H. Liu, A. Srinivasan, K. Streetz, K.L. Valentino, and M.J. Czaja. 2000. Hepatocytes sensitized to tumor necrosis factor- $\alpha$  cytotoxicity undergo apoptosis through caspase-dependent and caspase-independent pathways. *J. Biol. Chem.* 275:705–712.
- Jones, S.J., E.C. Ledgerwood, J.B. Prins, J. Galbraith, D.R. Johnson, J.S. Pober, and J.R. Bradley. 1999. TNF recruits TRADD to the plasma membrane but not the trans-Golgi network, the principal subcellular location of TNF-R1. *J. Immunol.* 162:1042–1048.
- Kellihier, M.A., S. Grimm, Y. Ishida, F. Kuo, B.Z. Stanger, and P. Leder. 1998. The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. *Immunity*. 8:297–303.
- Ko, Y.G., Y.S. Kang, H. Park, W. Seol, J. Kim, T. Kim, H.S. Park, E.J. Choi, and S. Kim. 2001. Apoptosis signal-regulating kinase 1 controls the proapoptotic function of death-associated protein (Daxx) in the cytoplasm. *J. Biol. Chem.* 276:39103–39106.
- Koumenis, C., R. Alarcon, E. Hammond, P. Sutphin, W. Hoffman, M. Murphy, J. Derr, Y. Taya, S.W. Lowe, M. Kastan, and A. Giaccia. 2001. Regulation of p53 by hypoxia: dissociation of transcriptional repression and apoptosis from p53-dependent transactivation. *Mol. Cell. Biol.* 21:1297–1310.
- Li, X., Y. Wang, K.M. Debatin, and H. Hug. 2000. The serine/threonine kinase HIPK2 interacts with TRADD, but not with CD95 or TNF-R1 in 293T cells. *Biochem. Biophys. Res. Commun.* 277:513–517.
- Lin, Y., A. Devin, Y. Rodriguez, and Z.G. Liu. 1999. Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. *Genes Dev.* 13:2514–2526.
- Locksley, R.M., N. Killeen, and M.J. Lenardo. 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell*. 104:487–501.
- Luschen, S., S. Ussat, G. Scherer, D. Kabelitz, and S. Adam-Klages. 2000. Sensitization to death receptor cytotoxicity by inhibition of fas-associated death domain protein (FADD)/caspase signaling. Requirement of cell cycle progression. *J. Biol. Chem.* 275:24670–24678.
- Maul, G.G., D. Negorev, P. Bell, and A.M. Ishov. 2000. Properties and assembly mechanisms of ND10, PML bodies, or PODs. *J. Struct. Biol.* 129:278–287.
- Miyashita, T., and J.C. Reed. 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell*. 80:293–299.
- Monney, L., I. Otter, R. Olivier, H.L. Ozer, A.L. Haas, S. Omura, and C. Borner. 1998. Defects in the ubiquitin pathway induce caspase-independent apoptosis blocked by Bcl-2. *J. Biol. Chem.* 273:6121–6131.
- Morgan, M.J., J. Thorburn, L. Thomas, T. Maxwell, A.R. Brothman, and A. Thorburn. 2001. An apoptosis signaling pathway induced by the death domain of FADD selectively kills normal but not cancerous prostate epithelial cells. *Cell Death Differ.* 8:696–705.
- Nakano, K., and K.H. Vousden. 2001. PUMA, a novel proapoptotic gene, is induced by p53. *Mol. Cell.* 7:683–694.
- Oda, E., R. Ohki, H. Murasawa, J. Nemoto, T. Shibue, T. Yamashita, T. Tokino, T. Taniguchi, and N. Tanaka. 2000a. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science*. 288:1053–1058.
- Oda, K., H. Arakawa, T. Tanaka, K. Matsuda, C. Tanikawa, T. Mori, H. Nishimori, K. Tamai, T. Tokino, Y. Nakamura, and Y. Taya. 2000b. p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell*. 102:849–862.
- Okuno, S., S. Shimizu, T. Ito, M. Nomura, E. Hamada, Y. Tsujimoto, and H. Matsuda. 1998. Bcl-2 prevents caspase-independent cell death. *J. Biol. Chem.* 273:34272–34277.
- Park, A., and V.R. Baichwal. 1996. Systematic mutational analysis of the death domain of the tumor necrosis factor receptor 1-associated protein TRADD. *J. Biol. Chem.* 271:9858–9862.
- Quignon, F., F. De Bels, M. Koken, J. Feunteun, J.C. Ameisen, and H. de The. 1998. PML induces a novel caspase-independent death process. *Nat. Genet.* 20:220–222.
- Rego, E.M., Z.G. Wang, D. Peruzzi, L.Z. He, C. Cordon-Cardo, and P.P. Pandolfi. 2001. Role of promyelocytic leukemia (PML) protein in tumor suppression. *J. Exp. Med.* 193:521–529.
- Rochat-Steiner, V., K. Becker, O. Micheau, P. Schneider, K. Burns, and J. Tschoopp. 2000. FIST/HIPK3. A Fas/fadd-interacting serine/threonine kinase that induces fadd phosphorylation and inhibits fas-mediated jun nh(2)-terminal kinase activation. *J. Exp. Med.* 192:1165–1174.
- Rokhlin, O.W., A.V. Gudkov, S. Kwak, R.A. Glover, A.S. Gewies, and M.B. Cohen. 2000. p53 is involved in tumor necrosis factor- $\alpha$ -induced apoptosis in the human prostatic carcinoma cell line LNCaP. *Oncogene*. 19:1959–1968.
- Siegel, R.M., D.A. Martin, L. Zheng, S.Y. Ng, J. Bertin, J. Cohen, and M.J. Lenardo. 1998. Death-effector filaments: novel cytoplasmic structures that recruit caspases and trigger apoptosis. *J. Cell Biol.* 141:1243–1253.
- Shu, H.B., M. Takeuchi, and D.V. Goeddel. 1996. The tumor necrosis factor receptor 2 signal transducers TRAF2 and c-IAP1 are components of the tumor necrosis factor receptor 1 signaling complex. *Proc. Natl. Acad. Sci. USA*. 93:13973–13978.
- Torii, S., D.A. Egan, R.A. Evans, and J.C. Reed. 1999. Human Daxx regulates Fas-induced apoptosis from nuclear PML oncogenic domains (PODs). *EMBO J.* 18:6037–6049.
- Tsao, D.H., T. McDonagh, J.B. Telliez, S. Hsu, K. Malakian, G.Y. Xu, and L.L. Lin. 2000. Solution structure of N-TRADD and characterization of the interaction of N-TRADD and C-TRAF2, a key step in the TNFR1 signaling pathway. *Mol. Cell.* 5:1051–1057.

- Vercammen, D., G. Brouckaert, G. Denecker, M. Van de Craen, W. Declercq, W. Fiers, and P. Vandenaebale. 1998. Dual signaling of the Fas receptor: initiation of both apoptotic and necrotic cell death pathways. *J. Exp. Med.* 188:919–930.
- Wang, Y., K.M. Debatin, and H. Hug. 2001. HIPK2 overexpression leads to stabilization of p53 protein and increased p53 transcriptional activity by decreasing Mdm2 protein levels. *BMC Mol. Biol.* 2:8.
- Wang, Z.G., D. Ruggero, S. Ronchetti, S. Zhong, M. Gaboli, R. Rivi, and P.P. Pandolfi. 1998. PML is essential for multiple apoptotic pathways. *Nat Genet.* 20:266–272.
- Yeh, W.C., A. Shahinian, D. Speiser, J. Kraunus, F. Billia, A. Wakeham, J.L. de la Pompa, D. Ferrick, B. Hum, N. Iscove, P. Ohashi, M. Rothe, D.V. Goeddel, and T.W. Mak. 1997. Early lethality, functional NF-kappaB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. *Immunity.* 7:715–725.
- Yeh, W.C., J.L. Pompa, M.E. McCurrach, H.B. Shu, A.J. Elia, A. Shahinian, M. Ng, A. Wakeham, W. Khoo, K. Mitchell, W.S. El-Deiry, S.W. Lowe, D.V. Goeddel, and T.W. Mak. 1998. FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis. *Science.* 279:1954–1958.
- Zhong, S., P. Salomoni, and P.P. Pandolfi. 2000a. The transcriptional role of PML and the nuclear body. *Nat. Cell Biol.* 2:E85–E90.
- Zhong, S., P. Salomoni, S. Ronchetti, A. Guo, D. Ruggero, and P.P. Pandolfi. 2000b. Promyelocytic leukemia protein (PML) and Daxx participate in a novel nuclear pathway for apoptosis. *J. Exp. Med.* 191:631–640.