**The EMBO Journal (2005) 24,** 1886–1898 | © 2005 European Molecular Biology Organization | All Rights Reserved 0261-4189/05
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THE EMBO JOURNAL

# TNF-α induced c-IAP1/TRAF2 complex translocation to a Ubc6-containing compartment and TRAF2 ubiquitination

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**Signaling through tumor necrosis factor receptor 2 (TNF-R2) results in ubiquitination of TRAF2 by the E3 c-IAP1. In this report, we confirm that TRAF2 translocates to a Triton X-100 (TX)-insoluble compartment upon TNF-R2 engagement. Moreover, TRAF2 ubiquitination occurs in this compartment, from which TRAF2 is degraded in a proteasome-dependant manner. Confocal microscopy demonstrated that the TX-insoluble compartment is perinuclear and co-localizes with endoplasmic reticulum (ER) markers. The ER transmembrane Ubc6 bound to c-IAP1 and served as a cognate E2 for c-IAP1's E3 activity *in vitro*. Furthermore, Ubc6 co-localized with translocated TRAF2/c-IAP1 in the ER-associated compartment *in vivo*, and a catalytically inactive Ubc6 mutant inhibited TNF-α-induced, TNF-R2-dependent TRAF2 degradation. These results indicate that upon TNF-R2 signaling, translocation of TRAF2 and c-IAP1 to an ER-associated, Ubc6-containing perinuclear compartment is required for the ubiquitination of TRAF2 by c-IAP1. Therefore, the ER plays a key role in the TNF-R-mediated signal transduction cascade by acting as a site of assembly for E2/E3/substrate complexes.**
*The EMBO Journal* (2005) **24,** 1886–1898. doi:10.1038/sj.emboj.7600649; Published online 28 April 2005
*Subject Categories*: differentiation & death
*Keywords*: c-IAP1; intracellular translocation; TNFR; TRAF2; ubiquitination

## Introduction

Tumor necrosis factor α (TNF-α) affects many cellular functions including growth, differentiation, inflammation, immune responses, and apoptosis (Tracey and Cerami, 1994). It exerts its biological effects by interacting with two members of the TNF receptor (TNF-R) superfamily: TNF-R1 (p55/p60) and TNF-R2 (p75/p80) (Tartaglia and Goeddel, 1992; Armitage, 1994). After engagement with TNF-α, TNF-R1 recruits the death effector adaptor molecules TRADD and FADD through death domain interactions, with the subsequent activation of a caspase cascade that leads to apoptosis (Tschopp *et al*, 1999). Stimulation via TNF-R1 also results in activation of NF-κB and induction of NF-κB-regulated anti-apoptotic factors by a pathway involving TRADD, TRAF2, RIP, and MEKK3 (Wajant *et al*, 1999; Wajant *et al*, 2001; Yang *et al*, 2001). The activity of NF-κB plays an important role as a checkpoint in a cell's decision to survive or die (Micheau and Tschopp, 2003).

Whereas the mechanisms for TNF-R1-mediated cellular responses have been widely studied, the examination of signal transduction via TNF-R2 has been more limited. Interestingly, TNF-R2 has been shown to play a role in determining TNF-R1-mediated cellular responses, and its occupancy greatly potentiates TNF-R1-induced cell death (Erickson *et al*, 1994; Sarin *et al*, 1995; Vandenabeele *et al*, 1995; Zheng *et al*, 1995; Duckett and Thompson, 1997; Weiss *et al*, 1997; Declercq *et al*, 1998; Chan and Lenardo, 2000). This is surprising because, unlike TNF-R1, TNF-R2 does not possess a death domain. In the case of TNF-R2, stimulation results in direct binding of TNF Receptor-Associated Factor (TRAF)-2 and TRAF1 to its cytoplasmic tail, leading to recruitment of the cellular inhibitor of apoptosis proteins c-IAP1 and c-IAP2 (Rothe *et al*, 1994, 1995a). TRAFs are critical mediators of signal transduction by members of the TNF-R superfamily. In particular, TRAF2 is important in protecting cells from TNF-R1-induced apoptosis, as is clear from the observation that cells from TRAF2-deficient mice are much more sensitive to TNF-α-induced cell death mediated by TNF-R1 (Yeh *et al*, 1997). c-IAPs, like other IAP family members, are thought to inhibit cell apoptosis by interacting with caspases and downregulating their activity. Stimulation via TNF-R2 leads to a decrease in TRAF2 protein levels, which has been implicated in potentiation of TNF-R1-induced apoptosis by TNF-R2 (Duckett and Thompson, 1997; Declercq *et al*, 1998; Chan and Lenardo, 2000; Li *et al*, 2002). The decrease in TRAF2 levels induced by TNF-R2 was initially ascribed to the activity of a calpain-like protease (Duckett and Thompson, 1997), and subsequently to translocation of TRAF2 to a Triton X-100 (TX)-insoluble cellular compartment (Arch *et al*, 2000). We have reported that the TNF-R-associated IAPs are RING-containing ubiquitin (Ub) protein ligases (E3s) (Yang *et al*, 2000), and that signaling via TNF-R2 induces c-IAP1 to ubiquitinate TRAF2, thereby triggering its proteasome-dependent degradation and subsequent cessation of TRAF2-dependent signaling (Li *et al*, 2002). How TNF-R2 occupancy induces c-IAP1 Ub protein ligase activity is unknown.

In this study, we demonstrate that ligation of TNF-R2 causes TRAF2 and c-IAP1 to translocate from the cytosol to a TX-insoluble compartment. It is in this compartment, located at the perinuclear endoplasmic reticulum (ER)/nuclear envelope, that TRAF2 and c-IAP1 co-localize with the E2

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**Received: 17 September 2004; accepted: 22 March 2005; published online: 28 April 2005**

 

Ubc6, a cognate E2 for c-IAP1's E3 activity, and where TRAF2 ubiquitination occurs. This dynamic regulation of the intracellular location of the molecules involved in ubiquitination (substrate, E2, and E3) represents a potential mechanism for terminating signaling following cell surface receptor occupancy.

## Results

### *TNF-α stimulation via TNF-R2 induces TRAF2 ubiquitination in, and degradation from, a TX-insoluble compartment*

Signaling via TNF-R2 causes TRAF2 to translocate to a TX-insoluble compartment (Arch *et al*, 2000; Fotin-Mleczek *et al*, 2002). To investigate what relationship, if any, this phenomenon might have with TRAF2 ubiquitination, we studied TNF-α-stimulated Jurkat T cells (TNF-R1$^+$/TNF-R2$^-$) and a stable TNF-R2 transfectant, 4E3 (TNF-R1$^+$/TNF-R2$^+$) (Chan and Lenardo, 2000). As shown in Figure 1A, the vast majority of the TRAF2 in unstimulated Jurkat and 4E3 cells was located in the TX-soluble fraction. Treatment of TNF-R2$^-$ Jurkat cells with TNF-α did not cause any change in the level of expression of TRAF2 or in its distribution between the different detergent-soluble pools. In contrast, treatment of 4E3 cells with TNF-α for as little as 2 h resulted in a noticeable reduction in TRAF2 from the TX-soluble pool. Strikingly, the proteasome inhibitor MG-132 caused TRAF2 accumulation only in the TX-insoluble fraction. TNF-α treatment for 4 h led to a greater reduction of TRAF2, and again treatment with MG-132 restored its expression, but only in the TX-insoluble fraction. Similar results were found with another specific proteasome inhibitor, lactacystin (data not shown). As TNF-

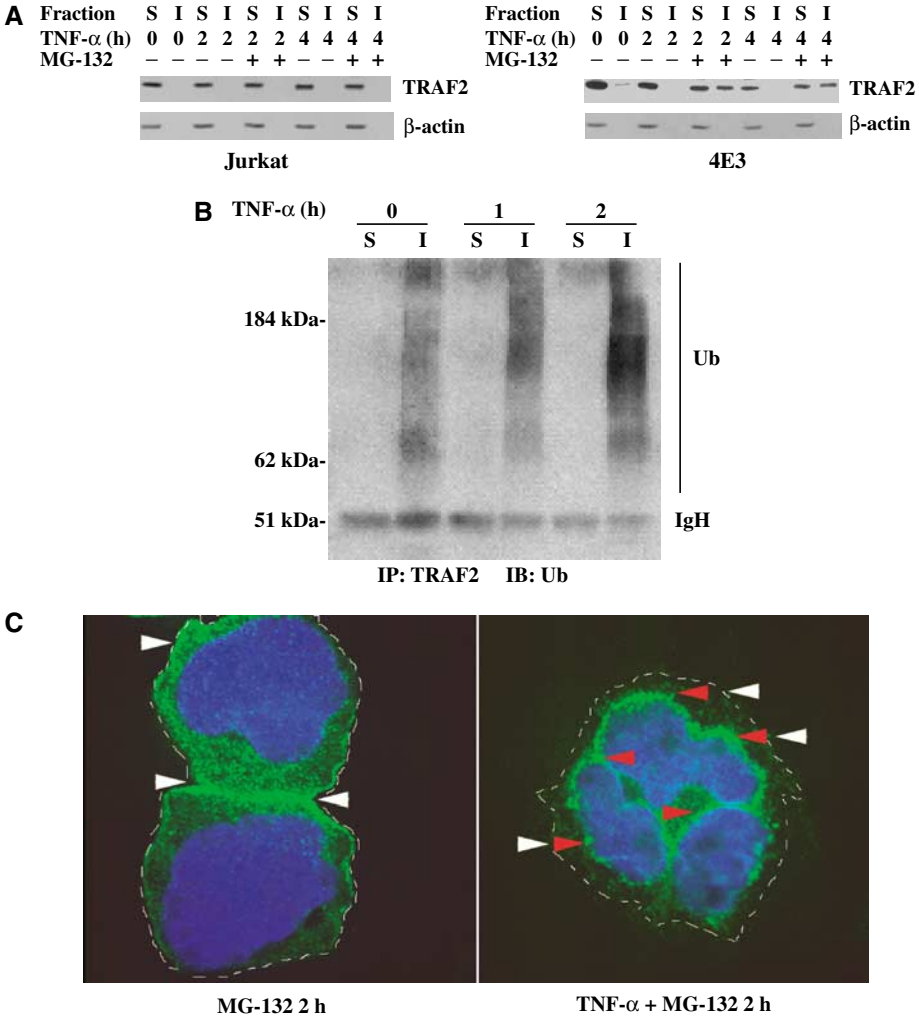


**Figure 1** TNF-α induces TNF-R2-dependent TRAF2 ubiquitination and degradation in a TX-insoluble compartment. (**A**) Jurkat cells (left panel) or Jurkat cells stably transfected with TNF-R2 (4E3 cells, right panel) were treated with 20 ng/ml TNF-α for the indicated times. Where indicated, cells were pretreated with 25 μM MG-132 for 30 min and cultured with the proteasome inhibitor until harvesting. Cells were lysed with 0.5% TX lysis buffer. Soluble (S) and insoluble (I) fractions were isolated as described previously (Arch *et al*, 2000), and were resolved on 10% SDS–PAGE. TRAF2 levels were detected by IB. (**B**) 4E3 cells were stimulated with 50 ng/ml TNF-α for the indicated times in the presence of lactacystin and lysed in 0.5% TX buffer (S) or 0.5% TX buffer containing 1% octylglucoside (I). Lysates were heated in 0.5% SDS to disrupt noncovalent molecular interactions, immunoprecipitated with anti-TRAF2, and immunoblotted with anti-Ub antibodies. (**C**) TNF-α stimulation induces TRAF2 translocation from the plasma membrane and cytosol to the perinuclear region in 4E3 cells. 4E3 cells were incubated with 10 μM MG-132 and then stimulated or not with 20 ng/ml TNF-α for 2 h. Cells were attached to slides by centrifugation, fixed with 4% paraformaldehyde, stained with anti-TRAF2 antibodies, and analyzed by confocal microscopy. Images were obtained with a Zeiss microscope equipped with an ULTRAVIEW (Perkin-Elmer) confocal microscope attachment.

induced TRAF2 degradation by proteasomes occurs secondarily to ubiquitination by c-IAP1 (Li *et al*, 2002), one possible explanation for this result is that it is in the TX-insoluble compartment that TRAF2 ubiquitination occurs. To test this, 4E3 cells were stimulated for the indicated times in the presence of lactacystin, lysed, heated in 0.5% SDS to disrupt noncovalent associations, and TRAF2 was immunoprecipitated from the TX-soluble and -insoluble fractions and immunoblotted with anti-Ub (Figure 1B). A small amount of ubiquitinated material was observed in resting cells and increased with time after stimulation with TNF-α. Notably, ubiquitinated species were only detectable in the TX-insoluble fraction, which is consistent with ubiquitination occurring in this fraction. These results indicate that TNF-α stimulation via TNF-R2 but not TNF-R1 induces TRAF2 translocation to a TX-insoluble compartment, from which it is degraded in a proteasome-dependent fashion.

We used fluorescence confocal microscopy to visualize the intracellular location of endogenous TRAF2 in 4E3 cells after culture with MG-132 (Li *et al*, 2002) in the presence or absence of TNF-α (Figure 1C). TRAF2 was distributed throughout the cytoplasm in the absence of TNF-α with some concentration near the plasma membrane (left panel, white arrowheads). When the cells were treated with TNF-α, however, TRAF2 was observed to accumulate in the perinuclear region (right panel, indicated by red arrowheads). Taken together, the data suggest that TNF-α causes a TNF-R2-dependent translocation of TRAF2 to a perinuclear and TX-insoluble compartment prior to degradation.

### *Translocation of a c-IAP1/TRAF2 complex into a TX-insoluble fraction precedes c-IAP1 E3-dependent TRAF2 degradation*

Owing to the difficulty in studying intracellular distribution in cytoplasm-poor lymphoid cells, studies of TRAF2 localization were pursued in adherent cell lines. Given the central role of c-IAP1 in TRAF2 ubiquitination, transfected myc-c-IAP1 and hemagglutinin (HA)-TRAF2 levels were analyzed in compartments with different detergent solubility in 293 cells (Figure 2A). When expressed alone, a low level of c-IAP1 was found in the TX-soluble, and very little in the insoluble, fraction. Whereas the E3-deficient c-IAP1 (c-IAP-mut) was expressed at a higher level, the majority was in the TX-insoluble pool (compare lanes 2 and 7). Like c-IAP1, when expressed alone TRAF2 was predominantly detected in the TX-soluble fraction. Co-expression of c-IAP1 decreased TRAF2 levels in the TX-soluble compartment, without any increase in the TX-insoluble fraction. In contrast, expression of c-IAP1-mut resulted in substantial translocation of TRAF2 from the TX-soluble to the TX-insoluble fraction. Thus, co-expression of c-IAP1 results in the transition of TRAF2 from the TX-soluble to the insoluble pool.

To detect and characterize c-IAP1/TRAF2 complexes, transfected cells were treated with lactacystin for 6 h to prevent proteasome-mediated degradation, lysed, and the TX-soluble and -insoluble fractions were analyzed by co-immunoprecipitation (IP) and immunoblotting (IB) with appropriate antibodies (Figure 2B). Although TRAF2 co-immunoprecipitated with c-IAP1 could be detected in both the TX-soluble and -insoluble fractions, the majority of the c-IAP1/TRAF2 complexes existed in the TX-insoluble pool. In addition, when TRAF2 was co-immunoprecipitated with c-IAP1, there was an immunoreactive species in the TX-insoluble, but not -soluble, fraction that was approximately 8 kDa larger than HA-TRAF2, consistent with monoubiquitination (lane 5). This was strongly supported by the observation that this band was not detected when TRAF2 was co-precipitated with the Ub protein ligase-defective c-IAP1-mut (lane 6). To confirm that c-IAP1-associated TRAF2 in the TX-insoluble fraction was ubiquitinated, lysates were heated in 0.5% SDS, and immunoprecipitated HA-TRAF2 proteins from the TX-soluble and -insoluble fractions were subjected to IB with anti-Ub. As shown in Figure 2C, ubiquitinated TRAF2 was detected in the presence of c-IAP1 but not c-IAP1-mut. Moreover, ubiquitinated TRAF2 was found predominantly in the TX-insoluble fraction. Therefore, c-IAP1/TRAF2 complexes migrate to a TX-insoluble compartment where c-IAP1-mediated TRAF2 ubiquitination occurs and from which they are degraded. The finding that c-IAP1-mut/TRAF2 complexes were also concentrated in this compartment indicates that ubiquitination does not precede the translocation, but rather occurs after translocation.

### *Co-localization of c-IAP1 and TRAF2 in a perinuclear/ER compartment*

Confocal microscopy was used to examine the subcellular location of TRAF2, c-IAP1, and c-IAP1/TRAF2 complexes (Figure 3A). When expressed in Cos7 cells alone, TRAF2 was widely distributed in the cytoplasm, with an occasional perinuclear aggregate. Ectopically expressed c-IAP1 was distributed throughout the cell, with a substantial amount present in the nucleus. This pattern of distribution is consistent with that observed by immunohistochemical staining for endogenous c-IAP1 in human cell lines (Imoto *et al*, 2002; McEleny *et al*, 2002). Although more cells had detectable expression (data not shown), c-IAP1-mut was distributed in the same manner as wild-type (wt) c-IAP1. In contrast, co-expression of HA-TRAF2 and myc-c-IAP1 resulted in the movement of TRAF2, and even more strikingly c-IAP1, to large perinuclear structures (middle row). These perinuclear aggregates were much more readily observed when HA-TRAF2 was co-expressed with myc-c-IAP1-mut because the loss of RING-related E3 activity resulted in higher expression of both proteins (bottom row).

To better define the nature of the c-IAP1/TRAF2-containing structures, cells transfected with cDNAs encoding each protein were co-stained for their peptide tags as well as markers for various potentially perinuclear intracellular structures and analyzed by confocal microscopy (Figure 3B). Lamp2 is a marker for late endocytic compartments, which include late endosomes and lysosomes (Chen *et al*, 1985). c-IAP1/TRAF2 and Lamp2 had very different patterns of distribution, and there was no co-localization (first row). Lipid bodies are accumulations of neutral lipids contained in a monolayer of phospholipids probably associated with the cytoplasmic leaf of the ER membrane, and are similar in size to the c-IAP1/TRAF2-associated structures we observed (Tauchi-Sato *et al*, 2002). To test the possibility that the aggregates containing c-IAP1/TRAF2 were lipid bodies, transfected cells were co-stained with Nile Red (second row). Although c-IAP1/TRAF2 and lipid bodies were superficially similar in morphological appearance, they did not co-localize. Moreover, the c-IAP1/TRAF2 structures did not co-localize with TGN46, a marker of the trans-Golgi network (third row), or filipin, which stains

 

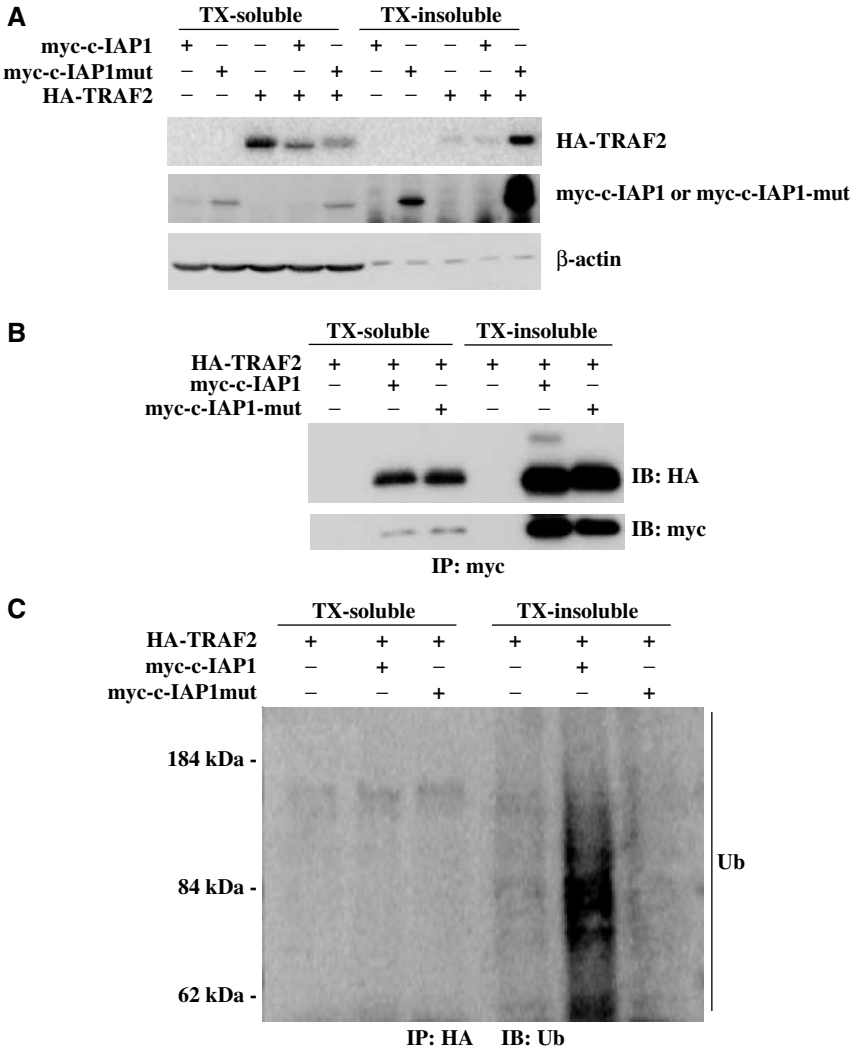


**Figure 2** Translocation of TRAF2–c-IAP1 complex into a TX-insoluble fraction is required for ubiquitination by c-IAP1 and degradation of TRAF2. (**A**) 293 cells were transfected with expression plasmids encoding HA-TRAF2, myc-c-IAP1, and myc-c-IAP1-mut in the indicated combinations. After 24 h, cells were lysed and TX-soluble and -insoluble fractions were prepared and resolved by 10% SDS–PAGE. IB was performed with anti-HA or anti-myc antibodies to detect TRAF2 and c-IAP1, respectively. β-actin was used as a loading control. (**B**) 293 cells were transfected and lysed as in panel A. The transfected cells were treated with 50 μM lactacystin for 6 h prior to lysis. The supernatants (soluble) were taken and the pellets (insoluble) were resuspended and lysed in lysis buffer containing 1% octylglucoside. Lysates from TX-soluble and -insoluble fractions were subjected to IP with anti-myc and IB with anti-HA and anti-myc. (**C**) 293 cells were transfected and lysed as in (B). Lysates from the TX-soluble and -insoluble fractions were heated in 0.5% SDS, immunoprecipitated with anti-HA, resolved on 8% SDS–PAGE, and immunoblotted with anti-Ub.

intracellular sterol depots (data not shown). In contrast to the other markers, the c-IAP1/TRAF2 co-localized with calreticulin (Figure 3B, bottom row), an ER-luminal chaperone with lectin-like properties (Michalak *et al*, 1999), and another ER-luminal chaperone, protein disulfide isomerase (PDI) (data not shown). We conclude, therefore, that c-IAP1/TRAF2 specifically translocates to a small aggregate-like structure associated or contiguous with the ER.

### *RING-deleted TRAF2 (ΔTRAF2)/c-IAP1 complexes do not translocate to the perinuclear ER and ΔTRAF2 is not degraded in vivo*

TRAF2 in which the N-terminal RING domain has been removed (ΔTRAF2) is a dominant negative for TNF-R2 signaling (Rothe *et al*, 1995b); the mechanism for its dominant-negative effect is unknown. To determine how a ΔTRAF2/c-IAP1 complex behaves *in vivo*, cDNAs encoding these two molecules were expressed in Cos7 cells and their intracellular localization was visualized by fluorescence confocal microscopy (Figure 4A). Like wt TRAF2, ΔTRAF2 was distributed in the cytosol. Unlike wt TRAF2, however, co-expression of c-IAP1 did not result in translocation of the complex to a perinuclear structure, but rather ΔTRAF2 and c-IAP1 co-localized in the cytosol. The observation prompted us to examine whether co-expression of c-IAP1 induces ΔTRAF2 degradation *in vivo*. The distribution of ΔTRAF2/c-IAP1 complexes into TX-soluble and insoluble compartments was assessed by expressing these molecules in 293 cells (Figure 4B). Like TRAF2, expression of ΔTRAF2 alone resulted in its appearance only in the TX-soluble fraction. Importantly, co-expression with c-IAP1 or c-IAP1-mut did not result in translocation to the TX-insoluble pool, and

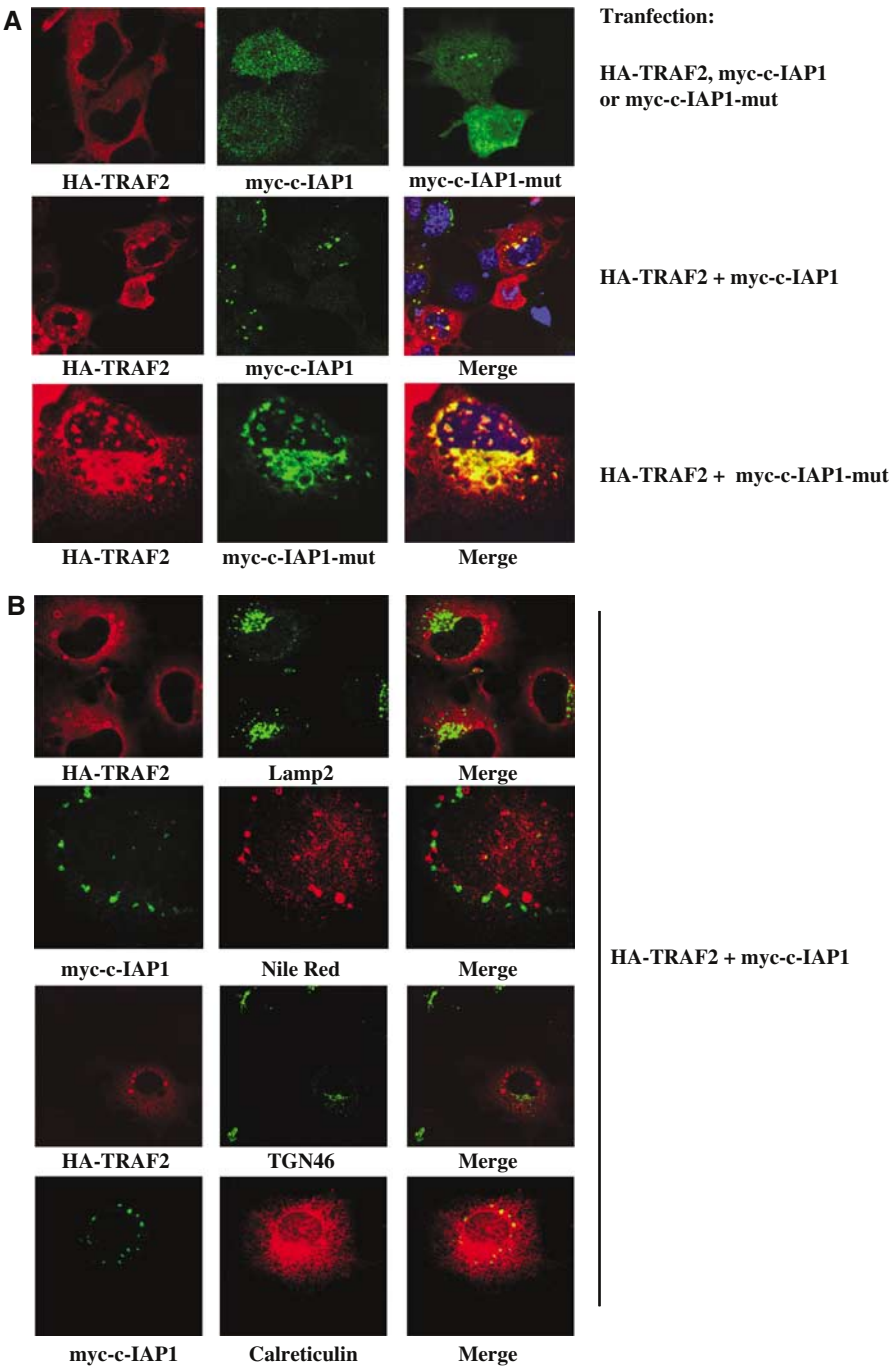


**Figure 3** (**A**) Overexpressed c-IAP1 and TRAF2 co-localize in perinuclear structures that are connected with the ER. Cos7 cells attached to coverslips were transfected with HA-TRAF2, myc-c-IAP1, or myc-c-IAP1-mut alone (first row), or the combination of HA-TRAF2 and myc-c-IAP1 (middle row), or HA-TRAF2 and myc-c-IAP1-mut (bottom row) for 24 h. Cells were fixed and stained with anti-HA antibody to detect TRAF2 (red), anti-myc antibody to detect c-IAP1 or c-IAP1-mut (green), and DAPI to label nuclei (blue). (**B**) TRAF2/c-IAP1-containing structures co-localize with ER. Cos7 cells were transfected with HA-TRAF2 and myc-c-IAP1 as in panel A, and stained with anti-HA (red) and anti-Lamp 2 (green) (first row), anti-myc (green) and Nile Red (red) (second row), anti-HA (red) and anti-TGN46 (green) (third row), or anti-myc (green) and anti-calreticulin (red) (bottom row).

c-IAP1 did not cause any loss of ΔTRAF2 expression. Furthermore, c-IAP1-mut, which is found predominantly in the TX-insoluble compartment when expressed alone (see Figure 2A), was found exclusively in the TX-soluble compartment when co-expressed with ΔTRAF2, and ΔTRAF2 eliminated the large difference in the level of expression between c-IAP1 and the E3-defective c-IAP1-mut (compare middle rows of Figures 2A and 4B). As we have previously shown that ΔTRAF2 binds to c-IAP1, and that *in vitro* ΔTRAF2 is a target for c-IAP1-mediated ubiquitination when an E2 is provided (Li *et al*, 2002), these results suggest that the ΔTRAF2/c-IAP-1 complex fails to interact with an appropriate E2, and are consistent with the possibility that intracellular translocation to an ER/perinuclear compartment is required

 

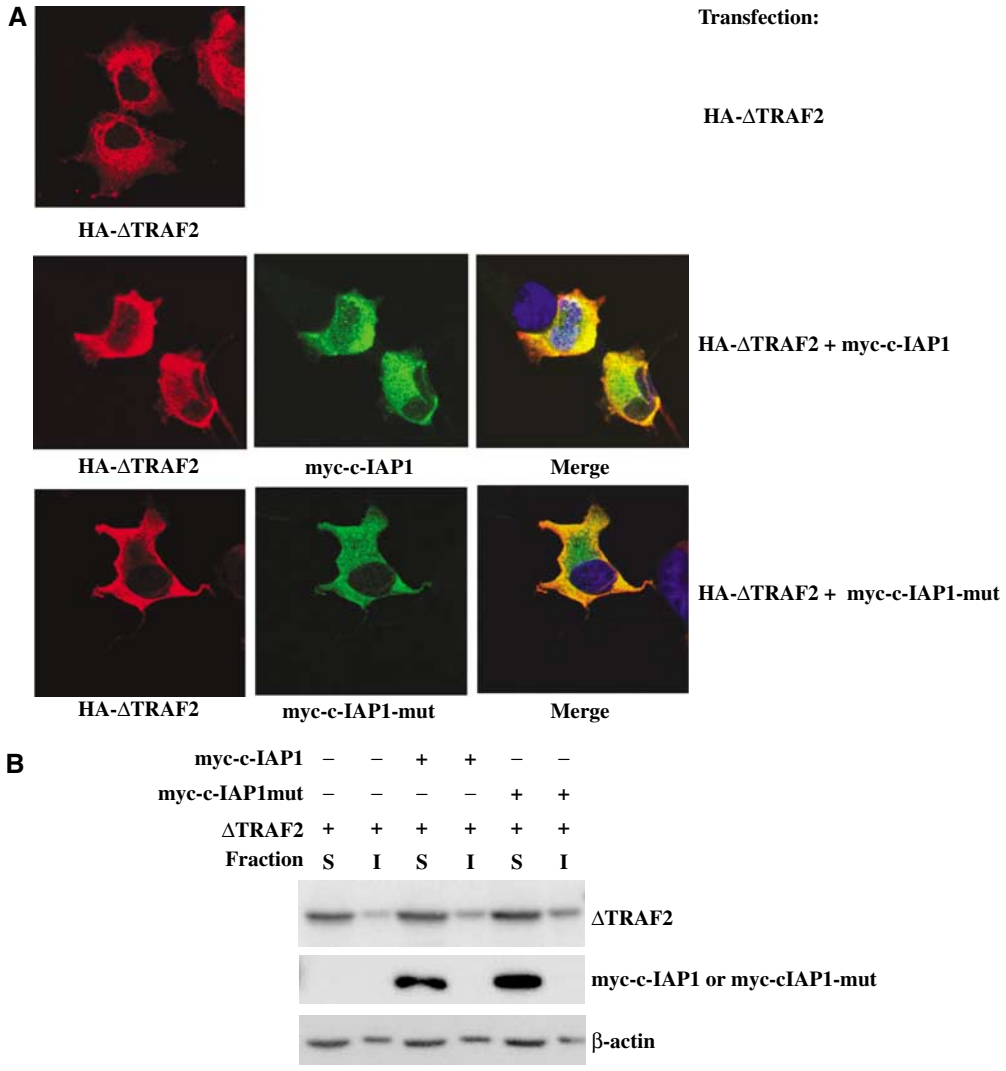


**Figure 4** c-IAP1 and ΔTRAF2 do not localize to the perinuclear region, and c-IAP1 does not induce ΔTRAF2 degradation. (**A**) Cos7 cells were transfected with HA-ΔTRAF2 (top row), the combination of HA-ΔTRAF2 and myc-c-IAP1 (middle row), or HA-ΔTRAF2 and myc-c-IAP1-mut (bottom row). After 24 h the cells were fixed and stained with anti-HA to detect ΔTRAF2 (red), anti-myc to detect c-IAP1 or c-IAP1-mut (green), and DAPI to label nuclei (blue). (**B**) 293 cells were transfected with expression plasmids encoding ΔTRAF2, myc-c-IAP1, and myc-c-IAP1-mut in the indicated combinations. After 24 h cells were lysed and TX-soluble (S) and -insoluble (I) fractions were prepared and resolved by 12% SDS–PAGE. IB was performed with anti-TRAF2 (Santa Cruz) or anti-myc antibodies to detect ΔTRAF2 and c-IAP1, respectively. β-actin was used as a loading control.

for the formation of a TRAF-2/c-IAP1/E2 complex and subsequent ubiquitination and degradation.

### *Ubc6 is a cognate E2 for c-IAP1*

c-IAP1 E3 activity requires the cooperation of a Ub conjugating enzyme (Ubc, or E2). There are two ER-associated E2s: Ubc6 and Ubc7. Ubc6 is an ER transmembrane protein whose catalytic domain resides in the cytoplasm (Sommer and Jentsch, 1993; Tiwari and Weissman, 2001). Ubc7 does not span membranes, but is recruited to the cytoplasmic face of the ER by binding the ER transmembrane protein Cue1p (Biederer *et al*, 1997; Tiwari and Weissman, 2001). As our results suggested that c-IAP1 ubiquitinates TRAF2 in an ER-associated compartment, we asked if Ubc6 and/or Ubc7 might be a cognate E2 for c-IAP1. A glutathione *S*-transferase (GST)–c-IAP1 fusion protein was used to pull-down interacting proteins. GST–c-IAP1 bound to glutathione-Sepharose beads was incubated with the lysates from 293 cells that had been transfected with plasmids encoding various myc-tagged E2s (Figure 5A and B). The human c-IAP1 fused with GST did not bring down human UbcH6, a close homolog of UbcH5 (Nuber *et al*, 1996), human UbcH7 (Nuber *et al*, 1996), a cytosolic E2 that has been shown to interact with at least eight E3s, and is functionally important for Ub protein ligase activity of Cbl (Yokouchi *et al*, 1999) and Parkin (Shimura *et al*, 2000), or human UbcH8, an E2 highly related to UbcH7 (Kumar *et al*, 1997). On the other hand, c-IAP1 binding to human Ubc6 (hUbc6e) was easily detected (Figure 5A). Similarly, GST–c-IAP1 pulled down murine Ubc6 (MmUbc6), but not the other ER-associated E2, murine Ubc7 (MmUbc7) (Figure 5B).

An *in vitro* ubiquitination assay was employed to test if Ubc6 actually functions as an E2 for c-IAP1's Ub protein ligase activity. Autoubiquitination was assessed by incubat-

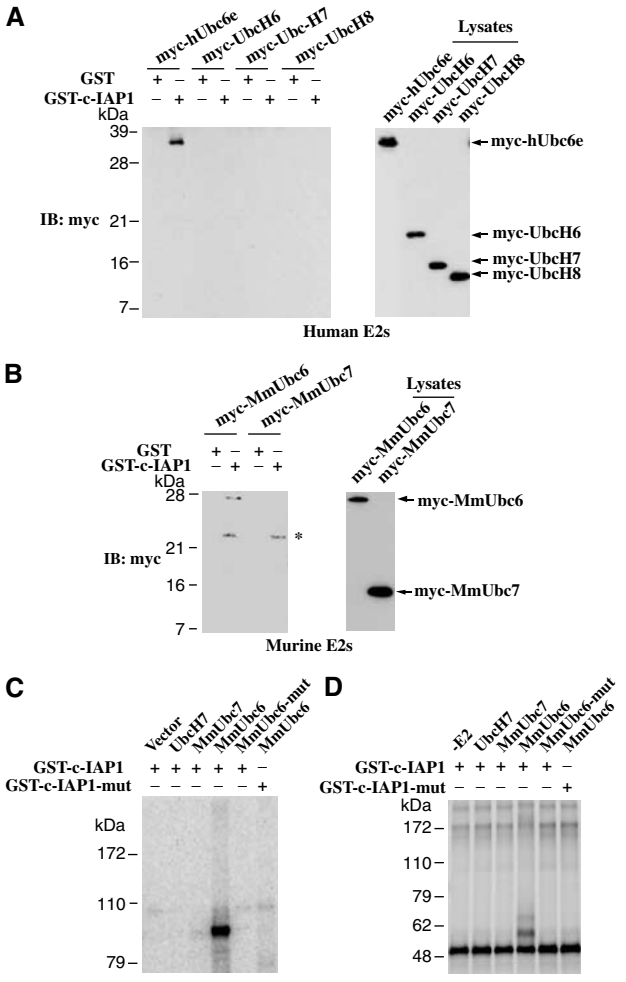


**Figure 5** Ubc6 is a cognate E2 for c-IAP1. Equal amounts of bacterially expressed GST or GST–c-IAP1 bound to Glutathione-Sepharose beads were incubated at 4°C for 2 h with lysates of 293 cells that had been transfected with (**A**) myc-hUbc6e, myc-UbcH6, myc-UbcH7, or myc-UbcH8, or (**B**) myc-MmUbc6 or myc-MmUbc7. After extensive washing, bead-bound proteins were eluted by boiling in SDS sample buffer and analyzed by IB with anti-myc antibody (left panels). The same amounts of cell lysate were also subjected to IB to determine the protein expression levels (right panels). *Nonspecific band. Equal amounts of GST–c-IAP1 were used in each lane, as confirmed by staining with Coomassie blue (data not shown). (**C**) Ubc6 assists c-IAP1 autoubiquitination. In all, 2.5 pmol of GST–c-IAP1 or GST–c-IAP1-mut was incubated with 100 ng rabbit E1 in combination with 2 μl of bacteria lysates containing PET28a vector or PET28a encoding UbcH7, MmUbc7, or MmUbc6. After incubation, reaction mixtures were separated by 8% SDS–PAGE and $^{32}$P-Ub-labeled species were visualized with a Storm PhosphorImager. One representative experiment of four is shown. (**D**) Ubc6 promotes ubiquitination of TRAF2 by c-IAP1. *In vitro*-translated and $^{35}$S-labeled TRAF2 was incubated with beads coated with GST–c-IAP1. The beads were washed and subjected to an *in vitro* ubiquitination assay using 1 μg of the indicated purified His-E2s. After incubation, reactions were resolved by 10% SDS–PAGE. $^{35}$S-TRAF2 was visualized with a PhosphorImager. One representative experiment of four is shown.

ing purified GST–c-IAP1 with various bacterially expressed human or murine E2s in the presence of $^{32}$P-labeled Ub (Figure 5C). Ubiquitinated c-IAP1 was observed only when murine MmUbc6 was included in the reaction. In contrast, ubiquitination was barely detectable in the absence of E2, or in the presence of human UbcH7 or murine Ubc7. As previously observed (Yang *et al*, 2000), c-IAP1-mut was unable to autoubiquitinate. Importantly, the use of MmUbc6 containing a single amino-acid substitution in the conservative cysteine in its Ubc domain (Tiwari and Weissman, 2001) resulted in dramatically reduced c-IAP1 autoubiquitination. The ability of Ubc6 to act as an E2 for a physiologically relevant substrate was tested with *in vitro*-translated and metabolically labeled TRAF2 (Figure 5D). Ubiquitinated species were observed in the presence of MmUbc6, but not MmUbc7 or UbcH7. Furthermore, TRAF2 ubiquitination was not detected when c-IAP1-mut was used or when MmUbc6-mut was included in the reactions.

### *Co-localization of c-IAP1 and Ubc6 in the perinuclear ER*

Further characterization of the intracellular localization of c-IAP1/Ubc6 complexes was performed with confocal microscopy (Figure 6A and B). As described above, myc-c-IAP1 was distributed in both cytosol and the nucleus, the relatively low levels of c-IAP1 being due to autoubiquitination and degradation (Yang *et al*, 2000). Treatment with lactacystin led to an accumulation of c-IAP1 in the perinuclear region, where it co-localized with calreticulin (Figure 6A, bottom row). When c-IAP1 was co-transfected with MmUbc6, the E2 was found in a pattern typical of ER expression, but very few cells co-stained for c-IAP1. In fact, cells positive for exogenous c-IAP1 were always negative for co-transfected MmUbc6 (Figure 6B, upper row), confirming the observation made with IB that co-transfection of MmUbc6 dramatically reduced c-IAP levels (data not shown). When the co-transfected cells were treated with lactacystin, there was a large increase in c-IAP1 expression in a cytosolic and largely non-nuclear distribution (Figure 6B, bottom row, first panel), a pattern quite different from that seen when c-IAP1 was expressed alone in the absence of lactacystin (Figure 6A, upper row, first panel). Moreover, clear co-localization of c-IAP1 and Ubc6 was seen in the perinuclear region (Figure 6B, bottom row, last two panels). Thus, c-IAP1 co-localizes with the ER/nuclear envelope E2 Ubc6, and it is from this compartment that proteasomal degradation occurs.

### *Translocation of endogenous TRAF2/c-IAP1 complexes and association of endogenous TRAF2 with ER in response to TNF-R2 signaling*

Overexpression of cellular proteins allows one to more easily visualize intracellular localization and intermolecular interactions, but the sheer abundance of protein expressed has the potential to create artifacts. We therefore sought to follow the intracellular localization of endogenous TRAF2 and c-IAP1 in response to TNF-α. HeLa cells that stably express TNF-R2 were treated with or without TNF-α for 20 min, fixed, and stained for TRAF2 and c-IAP1. As shown in Figure 7A, in the absence of TNF-α (upper panels), TRAF2 had a widespread distribution in the cytosol. c-IAP1 had a similar pattern, with some localized in the nucleus. Upon stimulation with TNF-α for 20 min, both TRAF2 and c-IAP1 moved to the perinuclear region (lower panels), with the appearance of large structures in which endogenous TRAF2 and c-IAP1 appear in the same region and showed substantial co-localization (selected examples indicated by arrowheads). These structures were most apparent in a focal plane that placed them adjacent to and above the nucleus.

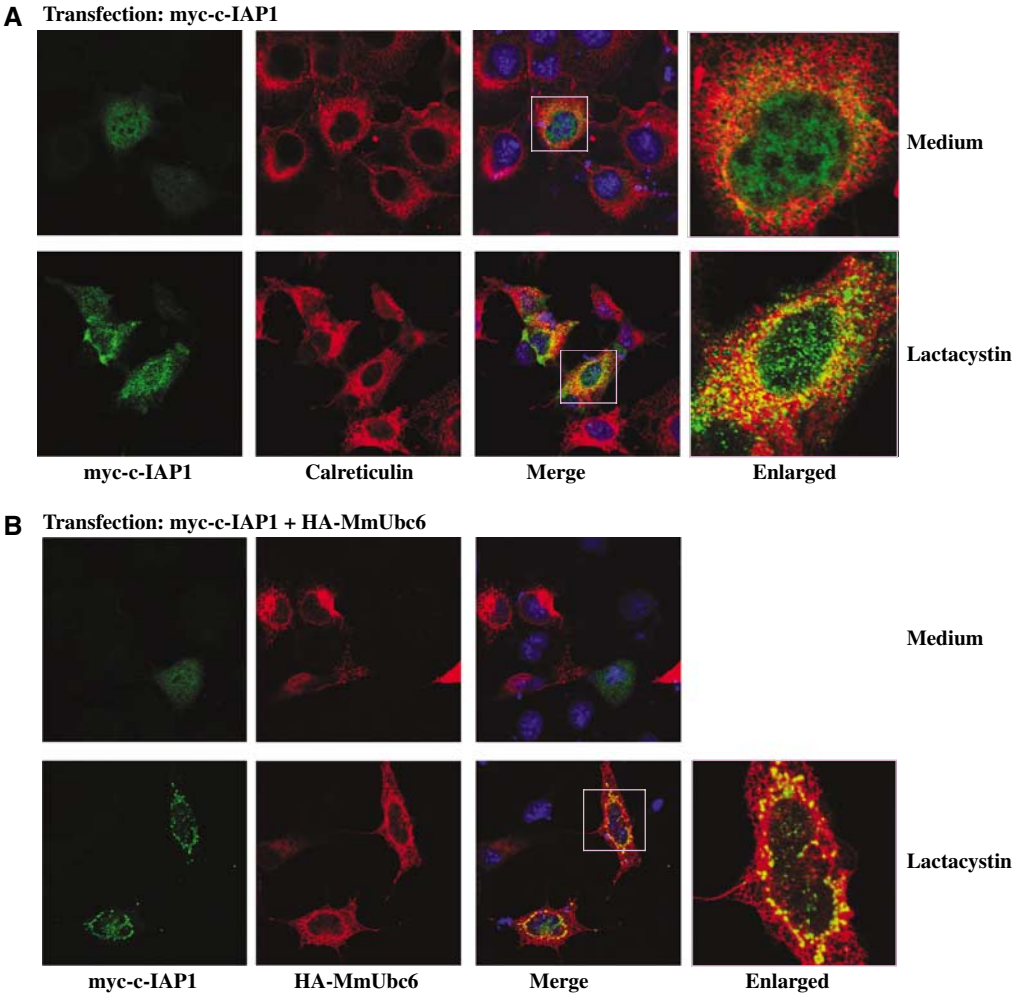


**Figure 6** c-IAP1 and Ubc6 co-localize to a perinuclear structure. Cos7 cells plated onto coverslips were transfected with (**A**) myc-c-IAP1 or (**B**) myc-c-IAP1 and HA-MmUbc6. After 24 h the cells were fixed and stained with anti-myc to stain c-IAP1 (green), anti-calreticulin to stain ER (red, panel A), or anti-HA to stain HA-MmUbc6 (red, panel B). DAPI was used to stain nuclei (blue). Cells were treated with vehicle alone (medium) or 50 μM lactacystin dissolved in DMSO (lactacystin), respectively, for 7 h before fixation. The panels labeled 'Enlarged' show the indicated cells in the preceding panels at higher magnification.

The interaction of endogenous TRAF2 with Ubc6 was also examined in TNF-R2$^+$ HeLa cells. As available reagents do not visualize endogenous Ubc6 well, we transfected the cells with myc-tagged Ubc6, and after 36 h treated them with or without TNF-α for 20 min. As shown in Figure 7B, in the presence of TNF-α (lower panels), TRAF2-containing structures were found in a region above the nucleus associated with Ubc6, as indicated by the merged images marked with an arrowhead. Nearly complete co-localization was observed. In the absence of TNF-α (upper panels), the TRAF2-containing structures were rarely observed in the perinuclear region and the few structures present showed no co-localization with Ubc6. Therefore, endogenous TRAF2 responds to TNF-α by associating with the perinuclear ER/nuclear envelop in close proximity to the E2 Ubc6.

### *Catalytically inactive Ubc6 inhibits TNF-R2-dependent TRAF2 degradation*

293 cells lack cell surface TNF-R2, which is required for TNF-α-induced TRAF2 degradation (Li *et al*, 2002). We generated 293 cells that stably expressed human TNF-R2. One such clone, clone 3, is shown in Figure 7C, left panel. Clone 3 cells were transfected with cDNAs encoding GFP with or without myc-tagged MmUbc6 or an enzymatically inactive myc-tagged MmUbc6 mutant (myc-MmUbc6-mut). The GFP-positive cells were isolated by cell sorting, stimulated with TNF-α for 6 h, lysed in sample buffer, and endogenous TRAF2 expression assessed by IB with anti-TRAF2 (Figure 7C, right panel). TNF-α caused a large decrease in TRAF2 levels in cells transfected with or without MmUbc6. In contrast, the enzymatically inactive MmUbc6 acted as a dominant negative, blunting the effect of TNF-α on TRAF2 degradation. Taken together, these results demonstrate that TRAF2/c-IAP1 co-localizes in the perinuclear ER with Ubc6, which provides the Ub conjugating function for c-IAP1 activity.

## Discussion

The sequelae of signaling through TNF-R2 are less well documented than those for TNF-R1. Signaling via TNF-R2 chimeric molecules has been shown to cause TRAF2 translocation to the perinuclear region, corresponding to its

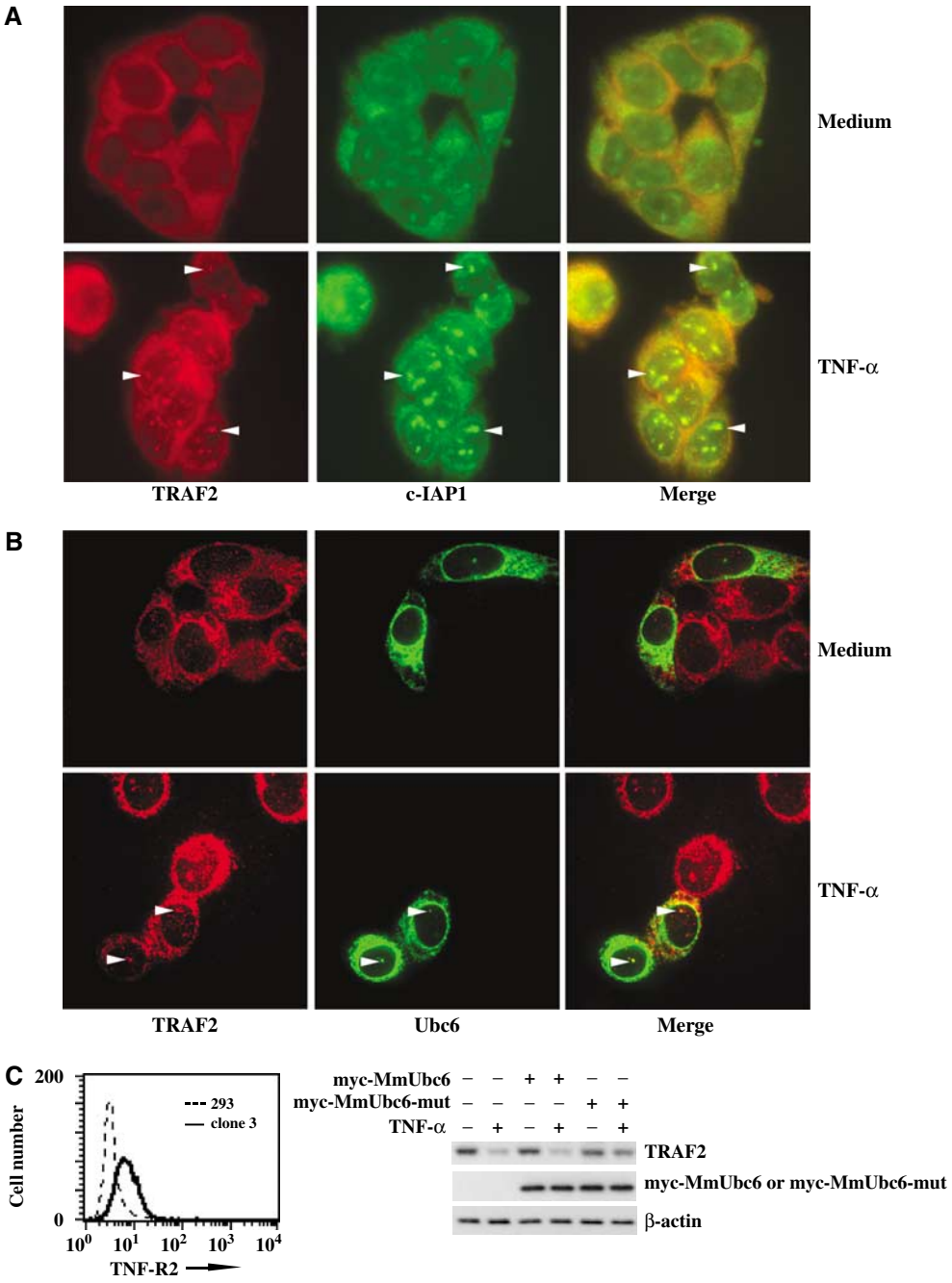


**Figure 7** Endogenous c-IAP1 and TRAF2 in HeLa/TNF-R2 cells co-localize near the nucleus in response to TNF-α, and Ubc6 functions *in vivo* in TNF-R2-mediated TRAF2 degradation. (**A**) HeLa/TNF-R2 cells or (**B**) Hela/TNF-R2 cells transfected with myc-MmUbc6 were cultured in medium (upper rows) or 10 ng/ml TNF-α (lower rows) for 20 min. Cells were fixed and stained with anti-TRAF2 (red) and anti-c-IAP1 (green) (A), or anti-TRAF2 (red) and anti-myc (green) (B). (**C**) A catalytically inactive Ubc6 mutant inhibits TNF-α-stimulated, TNF-R2-dependent TRAF2 degradation. (Left panel) 293 cells stably transfected with TNF-R2 were analyzed for TNF-R2 expression by flow cytometry. TNF-R2 expression on parental 293 cells and one stable transfectant, Clone 3, is shown. (Right panel) 293 Clone 3/TNF-R2$^+$ cells were transfected with expression vectors encoding EGFP plus myc-MmUbc6, myc-MmUbc6-mut, or empty vector. After 24 h, the transfected cells were harvested and sorted for GFP expression. $5 \times 10^5$ GFP-positive cells were re-plated and treated with or without 10 ng/ml of TNF-α for 6 h, then lysed with SDS sample buffer. Cell lysates were resolved on SDS–PAGE and immunoblotted with anti-TRAF2. Ubc6 expression was determined by IB with anti-myc.

depletion from the TX-soluble pool (Arch *et al*, 2000; Fotin-Mleczek *et al*, 2002). In addition, c-IAP1 acquires the ability to ubiquitinate TRAF2 upon TNF-R2 occupancy (Li *et al*, 2002). The results of this study demonstrate that these are related events, and in fact indicate that the former provides a mechanistic basis for the latter.

Ubc6 comprises a group of ER-resident E2s important for ER-associated degradation (ERAD). There are two classes of mammalian Ubc6 (Tiwari and Weissman, 2001; Lenk *et al*, 2002). The first is named MmUbc6, and comprises cDNAs encoding proteins with a high degree of overall sequence identity with yeast Ubc6p (approximately 40% at

 

the amino-acid level) and a similar calculated molecular weight (28 kDa). The second is called Ubc6ep, which displays less similarity to yeast Ubc6p (approximately 25% amino acid identity) and contains a longer C-terminal region containing the transmembrane segment. Both classes localize to the ER and participate in ERAD. It should be noted that in the present report the mouse MmUbc6 is of the Ubc6p class, and the human hUbc6e is of the Ubc6ep class. Therefore, both classes of Ubc6 interact with c-IAP1. We have recently cloned the cDNA of the human Ubc6p homolog from HeLa cells, and have found that mouse Ubc6p and the postulated human Ubc6p cDNA sequence (identical to GenBank accession number NM_058167) are almost identical in amino-acid composition, with more than 97% homology. Ubiquitination assays indicated that Ubc6, but not other E2s tested, has the capacity to potentiate c-IAP1's ubiquitination of both itself and TRAF2. Importantly, this suggests that migration of TRAF2 and c-IAP1 to the ER/nuclear envelope, where the complex can interact with Ubc6, is an essential step in the TNF-R2-mediated TRAF2 ubiquitination/degradation.

Intracellular trafficking of signaling molecules to different destinations may determine the sites of protein action and the fate of these molecules. In the case of ubiquitination, a process that results in the modification of target protein activity or longevity, different subcellular structures are involved in different outcomes. For instance, ligand binding to the IgE receptor induces the phosphorylation and clustering of the receptor in lipid rafts formed on the plasma membrane to which two E3s (Cbl and Nedd4) are recruited, resulting in the ubiquitination of the IgE receptor (Lafont and Simons, 2001). In the EGF signaling pathway, ligand-induced degradation of EGF receptor is mediated by its internalization and recruitment of the E3 c-Cbl to the same endosome (Levkowitz *et al*, 1998). In yeast, protein turnover of the transcriptional repressor Matα2 is much more rapid in the nucleoplasm than in the cytoplasm because the subunits of the cognate E3 are mainly found in the nucleoplasm, and the 26S proteasome required for degradation is concentrated in and around the nucleus (Enenkel *et al*, 1998; Lenk and Sommer, 2000). These examples suggest that intracellular transport and compartmentalization of signaling molecules may be critical for targeted ubiquitination. In addition, selective physical interactions between E2s and E3s may form the basis of specificity for functionally distinct E2:E3 combinations (Kumar *et al*, 1997). TNF-R2-mediated signaling represents another situation, one in which receptor-stimulated translocation of c-IAP1 (E3) and TRAF2 (substrate) to ER/nuclear envelope brings them into proximity with Ubc6 (E2), allowing ubiquitination to occur.

The ER provides protein quality control for newly synthesized and ER-resident proteins via ERAD (Brodsky and McCracken, 1997; Weissman, 2001). This process involves protein ubiquitination, either in the ER membrane or in the cytosol (also known as retrograde ERAD). The substrates for ERAD comprise a wide variety of both misfolded proteins and normally regulated ER proteins (Brodsky and McCracken, 1999). The aggregation (usually detergent-insoluble) of various abnormal proteins that escape ERAD due to either ER dysfunction or proteasome impairment contributes to the pathology of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Imai *et al*, 2001; Paschen and Frandsen, 2001; Shastry, 2003). Ubc6 and Ubc7 are important E2s for the ubiquitination of targeted proteins in ERAD. Specificity in the highly selective Ub–proteasome pathway is achieved by the cooperation of diverse E2s with E3s and other ancillary factors (Weissman, 2001). Mammalian Ubc6 and Ubc7 have been shown to cooperate with a number of different E3s to ubiquitinate distinct substrates for ERAD. For example, Ubc6 and Ubc7 can be used by Parkin to ubiquitinate Pael receptor, which tends to be unfolded and detergent-insoluble. Loss of Parkin's E3 activity results in the accumulation of Pael receptor in the brains of patients with autosomal recessive juvenile Parkinsonism (Imai *et al*, 2001). The tumor autocrine motility factor receptor gp78 can act as a Ub protein ligase that uses Ubc7 but not Ubc6 to ubiquitinate itself and TCR CD3-δ in the ER (Fang *et al*, 2001). Ubc6 is stimulated by PML-RARα to ubiquitinate N-CoR, resulting in a reduction in the level of soluble N-CoR in the nucleus, and perhaps contributing to PML-RARα-mediated leukemogenesis (Khan *et al*, 2004). The data in the present report find that ER-associated ubiquitination is not restricted to the quality control of proteins generated in or resident to the ER. Rather, it is a site of TNF-R2-regulated ubiquitination of a normally cytosolic signaling molecule, TRAF2.

In addition to targeting for proteasomal degradation, TRAF2 ubiquitination has been implicated in the TNF signaling cascade. Ubiquitination of TRAF2 with chains containing a lysine 63 linkage is required for TNF-α-induced JNK activation, and this ubiquitination is dependent on a specific E2 complex, Ubc13/Uev1A (Shi and Kehrl, 2003; Habelhah *et al*, 2004). Therefore, it is conceivable that TRAF2's co-localization with different E2/E3 complexes is important for determining distinct TRAF2 modifications with different functional consequences. It has been suggested that TNF-α stimulation results in Ubc13-dependent ubiquitination and translocation of TRAF2 to a detergent-insoluble membrane/cytoskeletal fraction, which may be important for the propagation of signals for JNK activation (Habelhah *et al*, 2004). In this case, however, nuclei were not removed prior to density separation, and therefore the results are just as compatible with the translocation we have observed to a perinuclear ER-associated compartment. Owing to subsequent degradation, it seems likely that Ub K48 polyubiquitin linkages are formed there. The finding that ΔTRAF2, a functional dominant negative, did not translocate to the perinuclear ER with c-IAP1 raises the interesting question of whether signaling might occur in that compartment as well. Whether TRAF2 K63 linkages, which correlate with alterations of function, are formed in the cytosol or in this compartment and contribute to TRAF2 translocation/activation is yet to be determined.

TRAF2 is not only an indispensable mediator in the activation of JNK by TNF-α (Yeh *et al*, 1997) but also a key player of ER stress-induced JNK activation (Urano *et al*, 2000). Misfolded proteins in the ER induce cellular stress and activate JNK via an ER transmembrane protein kinase IRE1. ER stress causes oligomerization and activation of IRE1 and leads to clustering of TRAF2 bound to the COOH-terminal cytoplasmic portion of the IRE1 (Urano *et al*, 2000). The IRE1–TRAF2 complex initiates signals, such as JNK activation, required for ER stress-induced cell death (Nishitoh *et al*,

2002), and has also been suggested to be responsible for ER stress-induced NF-κB activation (Leonardi *et al*, 2002). In contrast to its antiapoptotic role in TNF-R1-mediated apoptosis, TRAF2 is actually required for TNF-R2 enhancement of TNF-α-induced apoptosis via TNF-R1 (Weiss *et al*, 1998), as it is required in ER-stress-induced cell death. It is possible, therefore, that there may be a signaling and/or functional connection between ER localization of TRAF2 in response to TNF-R2 occupancy and that induced by ER stress.

TNF-α has a remarkable functional duality, inducing both cell apoptosis and cell survival/proliferation. A recent study has suggested that TNF-R1 signaling complexes are dynamic in cellular location and physical composition, and these changes are important for determining whether cells undergo apoptosis or not in response to TNF-α stimulation (Micheau and Tschopp, 2003). The translocation of TRAF2/c-IAP1 complexes to the ER/nuclear envelope in response to TNF-R2 occupancy may represent another mechanism involving the intracellular localization of signaling complexes in determining the consequences of TNF-α signaling, in this case by bringing together the necessary components for ubiquitination.

## Materials and methods

### *Cells, antibodies, and chemicals*

Human kidney embryo fibroblast (293), Cos7, and Jurkat cells were from the American Type Culture Collection (ATCC). Jurkat cells stably transfected with human TNF-R2 (4E3 cells) were kindly provided by Dr Michael Lenardo (NIAID) (Chan and Lenardo, 2000). HeLa cells stably transfected with human TNF-R2 were kindly provided by Dr Harald Wajant (University of Stuttgart, Germany). Rabbit anti-TRAF2, anti-myc, polyclonal rabbit anti-HA, and anti-Ub antibodies were purchased from Santa Cruz, anti-actin from Sigma, mouse anti-TRAF2 and anti-c-IAP1 from BD Pharmingen, anti-calreticulin and anti-PDI from Affinity BioReagents, and anti-TGN46 from Serotec (Oxford, UK). Human TNF-α was purchased from R&D Systems, and MG-132 and lactacystin from Calbiochem.

### *Plasmids*

Expression vectors encoding myc-c-IAP1, myc-c-IAP1-H588A (E3-defective: c-IAP1-mut), GST–c-IAP1, GST–c-IAP1-mut, and HA-TRAF2 have been described (Yang *et al*, 2000; Li *et al*, 2002). pMSCVpuro-TNF-R2 was derived by subcloning TNF-R2 cDNA from pCDM8-TNFRII (Weiss *et al*, 1997) into the pMSCVpuro vector (Clontech) using *Xho*I restriction sites. HA-tagged mouse Ubc6 (HA-MmUbc6) and its mutant (HA-MmUbc6 C91S) in the PCI vector, and myc-tagged mouse MmUbc7 in pcDNA3 were kindly provided by Allan Weissman (NCI) (Tiwari and Weissman, 2001). pcDNA3-myc encoding UbcH6, UbcH7, and UbcH8 were kindly provided by Dr Ryosuke Takahashi (RIKEN Brain Science Institute, Japan). pCMVPLD containing myc-tagged MmUbc6, MmUbcC91S, hUbc6e, or hUbc6e C94S mutant were provided by Dr Thomas Sommer (The Max-Delbruck-Centrum fur Molekulare Medizin, Germany). To generate bacterial expression plasmids encoding His-tagged E2s, MmUbc6, MmUbc6C91S, and MmUbc7, pCI-MmUbc6, pCI-MmUbc6C91S, and pcDNA3-MmUbc7 were digested with *Eco*RI and *Not*I and subcloned into pET28a. For bacterial expression, UbcH7 was subcloned into pET28a by PCR followed by *Eco*RI and *Xho*I digestion.

### *Cell transfection, separation of subcellular compartments, IP, and IB*

Cos7, 293, and HeLa cells were cultured in DMEM (BioSource International) supplemented with 10% fetal calf serum, 250 μg/ml gentamicin, 100 U/ml penicillin, and 4 mM glutamine. Jurkat and 4E3 cells were maintained in RPMI (BioSource) containing the same supplements. Cells were transfected with expression vectors as indicated using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions. Cells were lysed for 30 min on ice in lysis buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, 2 mM EDTA, 0.5% TX, and protease inhibitors. TX-soluble and -insoluble compartments were separated as described (Arch *et al*, 2000). For IP, the TX-soluble fraction was prepared as above and the pellets suspended in the same lysis buffer containing 1% octylglucoside to extract TX-insoluble proteins. Both the TX-soluble and -insoluble samples were subjected to IP, resolved on SDS–PAGE, and immunoblotted with antibodies against the target proteins. Signals were detected by enhanced chemiluminescence following the manufacturer's instructions (Pierce). In some experiments, signals were visualized with a Storm PhosphorImager (Molecular Dynamics) after being developed with $^{125}$I-protein A (ICN).

### *Protein–protein interaction assay*

293 cells were transfected with expression vectors as indicated using Lipofectamine 2000. GST–c-IAP1 fusion proteins were expressed in *E. coli* BL-21 (DE3) (Novagen), and purified from bacterial lysates with glutathione Sepharose (GS) 4B beads (Amersham Pharmacia). The GST- or GST–c-IAP1-bound GS beads were incubated with cell lysates of 293 cells, which were transfected with various myc-tagged E2s, at 4°C for 2 h. After washing, proteins were eluted by boiling beads in SDS sample buffer and analyzed by IB.

### *In vitro ubiquitination assays*

GST–c-IAP1 or GST–c-IAP1-mut expressed from bacteria was immobilized on GS beads. c-IAP1 auto-ubiquitination assays were carried out by adding 100 ng recombinant rabbit E1, 2 μl of bacterial lysate from BL-21 cells transformed with the indicated E2s, and $2 \times 10^4$ cpm of $^{32}$P-labeled Ub in ubiquitination buffer containing 50 mM Tris, pH 7.4, 2 mM ATP, 5 mM $MgCl_2$, and 2 mM DTT. Reactions in 30 μl were incubated for 60 min at 37°C with agitation in an Eppendorf Thermomixer. Reactions were stopped by adding SDS sample buffer and resolved by 8% SDS–PAGE. Ubiquitinated products were detected with a Storm PhosphorImager. For TRAF2 ubiquitination assays, the various bacterially expressed His-tagged E2s were purified using Talon metal affinity beads (Clontech). TRAF2 was *in vitro* translated and metabolically labeled with $^{35}$S-methionine using the TNT kit (Promega). The labeled TRAF2 was bound to GST–c-IAP1 proteins in GST binding buffer as described (Li *et al*, 2002) and incubated with rabbit E1, purified E2s, and unlabeled Ub in ubiquitination buffer for 60 min at 37°C. The material was eluted by heating in SDS sample buffer, resolved by 10% SDS–PAGE, and visualized with a Storm PhosphorImager.

### *Immunofluorescence and confocal microscopy*

Cos7 cells were grown on coverslips and transfected using Fugene 6 (Roche) according to the manufacturer's protocol. 4E3 cells were attached to poly-L-lysine-coated slides before fixation with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min. The fixed cells were permeabilized with 0.5% TX for 5 min and then incubated with primary antibodies for 1 h at room temperature or 18 h at 4°C. For double staining, the antibody incubations were performed sequentially. After washes with PBS, the coverslips were incubated with Oregon Green 488- or AlexaFluor 594-conjugated secondary antibodies. 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI, from Sigma) was used to stain nuclei. Images were obtained either on a Leica or an Ultraview (Perkin-Elmer) confocal microscope using an $\times 63$ or $\times 100$ objective.

## Acknowledgements

We thank Tatiana Karpova and James McNally of the NCI Core Fluorescence Imaging Facility and Barbara Taylor from NCI FACS Core Laboratory for providing access and advice, Allan Weissman (NCI-Fredrick) for helpful advice and critical reading of the manuscript, Shengyun Fang for helpful discussions, and Bei Dong and Tao Li for technical support. We are grateful to all who provided us with cells and constructs.

 

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