3374–3378 (1991).
19. Kuzin, I. I. *et al.* Tetracyclines inhibit activated B cell function. *Int. Immunol.* **13,** 921–931 (2001).
20. Fagarasan, S., Kinoshita, K., Muramatsu, M., Ikuta, K. & Honjo, T. *In situ* class switching and differentiation to IgA-producing cells in the gut lamina propria. *Nature* **413,** 639–643 (2001).
21. Gu, H., Zou, Y. R. & Rajewsky, K. Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. *Cell* **73,** 1155–1164 (1993).
22. Jung, S., Rajewsky, K. & Radbruch, A. Shutdown of class switch recombination by deletion of a switch region control element. *Science* **259,** 984–987 (1993).
23. Xu, L. *et al.* Replacement of germ-line e promoter by gene targeting alters control of immunoglobulin heavy chain class switching. *Proc. Natl Acad. Sci. USA* **90,** 3705–3709 (1993).
24. Zhang, J., Bottaro, A., Li, S., Stewart, V. & Alt, F. W. A selective defect in IgG2b switching as a result of targeted mutation of the Iγ2b promoter and exon. *EMBO J.* **12,** 3529–3537 (1993).
25. Dunnick, W., Hertz, G. Z., Scappino, L. & Gritzmacher, C. DNA sequences at immunoglobulin switch region recombination sites. *Nucleic Acids Res.* **21,** 365–372 (1993).
26. Iwasato, T., Arakawa, H., Shimizu, A., Honjo, T. & Yamagishi, H. Biased distribution of recombination sites within S regions upon immunoglobulin class switch recombination induced by transforming growth factor β and lipopolysaccharide. *J. Exp. Med.* **175,** 1539–1546 (1992).
27. Obata, M. *et al.* Structure of a rearranged γ 1 chain gene and its implication to immunoglobulin class-switch mechanism. *Proc. Natl Acad. Sci. USA* **78,** 2437–2441 (1981).
28. Winter, E., Krawinkel, U. & Radbruch, A. Directed Ig class switch recombination in activated murine B cells. *EMBO J.* **6,** 1663–1671 (1987).
29. Cubitt, A. B. *et al.* Understanding, improving and using green fluorescent proteins. *Trends Biochem. Sci.* **20,** 448–455 (1995).
30. Kitamura, T. New experimental approaches in retrovirus-mediated expression screening. *Int. J. Hematol.* **67,** 351–359 (1998).

Acknowledgements

We are grateful to H. Yagita and Targeted Genetics Co. for providing the HM40-3 monoclonal antibody and the tgCMV/HyTK plasmid, respectively. We thank Y. Sakakibara, K. Ikuta, H. Nagaoka, S. Fagarasan and T. Okazaki for their critical reading of the manuscript; T. Muto, T. Eto and T. Doi for discussions; Y. Tabuchi, T. Toyoshima and K. Yurimoto for their technical assistance; and T. Nishikawa for secretarial help. This work was supported by a Center of Excellence Grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan. I.O. is a research fellow of the Japan Society for the Promotion of Science.

Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to T.H. (e-mail: honjo@mfour.med.kyoto-u.ac.jp).

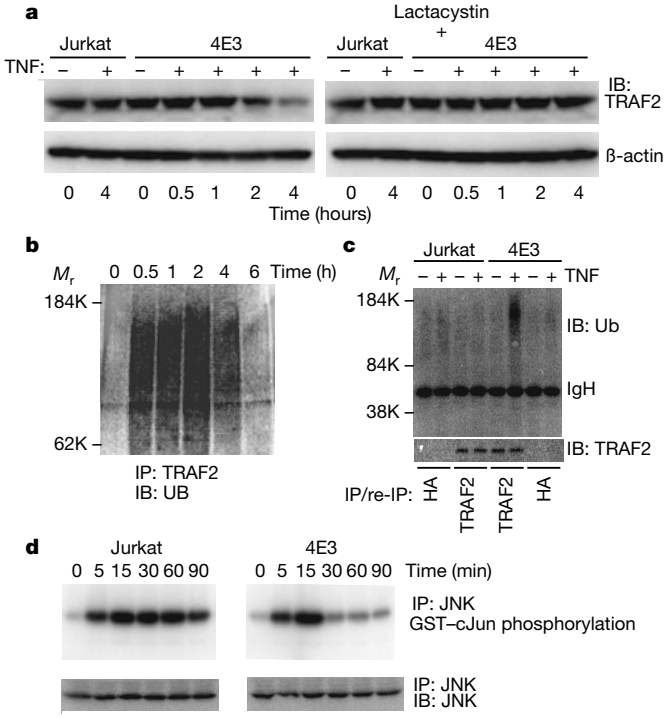

Figure 1 TNF-α induces ubiquitination and proteasome-mediated degradation of TRAF2. **a**, Jurkat and 4E3 cells were cultured in medium (left panel) or pretreated with lactacystin (50 μM) (right panel) for 60 min and stimulated with TNF-α (20 ng ml^{-1}) for the indicated times. Expression of TRAF2 was detected by immunoblotting (IB). **b**, 4E3 cells were pretreated with MG-132 (40 μM) for 30 min and stimulated with TNF-α (50 ng ml^{-1}) for the indicated times, at which the cells were lysed, immunoprecipitated (IP) with anti-TRAF2, and immunoblotted with anti-ubiquitin (Ub). **c**, Jurkat and 4E3 cells were pretreated with MG-132 and stimulated with TNF-α as in **b**. After 2 h the cells were lysed and immunoprecipitation was performed with anti-TRAF2 or and-HA (specificity control). The immunoprecipitated material was eluted from the beads by heating in the presence of 0.5% SDS, and re-immunoprecipitated (re-IP) with the same antibodies. After separation by SDS–PAGE and transfer, the membranes were blotted with anti-ubiquitin (top) or anti-TRAF-2 (bottom). IgH indicates immunoglobulin heavy chain. **d**, Jurkat and 4E3 cells were stimulated with TNF-α for the indicated times. Cell lysates were subjected to immunoprecipitation with anti-JNK antibody. Half of the immunoprecipitated material was used for a kinase assay (upper panel) and half was used for immunoblotting with anti-JNK (lower panels).

TNF-RII and c-IAP1 mediate ubiquitination and degradation of TRAF2

Xiaoming Li, Yili Yang & Jonathan D. Ashwell

Laboratory of Immune Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA

Tumour necrosis factor-α (TNF-α) is a proinflammatory mediator that exerts its biological functions by binding two TNF receptors (TNF-RI and TNF-RII), which initiate biological responses by interacting with adaptor and signalling proteins. Among the signalling components that associate with TNF receptors are members of the TNF-R-associated factor (TRAF) family[1,2]. TRAF2 is required for TNF-α-mediated activation of c-Jun N-terminal kinase (JNK), contributes to activation of NF-κB, and mediates anti-apoptotic signals[3,4]. TNF-RI and TNF-RII signalling complexes also contain the anti-apoptotic ('inhibitor of apoptosis') molecules c-IAP1 and c-IAP2 (refs 5, 6), which also have RING domain-dependent ubiquitin protein ligase (E3) activity[7]. The function of IAPs in TNF-R signalling is unknown. Here we show that binding of TNF-α to TNF-RII induces ubiquitination and proteasomal degradation of TRAF2. Although c-IAP1 bound TRAF2 and TRAF1 *in vitro*, it ubiquitinated only TRAF2. Expression of wild-type c-IAP1, but not an E3-defective mutant, resulted in TRAF2 ubiquitination and degradation. Moreover, E3-defective c-IAP1 prevented TNF-α-induced TRAF2 degradation and inhibited apoptosis. These findings identify a physiologic role for c-IAP1 and define a mechanism by which TNF-RII-regulated ubiquitin protein ligase activity can potentiate TNF-induced apoptosis.

TNF-α induces apoptosis mainly through TNF-RI, which contains an intracellular death domain that recruits death effector adaptor molecules like TRADD and FADD, with the subsequent activation of a caspase cascade[8]. Given that TNF-RII does not contain a death domain and that NF-κB and the IAPs have anti-apoptotic activity[9–12], it is paradoxical that signalling via TNF-RII potentiates the pro-apoptotic effects of concomitant TNF-RI signals[13,14]. Signalling through TNF-RII results in decreased TRAF2 recovery in Triton X-100 detergent lysates. It was initially suggested that this is due to calpain-like protease activity[15], although in a subsequent study inhibition of calpain activity did not prevent the loss of TRAF2 (ref. 16). More recently it was shown that signalling

through the intracellular domain of some TNF-R family members causes redistribution of TRAF2 to a Triton X-100-insoluble compartment[17]. To explore the mechanism of TRAF2 protein down-regulation, Jurkat T cells (which bear TNF-RI but very little TNF-RII) and 4E3 cells (Jurkat T cells stably transfected with TNF-RII) were stimulated with TNF-α, and at different times the cells were lysed in RIPA buffer, which leaves relatively little insoluble material (Fig. 1a). Stimulation of the parent Jurkat cell line had no effect on TRAF2 levels. However, as previously reported after solubulization with Triton X-100 (ref. 16), TNF-α stimulation of 4E3 cells resulted in the progressive loss of TRAF2. No TRAF2 was found in the small amount of RIPA-insoluble material from 4E3 cells, excluding protein partitioning as a cause for the decreased recovery (data not shown). To test the possibility that TRAF2 was being degraded in proteasomes, Jurkat and 4E3 cells were stimulated with TNF-α in the presence of the proteasome inhibitor lactacystin. In this case, no decrease in TRAF2 was observed. The TNF-α-induced decrease in TRAF2 was not prevented by ZVAD (a broad-spectrum caspase inhibitor that prevents apoptosis) or the calpain inhibitor E64d (data not shown). Because proteins are targeted for proteasome-mediated degradation by modification with polyubiquitin chains, 4E3 cells were stimulated with TNF-α in the presence of the proteasome inhibitor MG-132, to allow the accumulation of ubiquitinated species. Within 30 min TNF-α induced a large increase in the amount of polyubiquitinated material immunoprecipitated with anti-TRAF2, with the levels falling 4–6 h after stimulation (Fig. 1b). To address the possibility that the ubiquitinated material might represent proteins co-precipitated with TRAF2 rather than TRAF2 itself, a reprecipitation experiment was performed (Fig. 1c). Jurkat and 4E3 cells were stimulated with TNF-α and after 2 h the

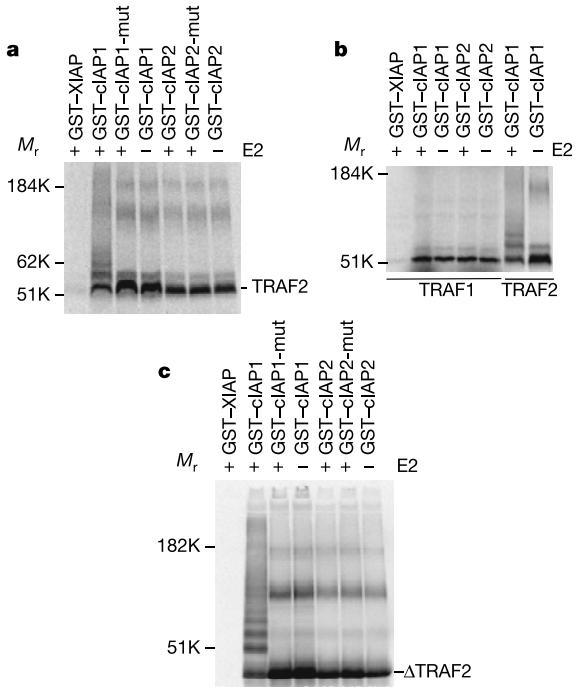

Figure 2 Ubiquitination of TRAFs by IAPs *in vitro*. **a**, c-IAP1 but not c-IAP2 ubiquitinates TRAF2. *In vitro* translated and ^{35}S-labelled TRAF2 was incubated with beads coated with GST–IAP fusion proteins as indicated for 2 h at 4 °C. The beads were washed and subjected to an *in vitro* ubiquitination assay. The bound material was eluted and resolved by SDS–PAGE. One representative experiment of five is shown. **b**, TRAF1 is not a substrate for either c-IAP1 or c-IAP2. ^{35}S-labelled TRAF1 or TRAF2 was incubated with beads coated with the indicated GST–IAP fusion proteins and *in vitro* ubiquitination was performed. Ubiquitination of TRAF2 was used as a positive control. One representative experiment of three is shown. **c**, The RING domain of TRAF2 is dispensable for its ubiquitination by c-IAP1. TRAF2 and ΔTRAF2, which lacks the N-terminal RING domain, were subjected to an *in vitro* ubiquitination assay as above. One representative experiment of three is shown.

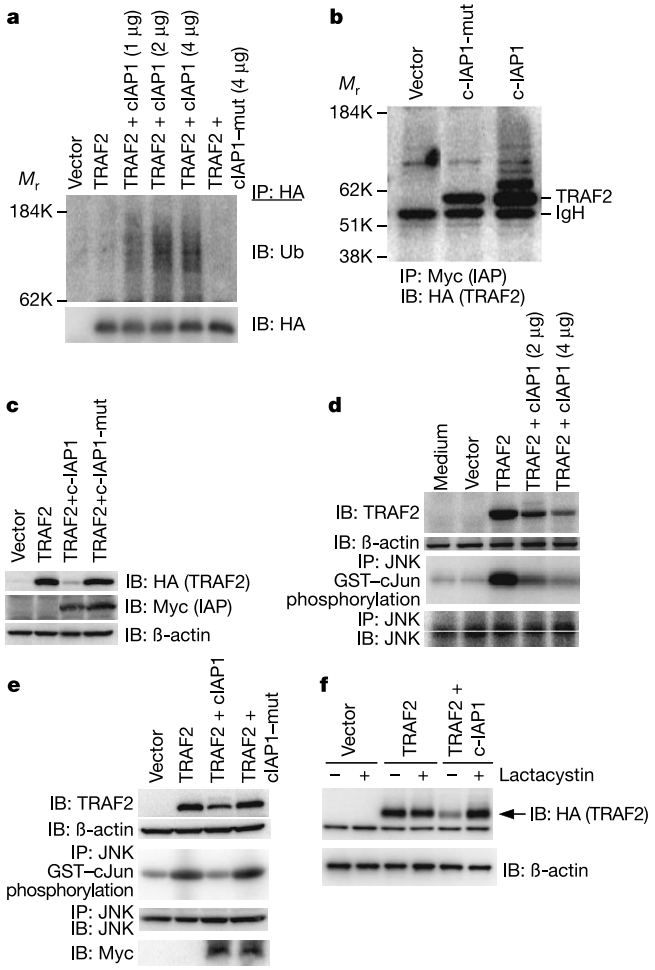

Figure 3 Ubiquitination and proteasome-mediated degradation of TRAF2 by c-IAP1 *in vivo*. **a**, 293 cells were transfected with indicating amounts of expression vectors encoding HA-tagged TRAF2 and Myc-tagged c-IAP1 or c-IAP1-mut (E3-inactive). After 24 h cells were harvested and lysates immunoprecipitated with anti-HA, resolved by SDS–PAGE, and immunoblotted with anti-ubiquitin (upper panel) or anti-HA (lower panel). **b**, 293 cells were transfected with 1 μg of HA-TRAF2 with or without 4 μg of Myc-tagged c-IAP1 or c-IAP-mut. After 20 h, ubiquitin aldehyde (3 μM) was added to inhibit deubiquitination, and 4 h later the cells were lysed, immunoprecipitated with anti-Myc, and immunoblotted with anti-HA to detect c-IAP1-associated HA-TRAF2. **c**, 293 cells were transfected with 1 μg of HA-TRAF2 with or without 4 μg of Myc-tagged c-IAP1 or c-IAP1-mut. After 36 h the samples were split and immunoblotted with anti-HA (to detect TRAF2) or anti-Myc (to detect the IAPs). The membrane used for the anti-HA blot was stripped and reblotted with anti-β-actin antibodies. Results from one of four representative experiments are shown. **c**, 293 cells were transfected with the indicated expression vectors. After lysis, a portion of the material was used for immunoblotting with anti-HA for HA-TRAF2 expression and anti-β-actin, and the rest was subjected to immunoprecipitation with anti-JNK antibodies followed by a JNK kinase assay. The immunoprecipitate was also immunoblotted with anti-JNK. **e**, 293 cells were transfected with the indicated expression vectors and analysed as in **d**, except that anti-Myc was used to detect the transfected IAPs. **f**, 293 cells were transfected with expression vectors encoding HA-TRAF2 and Myc-tagged c-IAP1 as indicated. Twenty-four hours after transfection, lactacystin (50 μM) was added to the medium and 8 h later expression of HA-TRAF2 was assessed by immunoblotting with anti-HA antibodies. β-actin levels were simultaneously measured. One representative experiment of five is shown.

lysates were immunoprecipitated with anti-TRAF2 or anti-haemagglutinin (HA) (nonspecific control) antibodies. The immunoprecipitated material was eluted from the beads, the eluate re-immunoprecipitated with the same antibodies, and the material was then resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotting with anti-ubiquitin antibodies. The TRAF2 isolated from TNF-α-stimulated 4E3 cells, but not Jurkat cells, had high relative-molecular mass (M_r) polyubiquitinated species, demonstrating that TRAF2 itself is ubiquitinated in response to TNF-α signalling. TRAF2 is required for TNF-α-induced JNK activation, leading us to ask whether the loss of TRAF2 in 4E3 cells results in premature termination of JNK activity (Fig. 1d). In both Jurkat and 4E3 cells JNK activity was increased within 5 min, reaching maximum levels at 15 min. In Jurkat cells, however, activity was sustained for up to 90 min, whereas in 4E3 cells it was reduced to near baseline by 30 min. Because stimulation with a TNF-RII agonist, but not with a TNF-RI agonist antibody, caused a decrease in TRAF2 recovery in 4E3 cells[16], and because we observed no TNF-α-induced increase in TRAF2 ubiquitination or decrease in TRAF2 levels in the TRF-RII-deficient parental Jurkat cells, it is likely that induction of degradation is initiated largely if not entirely by TNF-RII occupancy.

IAPs are members of TNF-RII signalling complexes[1,6] and have E3 activity[7,18], leading us to ask whether TRAF2 might be an IAP substrate. Beads coated with glutathione *S*-transferase (GST)–IAP fusion proteins were incubated with *in vitro*-translated and metabolically labelled TRAF2, and after washing were subjected to an *in vitro* ubiquitination assay (Fig. 2a). As expected, TRAF2 was not pulled down by GST–XIAP but was pulled down by GST–cIAP1 and GST–c-IAP2. Furthermore, in the presence but not the absence of an E2, c-IAP1 induced TRAF2 ubiquitination, as indicated by a decrease in the amount of the unmodified M_r 56,000 protein and the appearance of higher-M_r material. A c-IAP1 RING point mutant that has no E3 activity[7] bound TRAF2 but did not cause its ubiquitination. Although GST–c-IAP2 causes its own ubiquitination as potently as GST–c-IAP1 (data not shown) and binds TRAF2 (Fig. 2a), it caused little if any ubiquitination of TRAF2. GST–c-IAP1 and GST-c-IAP2 also bound *in vitro*-translated TRAF1, but unlike TRAF2 this molecule was not ubiquitinated by either IAP (Fig. 2b). TRAF2 itself has a RING domain, whereas TRAF1 does not[19]. To test the possibility that a RING–RING interaction might be required for TRAF2 ubiquitination by c-IAP1, a TRAF2 truncation mutant lacking amino acids 1–86 (ΔTRAF2)[20], and thus the RING domain, was tested as an IAP substrate. ΔTRAF2 behaved identically to wild-type TRAF2, failing to bind GST–XIAP but binding GST–c-IAP1 and GST–c-IAP2, and being ubiquitinated in an E2- and c-IAP1 RING-dependent manner (Fig. 2c). Therefore, the RING of TRAF2 is not required for its ubiquitination by c-IAP1.

To determine if c-IAP1 can ubiquitinate TRAF2 in cells, 293 cells (see Methods) (which express little endogenous TRAF2, data not shown) were co-transfected with a complementary DNA expression vector encoding HA-tagged TRAF2 and expression vectors encoding Myc-tagged c-IAP1 or the E3-inactive Myc-tagged c-IAP1 RING point mutant. TRAF2 immunoprecipitated from cells cotransfected with c-IAP1 was polyubiquitinated, but TRAF2 expressed alone or with E3-inactive c-IAP1 was not (Fig. 3a). The transfected HA-TRAF2 levels were similar amongst the groups, presumably because the amount of antibody used to immunoprecipitate was limiting. Ubiquitination of c-IAP1-associated TRAF2 was also evidenced by the appearance of a ladder of mono- and multiubiquitinated TRAF2 species (Fig. 3b, lane 3). Notably, TRAF2 associated with the E3-inactive c-IAP1 mutant was not ubiquitinated (lane 2). To determine whether TRAF2 levels were affected by c-IAP1, immunoblotting of cell lysates was performed. Corresponding with the appearance of ubiquitinated species, the level of TRAF2 protein decreased in cells cotransfected with wild-type c-IAP1 but not the E3-defective c-IAP1 (Fig. 3c). Expression of TRAF2 alone induces JNK activity[21], and JNK activity was indeed markedly increased in TRAF2-expressing 293 cells (Fig. 3d); co-expression of c-IAP1 resulted in a dose-dependent decrease in TRAF2-dependent JNK specific activity. Expression of the E3-defective c-IAP1 reduced neither TRAF2 levels nor JNK activity (Fig. 3e). Whereas expression of TRAF2 alone in 293 cells was not affected by lactacystin, the marked reduction in its levels caused by c-IAP1 was prevented by this proteasome inhibitor (Fig. 3f). Together, these data indicate that overexpressed c-IAP1 causes proteasome-mediated degradation of TRAF2.

To address the functional importance of c-IAP1-mediated TRAF2 ubiquitination in TNF-α signalling, attempts were made to use E3-defective c-IAP1 as a dominant negative. 4E3 cells were

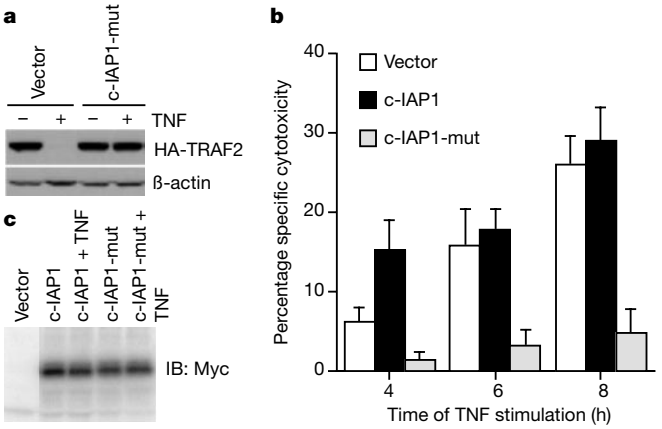

Figure 4 -IAP1-mut blocks TNF-α-induced degradation of TRAF2 and delays apoptosis. **a**, 4E3 cells were transfected with HA-TRAF2 plus empty vector or c-IAP1-mut. Twenty-four hours later, transfectants were stimulated with TNF-α and after 4 h cell lysates were immunoblotted with anti-HA (upper panel). β-actin blotting of the same membrane was performed (lower panel). **b**, 4E3 cells were transfected with a β-galactosidase reporter plasmid with or without expression vectors for Myc-c-IAP1 or Myc-c-IAP1-mut, or the expression vector alone. Twenty-four hours later each group was split in two, with one maintained in medium and the other treated with TNF-α. At the indicated times the per cent specific cytotoxicity of independent transfections was determined by loss of β-galactosidase activity (left). The arithmetic means of three (6 h) and five (4 h and 8 h) independent experiments are shown. Expression of c-IAP1 or c-IAP1-mut did not affect the level of co-transfected β-gal (data not shown). **c**, 4E3 cells transfected as in **b** and cultured for 24 h. The cells were then cultured for an additional 6 h in the absence or presence of TNF-α, after which time they were analysed for expression of Myc-c-IAP1 or Myc-c-IAP1-mut by immunoblotting with anti-Myc. One representative experiment of three is shown. Immunoblotting for β-actin confirmed that equal amounts of protein were loaded between the groups (data not shown).

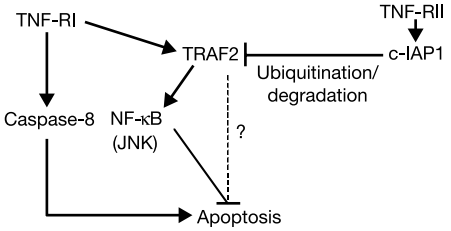

Figure 5 Simple model of TNF-R signalling and regulation by c-IAP1. TNF-RI occupancy induces apoptosis by activating a caspase cascade. TNF-RI also signals through TRAF2, which inhibits apoptosis through activation of NF-κB and/or possibly other mechanisms mediated by TRAF2. Occupancy of TNF-RII induces c-IAP1-mediated ubiquitination and subsequent degradation of TRAF2, thus enhancing TNF-RI-induced apoptosis.

transfected with HA-TRAF2 with or without E3-defective c-IAP1 and treated with TNF-α. Notably, the TNF-α-induced degradation of TRAF2 was prevented by co-expression of the c-IAP1 RING mutant (Fig. 4a). The E3-inactive c-IAP1 mutant also rescued TRAF2 protein expression in 293 cells and HeLa cells transfected with TNF-RII and treated with TNF-α (data not shown). TRAF2 protects cells from TNF-α-induced death[22,23], and thus the prevention of TRAF2 degradation by the E3-defective c-IAP1 might be expected to have a similar effect. Whereas TNF-α did not induce the death of Jurkat cells (ref. 16 and our unpublished observations), TNF-α did kill 4E3 cells (Fig. 4b). Although in some instances c-IAP1 has anti-apoptotic activity by virtue of its BIR-containing amino-terminal region, it did not protect 4E3 cells from TNF-α-induced apoptosis. In fact, overexpression of c-IAP1 had a small but reproducible enhancing effect on TNF cytoxocity, especially at early time points. The E3-inactive c-IAP1 mutant, on the other hand, substantially inhibited TNF-α-induced apoptosis up to 8 h. The amount of cell death converged after this time, being similar among the groups by 12–16 h (data not shown). The levels of transfected c-IAP1 and c-IAP1-mut (RING mutant) in the 4E3 cells were similar and, unlike TRAF2, treatment with TNF-α did not cause an appreciable change in c-IAP1 or c-IAP1-mut levels (Fig. 4c). Thus, E3-inactive c-IAP1 appears to act in a dominant-negative fashion to prevent TNF-RII-mediated and TNF-α-induced down-regulation of TRAF2 and delay cell death. In preliminary studies we have found that unlike c-IAP1, c-IAP2 inhibits TNF-α-induced apoptosis of 4E3 cells (see Supplementary Information), consistent with its failure to ubiquitinate TRAF2 *in vitro*. That the enhancement of TNF-induced death by c-IAP1 was small presumably indicates that the level of endogenous c-IAP1 in 4E3 cells is not limiting for TRAF2 ubiquitination and degradation (see Fig. 1).

TRAFs are critical mediators of the cell activation and anti-apoptotic functions of the TNF-R superfamily. In particular, TRAF2 is important in protection from apoptosis, as is clear from the observation that cells from TRAF2-deficient mice are overly sensitive to TNF-α-induced apoptosis[4]. Although TNF-RII couples to the pro-survival TRAF2/NF-κB signalling pathway, cells from mice deficient in TRNF-II are actually less sensitive to TNF-α-induced apoptosis[24]. Furthermore, TNF-RII is required for TNF-α-induced apoptosis of normal T cells[13,14], and the binding of TRAF2 to TNF-RII is required for the potentiation of TNF-RI killing[22,23]. The data in the present study support a mechanism in which TNF-RII occupancy causes the ubiquitination of TRAF2 by c-IAP1 (Fig. 5). It is also possible that there are other as yet unidentified anti-apoptotic c-IAP1 substrates. We note that RING-less c-IAP1 was effective, while the full-length molecule was not, in preventing apoptosis induced by Sindbis virus, and that a carboxy-terminal (RING-containing) fragment of c-IAP1 actually induced apoptosis when expressed in cell lines[25]. In addition, other non-death-domain members of the TNF-R superfamily, such as CD30 and CD40, may share the ability to cause TRAF2 degradation, and in the latter case this has been reported to be proteasome-dependent[26,27]. Finally, mice lacking TNF-RII have enhanced TNF-RI-dependent inflammatory responses[28], consistent with a role for TNF-RII in decreasing TRAF2 levels. The results in this report indicate that the ubiquitin protein ligase c-IAP1 dynamically regulates the level and therefore function of TRAF2 in response to TNF-RII occupancy, and paradoxically can play a pro-apoptotic role in TNF-α signalling. □

Methods

Cell lines and reagents

Human embryonic kidney fibroblast 293 (293 cells), HeLa and Jurkat cells were obtained from the American Type Culture Collection. 4E3 cells were generated by stable transfection of Jurkat cells with human TNF-RII cDNA[16], provided by M. Lenardo. Constructs encoding GST–XIAP, GST–c-IAP1, and their mutant forms were generated as described[7]. GST–c-IAP2 and GST–c-IAP2-mut (encoding c-IAP2 residues 1–382, and thus lacking the C-terminal RING domain) were made by cloning the corresponding cDNAs into pGEX4T-2 with *Bam*HI and *Not*I sites. Plasmids used for *in vitro* translation and/or *in vivo* expression of human TRAFs were pBluescript KS(-)-TRAF1, pBluescript KS(-)-TRAF2, pCDNA3-ΔTRAF2, and pCDNA3-HA-tagged TRAF2 (provided by C. Duckett). Human c-IAP1, c-IAP1(H588A) (c-IAP1-mut), XIAP, and XIAP His(467) (XIAP-mut) were Myc-tagged at the 3′ end and cloned into pCDNA3 as described[7]. pCDNA3-TNF-RII cDNA plasmid was provided by P. Scheurich. The pCMV.SPORT-βgal reporter plasmid was purchased from GIBCO/BRL. Wheat E1 and the human E2 UbcH5B were expressed in *Escherichia coli*[7]. Ubiquitin was purchased from Sigma. To obtain radiolabelled ubiquitin, a GST–ubiquitin fusion protein (the construct provided by A. Weissman) was expressed in *E. coli*, and isolated protein phosphorylated with ^{32}P-γ-ATP. The GST-c-Jun (1–79) construct was provided by Z. Liu. Anti-Myc and anti-HA (mouse, immunoglobulin-γ1) monoclonal antibodies were derived from mouse hybridoma cell lines 12CA5 and 9E10, respectively. The anti-TRAF2 rabbit antiserum (C-20), and anti-TRAF2 monoclonal antibody (mouse, IgG1), an anti-ubiquitin monoclonal antibody (mouse, IgG1), and anti-JNK1 monoclonal antibodies were purchased from Santa Cruz Biotechnology. MG-132, lactacystin and ubiquitin aldehyde were purchased from CalBiochem. Human TNF-α was purchased from R&D Systems.

Cell cultures and transient transfections

293 cells and HeLa cells were grown in DMEM (BioSource) containing 10% heat-inactivated fetal calf serum, antibiotics, 4 mM glutamine, and 5×10^{-5} M β-mercaptoethanol. Jurkat and 4E3 cells were cultured in RPMI-1640 (BioSource) supplemented as above. Cells were transfected with the indicated expression vectors using Lipofectamine 2000 (GIBCO/BRL) according to the manufacturer's instructions.

Immunoprecipitation and immunoblotting

Cells were lysed in RIPA buffer (1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 0.5 mM AEBSF, leupeptin (20 μg ml^{-1}), and aprotinin (20 μg ml^{-1}) on ice. In some experiments the lysate was resolved on 10% SDS–PAGE, in others immunoprecipitation with the indicated antibodies was performed and, after boiling the beads, the eluted material was resolved by 8% or 10% SDS–PAGE. After transfer to nitrocellulose (Novex), the membranes were blotted with the indicated antibodies. For most experiments, blots were developed with ^{125}I-protein A (ICN) and visualized with a Storm PhosphorImager (Molecular Dynamics). In some experiments, visualization of immunoblots was performed by enhanced chemiluminescence (Amersham Pharmacia) according to the manufacturer's instructions.

In vitro ubiquitination assay

GST–IAP fusion proteins were expressed in *E. coli* BL-21 (DE3) (Novagen), and purified from bacterial lysates with glutathione Sepharose 4B beads (Amersham Pharmacia). Putative IAP substrates were translated *in vitro* and metabolically labelled with ^{35}S-methionine using the TNT kit (Promega). The labelled substrates were incubated with GST–IAP fusion proteins in GST-binding buffer (120 mM NaCl, 10% glycerol, 1% Triton X-100, 50 mM Tris, pH 7.5) at 4 °C for 2 h, after which time the beads were washed and the material bound to the GST fusion proteins was incubated in ubiquitination reaction buffer (50 mM Tris, pH 7.4, 0.2 mM ATP, 0.5 mM $MgCl_2$, 0.1 mM dithiothreitol (DTT), 1 mM creatine phosphate, 15 units of creatine phosphokinase) containing E1 (20 ng), E2 (20 ng), and 1 μg of unlabelled ubiquitin at 30 °C[29] for 1 h. The eluted material resolved by SDS–PAGE and visualized with a Storm PhosphorImager.

Determination of JNK activity

Activation of endogenous JNK was evaluated using GST-c-Jun (1–79) as a specific substrate. Briefly, JNK was immunoprecipitated from the lysates of Jurkat or 4E3 cells stimulated with TNF-α for the indicated times or 293 cells that had been transfected with HA-TRAF2 and the indicated amounts of Myc-tagged c-IAP1, and kinase reactions carried out at 30 °C for 30 min. The samples were boiled and the eluted material was resolved by 12.5% SDS–PAGE and visualized with a Storm PhosphorImager.

Quantitation of cell death

Death of transfected 4E3 cells was determined by release of β-galactosidase as described[30]. Briefly, 4E3 cells were transfected with pCMV.SPORT-βgal reporter plasmid (0.25 μg) with or without c-IAP1 (2.5 μg) or c-IAP1-mut (2.5 μg) expression vectors. After 24 h, the cells were split into two groups, one cultured without and one cultured with TNF-α (50 ng ml^{-1}), and at the indicated times the cells were lysed and β-galactosidase activity was measured. TNF-α had no effect on β-galactosidase levels when the cells were treated in the presence of Z-Val-Ala-Asp(O-methyl)-fluoromethylketone (ZVAD; Enzyme Systems Products) to prevent apoptosis (data not shown). Cell death was calculated with the formula: per cent specific cytotoxicity = 100 - [(experimental β-gal/medium β-gal) × 100].

Received 30 October; accepted 9 January 2001.

1. Rothe, M., Pan, M. G., Henzel, W. J., Ayres, T. M. & Goeddel, D. V. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. *Cell* **83,** 1243–1252 (1995).
2. Hsu, H., Shu, H. B., Pan, M. G. & Goeddel, D. V. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell* **84,** 299–308 (1996).
3. Lee, S. Y. *et al.* TRAF2 is essential for JNK but not NF-κB activation and regulates lymphocyte proliferation and survival. *Immunity* **7,** 703–713 (1997).
4. Yeh, W. C. *et al.* Early lethality, functional NF-κB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. *Immunity* **7,** 715–725 (1997).
5. Shu, H. B., Takeuchi, M. & Goeddel, D. V. 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 (1996).

6. Chan, F. K., Siegel, M. R. & Lenardo, J. M. Signaling by the TNF receptor superfamily and T cell homeostasis. *Immunity* **13,** 419–422 (2000).
7. Yang, Y., Fang, S., Jensen, J. P., Weissman, A. M. & Ashwell, J. D. Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science* **288,** 874–877 (2000).
8. Tschopp, J., Martinon, F. & Hofmann, K. Apoptosis: Silencing the death receptors. *Curr. Biol.* **9,** R381–R384 (1999).
9. Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R. & Verma, I. M. Suppression of TNF-α-induced apoptosis by NF-κB. *Science* **274,** 787–789 (1996).
10. Liu, Z. G., Hsu, H., Goeddel, D. V. & Karin, M. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. *Cell* **87,** 565–576 (1996).
11. Deveraux, Q. L. *et al.* IAPs block apoptotic events induced by caspase-8 and cytochrome *c* by direct inhibition of distinct caspases. *EMBO J.* **17,** 2215–2223 (1998).
12. Roy, N., Deveraux, Q. L., Takahashi, R., Salvesen, G. S. & Reed, J. C. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *EMBO J.* **16,** 6914–6925 (1997).
13. Sarin, A., Conan-Cibotti, M. & Henkart, P. A. Cytotoxic effect of TNF and lymphotoxin on T lymphoblasts. *J. Immunol.* **155,** 3716–3718 (1995).
14. Zheng, L. *et al.* Induction of apoptosis in mature T cells by tumour necrosis factor. *Nature* **377,** 348–351 (1995).
15. Duckett, C. S. *et al.* A conserved family of cellular genes related to the baculovirus iap gene and encoding apoptosis inhibitors. *EMBO J.* **15,** 2685–2694 (1996).
16. Chan, F. K. & Lenardo, M. J. A crucial role for p80 TNF-R2 in amplifying p60 TNF-R1 apoptosis signals in T lymphocytes. *Eur. J. Immunol.* **30,** 652–660 (2000).
17. Arch, R. H., Gedrich, R. W. & Thompson, C. B. Translocation of TRAF proteins regulates apoptotic threshold of cells. *Biochem. Biophys. Res. Commun.* **272,** 936–945 (2000).
18. Huang, H. *et al.* The inhibitor of apoptosis, cIAP2, functions as a ubiquitin-protein ligase and promotes *in vitro* ubiquitination of caspases-3 and -7. *J. Biol. Chem.* **275,** 26661–26664 (2000).
19. Rothe, M., Wong, S. C., Henzel, W. J. & Goeddel, D. V. A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell* **78,** 681–692 (1994).
20. Rothe, M., Sarma, V., Dixit, V. M. & Goeddel, D. V. TRAF2-mediated activation of NF-κB by TNF receptor 2 and CD40. *Science* **269,** 1424–1427 (1995).
21. Reinhard, C., Shamoon, B., Shyamala, V. & Williams, L. T. Tumor necrosis factor α-induced activation of c-jun N-terminal kinase is mediated by TRAF2. *EMBO J.* **16,** 1080–1092 (1997).
22. Weiss, T. *et al.* Enhancement of TNF receptor p60-mediated cytotoxicity by TNF receptor p80: requirement of the TNF receptor-associated factor-2 binding site. *J. Immunol.* **158,** 2398–2404 (1997).
23. Weiss, T. *et al.* TNFR80-dependent enhancement of TNFR60-induced cell death is mediated by TNFR-associated factor 2 and is specific for TNFR60. *J. Immunol.* **161,** 3136–3142 (1998).
24. Erickson, S. L. *et al.* Decreased sensitivity to tumour-necrosis factor but normal T-cell development in TNF receptor-2-deficient mice. *Nature* **372,** 560–563 (1994).
25. Clem, R. J. *et al.* c-IAP1 is cleaved by caspases to produce a pro-apoptotic C-terminal fragment. *J. Biol. Chem.* **276,** 7602–7608 (2000).
26. Duckett, C. S. & Thompson, C. B. CD30-dependent degradation of TRAF2: implications for negative regulation of TRAF signaling and the control of cell survival. *Genes Dev.* **11,** 2810–2821 (1997).
27. Brown, K. D., Hostager, B. S. & Bishop, G. A. Differential signaling and tumor necrosis factor receptor-associated factor (traf) degradation mediated by CD40 and the epstein-barr virus oncoprotein latent membrane protein 1 (Imp1). *J. Exp. Med.* **193,** 943–954 (2001).
28. Peschon, J. J. *et al.* TNF receptor-deficient mice reveal divergent roles for p55 and p75 in several models of inflammation. *J. Immunol.* **160,** 943–952 (1998).
29. Lorick, K. L. *et al.* RING fingers mediate ubiquitin conjugating enzyme (E2)-dependent ubiquitination. *Proc. Natl Acad. Sci. USA* **96,** 11364–11369 (1999).
30. Memon, S. A., Petrak, D., Moreno, M. B. & Zacharchuk, C. M. A simply assay for examining the effect of transiently expressed genes on programmed cell death. *J. Immunol. Methods* **180,** 15–24 (1995).

Supplementary Information accompanies the paper on *Nature*'s website (http://www.nature.com).

Acknowledgements

We are grateful to A. Weissman, C. Duckett, and Z. Liu for provision of reagents and reviews of this manuscript. X.L. is a visiting fellow from Bethune International Hospital, China.

Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to J.D.A. (e-mail: jda@pop.nci.nih.gov).

correction

Effects of experience and social context on prospective caching strategies by scrub jays

N. J. Emery & N. S. Clayton

Nature **414,** 443–446 (2001).

The exact number of species of American jays is a matter of debate[1]. This is particularly evident within the scrub-jay lineage of *Aphelocoma*[2]. Traditionally, *A. coerulescens* included the Florida scrub-jay and the western scrub-jay, among others. It has recently been brought to our attention that the Committee on Classification and Nomenclature of the American Ornithologists' Union now use *A. coerulescens* to refer exclusively to the Florida scrub-jay and *A. californica* to refer to the western scrub-jay[2]. The birds used in the Emery & Clayton[3] and Clayton & Dickinson[4] studies were western scrub-jays: *A. californica*, not *A. coerulescens*. We therefore wish to correct the oversight in this paper and our other papers using these birds, and apologize for any confusion that has arisen. We thank R. Curry, T. Langen and G. Woolfenden for alerting us to the change in nomenclature. □

1. Madge, S. & Burn, H. *Crows and Jays. A Guide to the Crows, Jays and Magpies of the World* (Houghton Mifflin, New York, 1999).
2. American Ornithologists' Union *Check-list of North American Birds* 7th edn (American Ornithologists' Union, Washington DC, 1998).
3. Emery, N. J. & Clayton, N. S. Effects of experience and social context on prospective caching strategies by scrub jays. *Nature* **414,** 443–446 (2001).
4. Clayton, N. S. & Dickinson, A. Episodic-like memory during cache recovery by scrub jays. *Nature* **395,** 272–278 (1998).

erratum

Tyrannosaurus was not a fast runner

John R. Hutchinson & Mariano Garcia

Nature **415,** 1018–1021 (2002).

In this Letter, the row in Table 1 corresponding to '*Gallus*, m_i ($\%m_{body}$)' should read: 1.1, 0.08, 2.0 and 1.5 for the hip, knee, ankle and toe respectively. □