Cell Death and Differentiation (2002) 9, 138–144

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# c-FLIP efficiently rescues TRAF-2$^{-/-}$ cells from TNF-induced apoptosis

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Received 2.4.01; revised 2.7.01; accepted 13.8.01
Edited by P Krammer

## Abstract

**For the past 20 years, it has been known that preparations of Tumor Necrosis Factor α (TNF) fail to induce apoptosis due to cytoprotective responses that render cells resistant to its cytotoxic activity. Here we show that TRAF-2$^{-/-}$ embryonic fibroblasts express reduced levels of the anti-apoptotic molecule c-FLIP due to extensive degradation of the protein. Reconstitution of TRAF-2$^{-/-}$ EF with c-FLIP is sufficient for resistance to TNF toxicity. Our results strengthen the role of c-FLIP in protecting cells from the cytotoxic effect of TNF and have implication for the treatment of inflammatory and proliferative disorders.**

*Cell Death and Differentiation* (2002) **9,** 138–144. DOI: 10.1038/sj/cdd/4400947

**Keywords:** TNF; apoptosis: c-FLIP; TRAF-2

**Abbreviations:** TNF, Tumor Necrosis Factor α; JNK, c-Jun N-Terminal Kinase 1; Z-L3VS, Carbxybenzyl-Leucyl-Leucyl-Leucine Vinyl Sulfone

## Introduction

TNF is a multifunctional cytokine that plays a significant role in the regulation of inflammation, one of the most important aspects of the host immune response.[1] However, excessive levels of TNF contribute to a variety of diseases such as septic shock, vascular thrombosis and extensive tissue cell death. A delicate molecular mechanism has evolved that protects normal cells from the cytotoxic effects of TNF. As a consequence, induction of apoptosis by TNF occurs efficiently only in the presence of inhibitors of protein synthesis, such as cycloheximide. Programmed cell death following TNF exposure also takes place in cells lacking molecules involved in signal transduction pathways activated by the TNF receptor complex. These include the RelA subunit of NF-κB transcription factor,[2–5] the serine/threonine kinase RIP, which is required for NF-κB activation upon TNF stimulation,[6] and TRAF-2, which is involved in the TNF-mediated activation of c-Jun N-terminal Kinase 1 (JNK).[7]

The signaling property of TNF receptor I is controlled by a series of modular interactions, which recruit to the receptor complex several signal transducer molecules, including TRADD, FADD and TRAF-2. Whereas FADD links the TNF receptor to apoptotic proteases, TRAF-2 has been implicated in both transcriptional-independent and transcriptional-dependent cytoprotective pathways.[8,9]

The FLICE-inhibitory protein c-FLIP, identified by several groups and variously named,[10–17] shares structural and sequence homology to caspase-8, a cysteine protease essential for cell death induced by TNF, Fas/Apo1 and DR3.[18] c-FLIP suppresses death receptors-induced apoptosis by inhibiting activation of caspases.[10,12,14–16] Similarly to RelA$^{-/-}$, RIP$^{-/-}$ and TRAF-2$^{-/-}$, c-FLIP$^{-/-}$ EF are also sensitive to cell death induced by TNF.[19] Here we show that c-FLIP expression is reduced in TRAF-2-deficient cells and re-introduction of c-FLIP in these cells restores resistance to TNF-induced apoptosis.

## Results and Discussion

The six mammalian protein TRAFs (TRAF 1–6) identified to date have been shown to possess distinct affinities for different members of the TNF Receptor gene superfamily. These proteins have been suggested to play a critical role in regulating signaling transduction pathways leading to activation of c-Jun and NF-κB transcription factor which, in turn, mediate transcription of genes promoting cell survival and proliferation. The TRAF-2$^{-/-}$ phenotype is characterized by sensitivity to TNF-induced cell death and reduction of JNK activation upon TNF stimulation.[7] As shown in Figure 1A, NF-κB activation in response to TNF stimulation is not impaired in TRAF-2$^{-/-}$ EF,[7] as assessed by phosphorylation and subsequent degradation of I-κB. We also examined the effect of the *TRAF-2* null mutation on the activity of other signaling pathways elicited by TNF stimulation. In the absence of TRAF-2, activation of p38 and ERK1/2 MAP kinases proceeds normally following TNF treatment (Figure 1A), whereas activation of JNK is strongly reduced (ref.[7] and Figure 1B). The defect in JNK is specific to the TNF signaling pathway, because other signals, such as IL-1 or anisomycin, evoke normal JNK activation (ref.[7] and Figure 1B).

To exclude the possibility that the sensitivity of TRAF-2$^{-/-}$ EF to TNF-induced apoptosis was an artifact of the *in vitro* culture conditions, we reconstituted these fibroblasts with retroviral constructs expressing TRAF-2 or a GFP control protein. As expected, TRAF-2$^{-/-}$ EF reconstituted with the cDNA encoding for TRAF-2, but not the GFP control, become resistant to TNF-induced cell death (Figure 2A,B). On the other hand, a dominant negative version of TRAF-2 (TRAF-

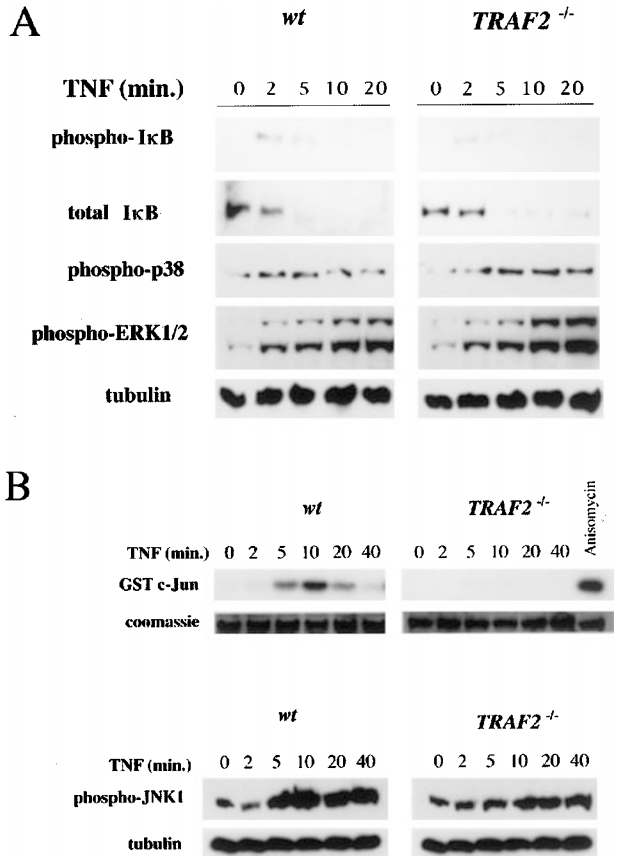



**Figure 1** (**A**) Activation of NF-κB, p38 and ERKs in TRAF-2$^{-/-}$ fibroblast. wt or TRAF-2$^{-/-}$ EF were stimulated with TNF (40 ng/ml) for the indicated time periods. Cells were then collected, lysed in RIPA buffer and examined by immunoblot analysis using the indicated phospho-specific (phospho-I-κB, phospho-p38 and phospho-ERK1/2) and phosphorylation state-independent antibodies (total I-κB and β-tubulin). (**B**) Impaired activation of JNK in TRAF-2$^{-/-}$ EF. wt or TRAF-2$^{-/-}$ EF were stimulated with TNF (40 ng/ml) for the indicated time periods. Cells were then collected, lysed and either subjected to immunoprecipitation with an antibody against JNK1 followed by *in vitro* kinase reaction using recombinant GST-c-Jun as substrate (upper panel); or (lower panel) analyzed by immunoblot using an anti-pan-phospho-JNKs. For loading control, recombinant GST-c-Jun was stained by coomassie in the *in vitro* kinase assay; anti-β-tubulin was used in the immunoblot experiments

$2^{266-501}$), which inhibits TNF-induced activation of JNK, was unable to protect TRAF-2$^{-/-}$ EF from TNF treatment. Other molecules of the TRAF family, such as TRAF-1 and TRAF-4, also failed to protect these cells from the cytotoxic activity of TNF (Figure 2C). Altogether, these experiments suggested that activation of JNK could be critical for protection from TNF cytotoxicity. To test for this, TRAF-2$^{-/-}$ EF were infected with retroviral constructs encoding for constitutive active forms of MEKK1 and MKK7, which are upstream activators of JNK. Forty-eight hours after infection, infected cells were treated with TNF and cellular viability was determined with a colorimetric assay. The results shown in Figure 2D indicate that, despite activation of JNK, TRAF-2$^{-/-}$ EF are still sensitive to TNF. We also observed that although JNK is normally activated in TRAF-2$^{-/-}$ EF following IL-1 stimulation, co-treatment of IL-1 and TNF does not restore resistance to TNF cytotoxicity (Figure 2D). From these experiments, we concluded that impaired activation of JNK might not be responsible for TRAF-2$^{-/-}$ sensitivity to TNF.

We next examined the expression level of molecules that might be involved in regulation of apoptosis in TRAF-2$^{-/-}$ EF upon exposure to TNF. In a panel of immunoblot experiments, we observed that these cells express lower amount of full-length c-FLIP, along with degradation products of the protein (Figure 3A). The specificity of the monoclonal antibody used for detection of c-FLIP in these experiments was assayed on cell lysates made from 293 cells transfected with a flagged version of c-FLIP. Both anti-c-FLIP and anti-FLAG antibodies recognize c-FLIP as single band migrating at approximately 56 kD (Figure 3B). Northern blot experiments revealed comparable transcriptional levels of c-FLIP in wt and TRAF-2$^{-/-}$ EF (Figure 3C), indicating that the reduction of c-FLIP observed in TRAF-2$^{-/-}$ cells is due to post-transcriptional mechanisms. To examine this possibility, pulse-chase experiments were carried out to determine the half-life of c-FLIP in TRAF-2$^{-/-}$ and wild type cells. wt and TRAF-2$^{-/-}$ EF were pulse-labeled for 2 h and chased for the indicated time periods in the presence of excess amounts of unlabeled amino-acids. Results shown in Figure 3D revealed a shorter life-span for c-FLIP in TRAF-2$^{-/-}$ EF. To further characterize this process of c-FLIP destabilization in the absence of TRAF-2, TRAF-2$^{-/-}$ EF were cultured in media supplemented with several protease inhibitors, including proteasome inhibitors, and the expression level of c-FLIP was determined by immunoblot assay. Data shown in Figure 3E indicate that none of the inhibitors tested in this assay was able to rescue the degradation of c-FLIP in TRAF-2$^{-/-}$.

The observation that TRAF-2$^{-/-}$ cells express lower amount of c-FLIP prompted us to investigate whether reconstitution of these cells with a retroviral construct expressing c-FLIP restores resistance to TNF-induced apoptosis. Since it has been reported that exaggerated expression of c-FLIP in fact promotes cell death in different cell types (Ref.[10,11,13,17] and data not shown), cell lysates from TRAF-2$^{-/-}$ EF were examined for c-FLIP expression by immunoblot assay 48 h after viral infection. Figure 4A shows that retrovirus-mediated reconstitution of TRAF-2$^{-/-}$ EF with c-FLIP cDNA results in TRAF-2$^{-/-}$ EF expressing c-FLIP at moderately higher level compared to wt cells. This level of expression of c-FLIP was not toxic to TRAF-2$^{-/-}$ EF, since no abnormal apoptosis was observed in these cultures (data not shown). Conversely, TRAF-2$^{-/-}$ EF reconstituted with c-FLIP were completely resistant to TNF-induced cell death (Figure 4B,C).

We next investigated the point at which TNF-induced cell death is suppressed in TRAF-2$^{-/-}$ EF reconstituted with c-FLIP. After 2 h of stimulation of TRAF-2$^{-/-}$ EF with TNF, both processing of pro-caspase-8 into the p20 fragment and proteolitic activity of caspase-8 were observed (Figure 4D,E). In the same condition, no evidence for pro-caspase-8 processing and activity was observed in wt cells. In contrast, processing and activity of pro-caspase-8 were suppressed in TRAF-2$^{-/-}$ cells reconstituted with c-FLIP cDNA, but not in the GFP-infected cells. Thus, in TRAF-2$^{-/-}$ EF expression level of FLIP is critical for regulation of caspase-8 activation and subsequent triggering of the cell death machinery.

Over the past two decades, many researchers have experienced that most normal and transformed cell types

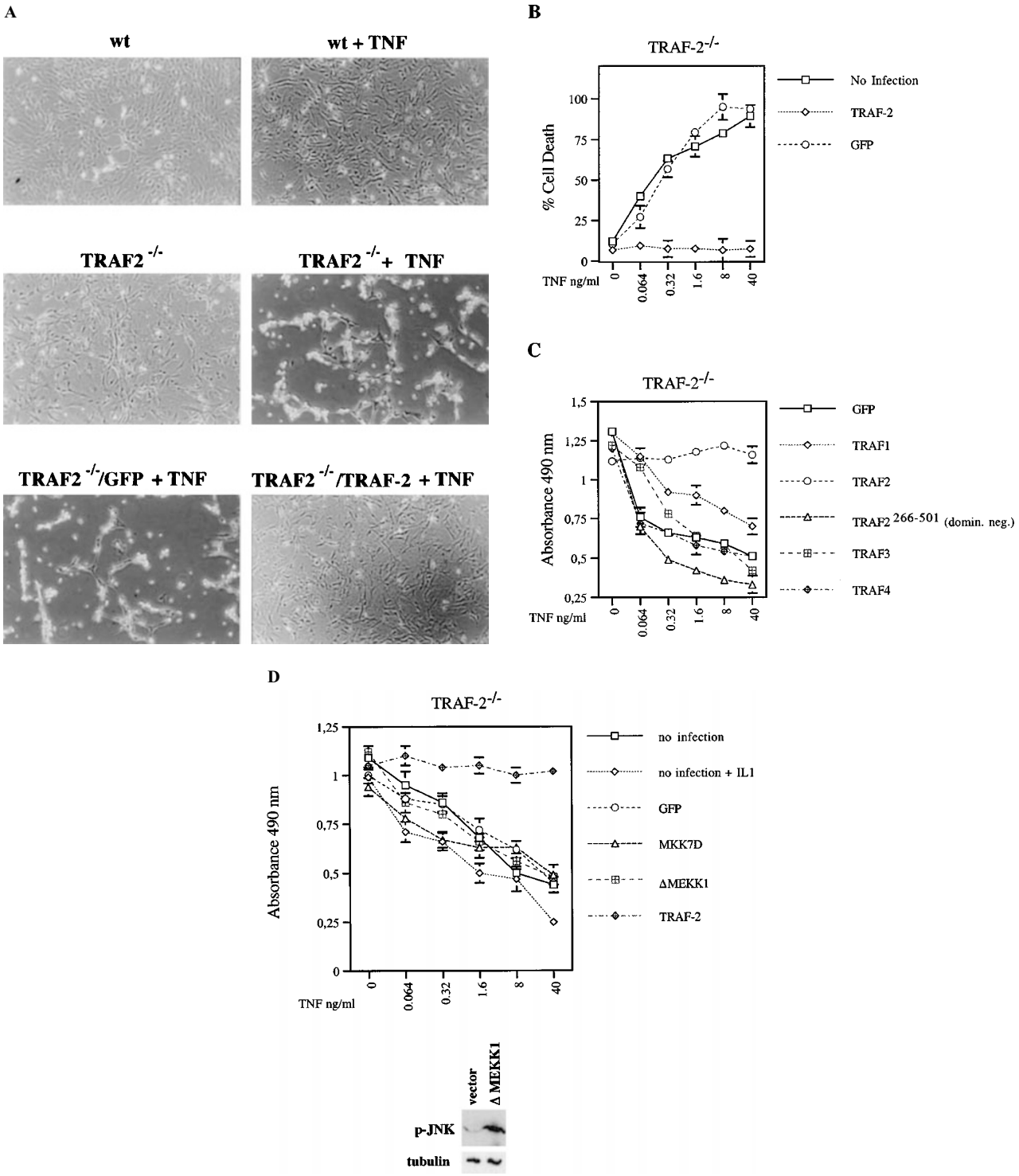



**Figure 2** (**A**) Phase-contrast micrographs of wt (upper panel), TRAF-2$^{-/-}$ (middle panel), or TRAF-2$^{-/-}$ fibroblast reconstituted with TRAF-2 cDNA or GFP (lower panel). Cells were left untreated or treated with TNF (where indicated, 20 ng/ml) for 16 h. (**B,C**) Reconstitution of TRAF-2$^{-/-}$ EF. Retroviral supernatants expressing the indicated cDNAs were used to infect TRAF-2$^{-/-}$ EF. Forty-eight hours after infection, cells were treated with TNF at the indicated concentration for 12–16 h and cell viability was then determined by trypan blue exclusion (**B**) or with an enzymatic assay (**C**). Data shown in (**C**) is representative of at least 20 independent triplicate experiments. (**D**) TRAF-2$^{-/-}$ EF uninfected or infected with retroviral supernatants expressing the indicated cDNAs were treated for 12–16 h with TNF at the indicated concentration alone or together with IL-1 (20 ng/ml) where indicated. Cell viability was then determined with an enzymatic assay. Activation of JNK upon expression of ΔMEKK1 was assessed by immunoblot using an antibody against phospho-JNK. Data is representative of at least 10 independent experiments done in triplicate

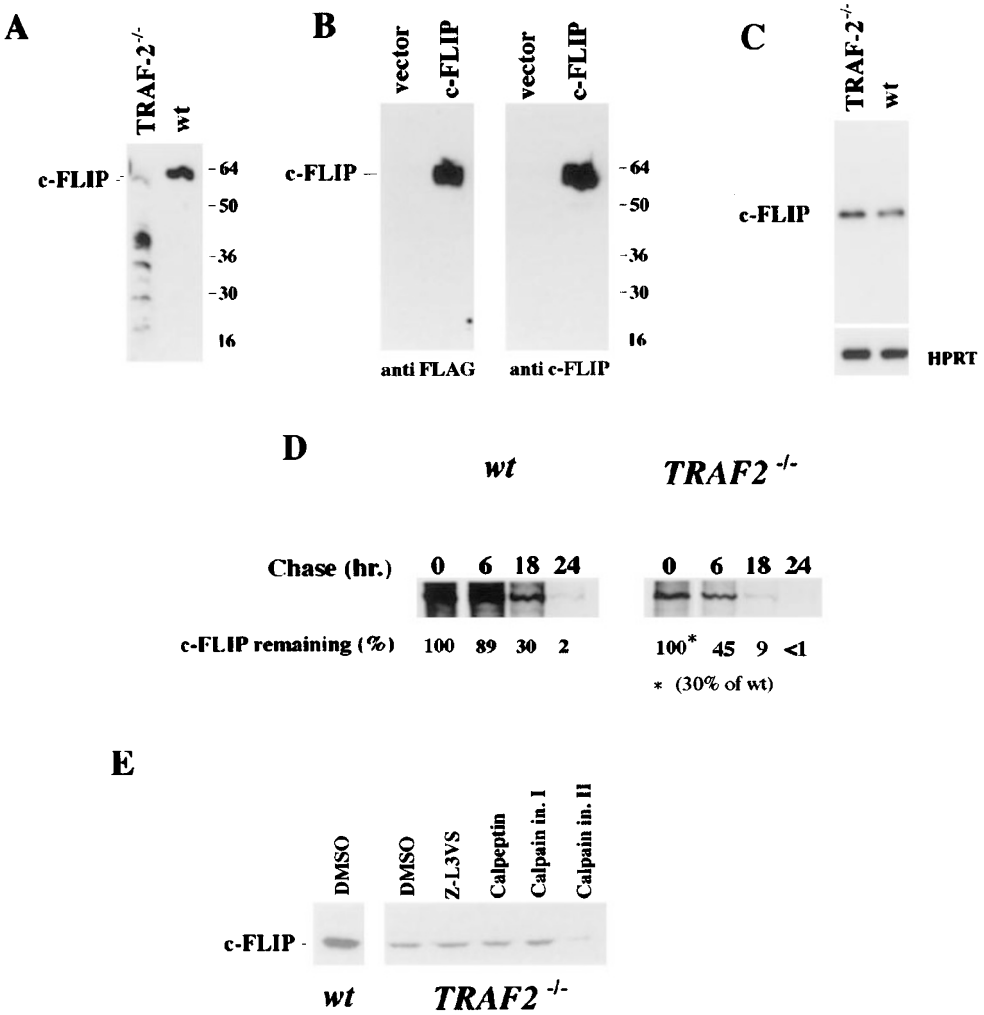



**Figure 3** (**A**) Degradation of c-FLIP in TRAF-2$^{-/-}$ EF. Cell extracts (20 $\mu$g) from TRAF-2$^{-/-}$ and wt EF were analyzed for c-FLIP expression by immunoblot analysis. (**B**) Specificity of the anti c-FLIP antibody used in this study. Cell lysates from 293 cells transfected with empty vector or a FLAG-tagged version of c-FLIP were analyzed by immunoblot assay with anti-FLAG (left panel) and anti-c-FLIP (right panel). (**C**) Northern blot analysis of c-FLIP expression. mRNA (2 $\mu$g) prepared from wt and TRAF-2$^{-/-}$ EF was separated by agarose electrophoresis and blotted on nylon membranes subsequently hybridized with a probe for c-FLIP. Filters were stripped and re-hybridized with a probe to HPRT for internal control. (**D**) wt and TRAF-2$^{-/-}$ EF were $^{35}$S-metabolically labeled for 3 h and chased for the indicated time periods. c-FLIP was immunoprecipitated from aliquots containing equal amount of protein and quantitative analysis was done with Phosphorimager. Quantities are relative to the amount of protein at time 0. (**E**) c-FLIP destabilization in TRAF-2$^{-/-}$ EF is not blocked by proteasome inhibitors. TRAF-2$^{-/-}$ EF were treated for 16 h with the indicated inhibitors each at a final concentration of 25 $\mu$M or with a DMSO solvent control. Normalized lysates were examined for c-FLIP by immunoblot analysis

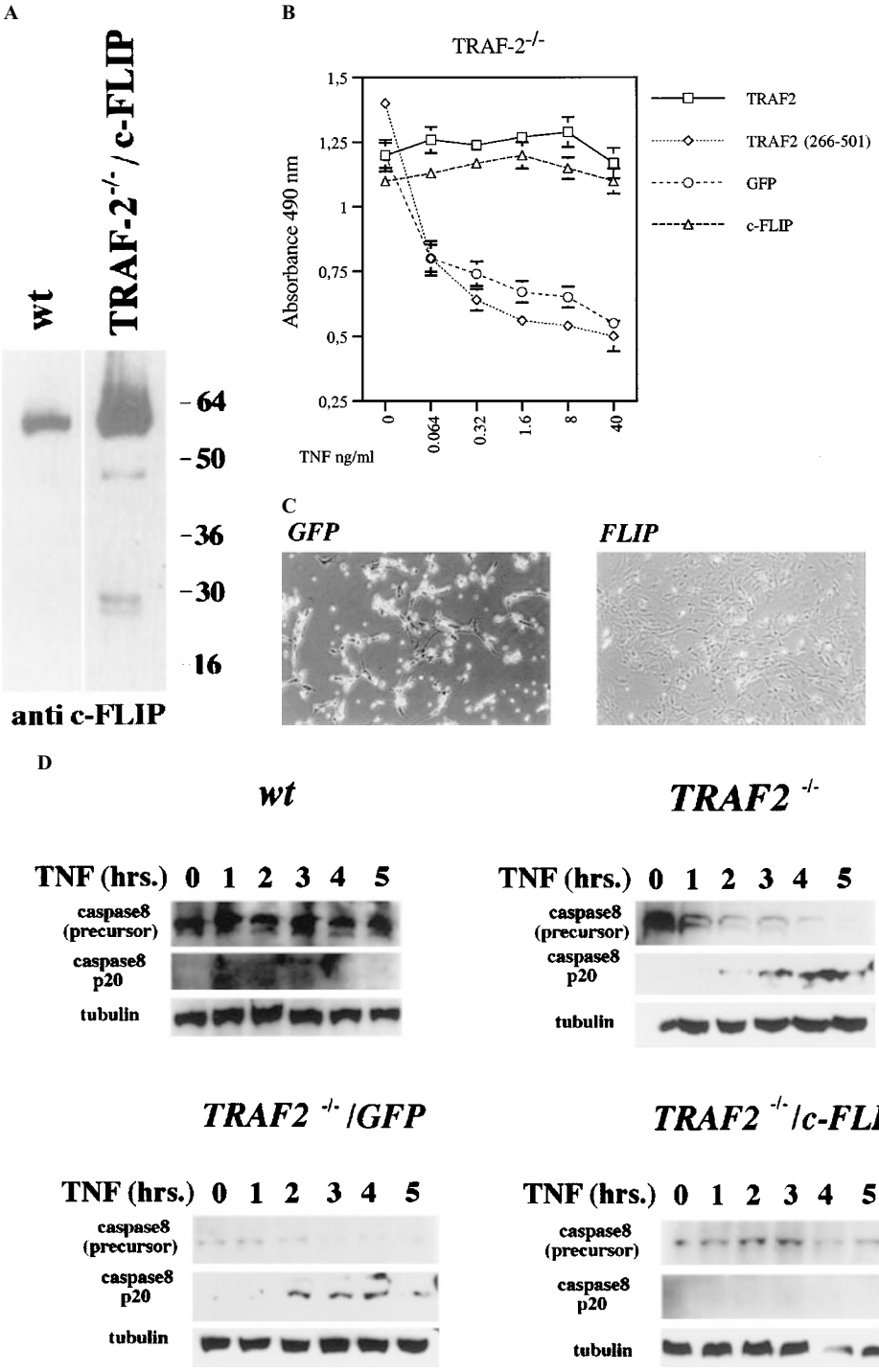
are resistant to TNF cytotoxicity unless treated with protein synthesis inhibitors. In addition, TNF pretreatment generally exerts positive effects on subsequent challenge with TNF+cycloheximide, indicating that cytoprotective pathways are turned on by TNF stimulation.[20] The transcription factor NF-$\kappa$B has emerged as a critical regulator of TNF-induced cytoprotective responses.[2–4] Retained in the cytoplasm by the inhibitory proteins I-$\kappa$Bs, NF-$\kappa$B translocates to the nucleus upon TNF stimulation and activates transcription of genes implicated in proliferation and cell survival, including anti-apoptotic proteins such as IAPs.[21]

However, both TRAF-2 and c-FLIP-deficient cells are sensitive to TNF-induced apoptosis, yet retaining normal NF-$\kappa$B activation. In addition, c-FLIP is normally expressed in RelA$^{-/-}$ cells, indicating that expression of this gene is not directly controlled by NF-$\kappa$B.[19] Data obtained from mutant mice generated by gene targeting, together with earlier biochemical observations,[9] have provided evidence for NF-$\kappa$B-independent cytoprotective mechanisms, which collaborate with the survival factors controlled by NF-$\kappa$B. In this regard, regulation of c-FLIP stability may play a crucial role in determining cell survival following TNF stimulation.

## Materials and Methods

### Cells and transfections

wt and TRAF-2$^{-/-}$ embryonic fibroblasts were cultured in MEM-Glutamax (Gibco BRL) supplemented with 10% FCS and 0.1 mM




sodium pyruvate. 293 cells were maintained in DMEM/10% FCS and transfected by calcium phosphate precipitation.

## Antibodies and reagents

Sources of antibodies were: anti c-FLIP (Alexis Corp.), anti $\beta$-tubulin (Roche Biochem.), phospho-I-$\kappa$B and phospho-ERK1/2 (New England Biolabs), phospho-p38, phospo-JNK and anti caspase-8 (SantaCruz) JNK1 (Pharmingen). Source of reagents were: TNF (Pharmingen, RD), IL-1 (Pharmingen). The inhibitors Calpain Inhibitor I, Calpain Inhibitor II and Calpeptin were purchased from Calbiochem. The proteasome inhibitor Z-L3VS (carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone) was kindly provided by Dr. M Bogyo.

For the cell death experiments, cell viability was determined by trypan blue exclusion and with CellQuant colorimetric assay kit

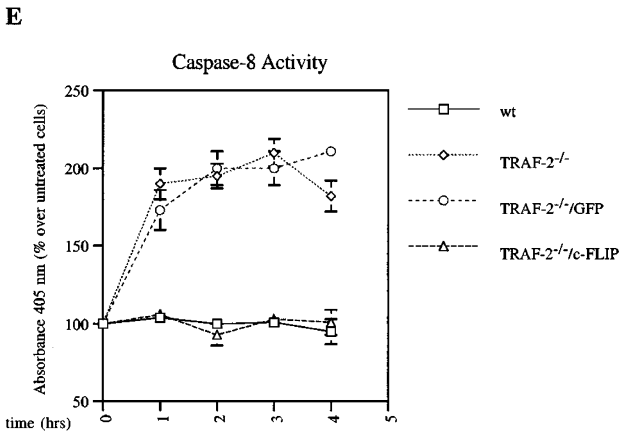



**Figure 4** (**A**) Expression level of c-FLIP in infected TRAF-2$^{-/-}$ EF. Retroviral supernatants expressing c-FLIP cDNA were used to infect TRAF-2$^{-/-}$ EF. Forty-eight hours after infection, cell lysates were prepared from uninfected EF (wt) and infected TRAF-2$^{-/-}$ EF (TRAF-2$^{-/-}$/c-FLIP). Protein concentration was determined by a Biorad Protein Assay and similar amounts of protein (20 $\mu$g) were analyzed for c-FLIP expression by immunoblot experiments. (**B**) Reconstitution of TRAF-2$^{-/-}$ EF with c-FLIP. Retroviral supernatants expressing the indicated cDNAs were made as indicated in Figure 2 and used to infect TRAF-2$^{-/-}$ EF. Forty-eight hours after infection, wt, uninfected and infected TRAF-2$^{-/-}$ EF were treated with TNF at the indicated concentration for 12–16 h and cell viability was then determined with an enzymatic assay. Data is representative of at least 10 independent triplicate experiments. (**C**) Phase-contrast micrographs of TRAF-2$^{-/-}$ fibroblasts reconstituted with c-FLIP or GPF cDNAs treated with TNF (20 ng/ml) for 16 h. (**D**) Processing of pro-caspase-8 in TRAF-2$^{-/-}$ EF. wt, TRAF-2$^{-/-}$, and TRAF-2$^{-/-}$ EF expressing c-FLIP or GFP were treated with TNF (20 ng/ml) for the indicated time. Cell extracts from treated cells were made in RIPA buffer, resolved by 14% SDS–PAGE and immunoblotted with an antibody recognizing both precursor and the active form (p20) of caspase-8. Filters were also probed with an antibody to $\beta$-tubulin. (**E**) Activity of caspase-8 is inhibited by c-FLIP in TRAF-2$^{-/-}$ EF. wt, TRAF-2$^{-/-}$, and infected TRAF-2$^{-/-}$ EF (TRAF-2$^{-/-}$/GFP and TRAF-2$^{-/-}$/c-FLIP) were treated with TNF (20 ng/ml) for the indicated time periods and caspase-8 activity was determined with an enzymatic assay

(Promega). Caspase-8 activity was determined with ApoAlert caspase-8 colorimetric assay kit (Clontech).

### Northern and immunoblot analysis

Poly(A) mRNA was prepared from wt and TRAF-2$^{-/-}$ EF using Poly (A) Quick mRNA columns (Stratagene), separated by agarose electrophoresis and blotted onto nylon membranes (Amersham-Pharmacia Biotech). For Western blot experiments, cells were lysed in RIPA buffer and protein concentration was determined with the Biorad Protein Assay (Biorad). Proteins were separated in a 11% polyacrylamide gel and electroblotted onto nylon membranes using a semidry device. Filters were incubated with primary antibodies of interest followed by a horseradish peroxidase-coupled secondary antibody (Promega). Enzyme activity was visualized by a chemiluminescence reaction (Pierce).

### Plasmid construction and retroviral stock production

LTR-driven retroviral expression constructs were made in pBMN vector using standard cloning techniques. PBMN vectors were then transfected in a packaging cell line by calcium phosphate precipitation. Twenty-four hours after transfection, secreted retrovirus was filtered, stocked, and used to infect TRAF-2$^{-/-}$ EF. Gene expression was examined 48 hr after infection by immunoblot analysis.

### Stability of c-FLIP *in vivo*

wt and TRAF-2$^{-/-}$ EF were starved in medium without methionine and cysteine for 2 h, then metabolically labeled with L-[$^{35}$S]methionine and L-[$^{35}$S]cysteine for 4 h. Cells were then chased in non-radioactive medium for the time periods indicated. Cells were lysed (150 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, 1% NP40 and a mixture of protease inhibitors) at 4°C for 20 min. Samples containing equal amount of protein were immunoprecipitated with an anti-c-FLIP antibody, collected with immobilized protein G-Sepharose (Amersham Pharmacia Biotech) and resolved on 12% SDS–PAGE. Gels were dried and protein visualized by autoradiography and quantified by a Phosphorimager (Molecular Dynamics).

## Acknowledgements

We thank Drs. D Liguoro and R Stilo for comments, Drs. A Leonardi and L D'Adamio for critical review of the manuscript. The Basel Institute for Immunology was founded and is supported by F Hoffmann-La Roche and Co. Ltd., CH-4005 Basel, Switzerland.

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