

TRAF2 Suppresses Basal IKK Activity in Resting Cells and TNF α Can Activate IKK in TRAF2 and TRAF5 Double Knockout Cells

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Tumor necrosis factor receptor (TNFR)-associated factor 2 (TRAF2) and TRAF5 are adapter proteins involved in TNF α -induced activation of the c-Jun N-terminal kinase and nuclear factor κ B (NF- κ B) pathways. Currently, TNF α -induced NF- κ B activation is believed to be impaired in TRAF2 and TRAF5 double knockout (T2/5 DKO) cells. Here, we report instead that T2/5 DKO cells exhibit high basal I κ B kinase (IKK) activity and elevated expression of NF- κ B-dependent genes in unstimulated conditions. Although TNF α -induced receptor-interacting protein 1 ubiquitination is indeed impaired in T2/5 DKO cells, TNF α stimulation further increases IKK activity in these cells, resulting in significantly elevated expression of NF- κ B target genes to a level higher than that in wild-type cells. Inhibition of NIK in T2/5 DKO cells attenuates basal IKK activity and restores robust TNF α -induced IKK activation to a level comparable with that seen in wild-type cells. This suggests that TNF α can activate IKK in the absence of TRAF2 and TRAF5 expression and receptor-interacting protein 1 ubiquitination. In addition, both the basal and TNF α -induced expression of anti-apoptotic proteins are normal in T2/5 DKO cells, yet these DKO cells remain sensitive to TNF α -induced cell death, due to the impaired recruitment of anti-apoptotic proteins to the TNFR1 complex in the absence of TRAF2. Thus, our data demonstrate that TRAF2 negatively regulates basal IKK activity in resting cells and inhibits TNF α -induced cell death by recruiting anti-apoptotic proteins to the TNFR1 complex rather than by activating the NF- κ B pathway.

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Abbreviations used: bTNF α , biotinylated TNF α ; cFLIP, cellular caspase-8 (FLICE)-like inhibitory protein; CHX, cycloheximide; cIAP, cellular inhibitor of apoptosis; DKO, double knockout; DN, dominant negative; GST, glutathione S-transferase; I κ B, inhibitor of κ B; IKK, I κ B kinase; IL, interleukin; JNK, c-Jun N-terminal kinase; KO, knockout; MEF, mouse embryonic fibroblast; mTNF α , mouse TNF α ; NF- κ B, nuclear factor κ B; RIP1, receptor-interacting protein 1; ROS, reactive oxygen species; siRNA, small interfering RNA; TNF α , tumor necrosis factor- α ; TNFR, TNF receptor; TRADD, TNFR-associated death domain; TRAF, TNFR-associated factor; WT, wild type.

Introduction

Tumor necrosis factor receptor (TNFR)-associated factors (TRAFs) are characterized by the presence of a TRAF domain at the C-terminus. Except for TRAF1, other TRAFs contain an N-terminal RING finger domain followed by five or seven zinc finger motifs.^{1,2} Whereas the RING and zinc finger domains are essential for TRAFs to activate downstream signaling pathways, the TRAF domain is required for their interactions with relevant receptors and downstream effectors.² TRAF2 is a prototypical member of the TRAF family and transduces signals from the TNFR superfamily members, leading to activation of the c-Jun N-terminal kinase (JNK) and the inhibitor of κ B (I κ B) kinase (IKK) pathways. JNK activates transcription factors of

the AP-1 group (e.g., c-jun/ATF2), and IKK activates those of the nuclear factor κ B (NF- κ B) group. These transcription factors in turn induce the expression of genes involved in inflammation, the immune response, cell proliferation, cell differentiation, and the suppression of death-receptor-induced apoptosis.^{3,4}

IKK is a kinase complex that consists of two catalytic subunits (IKK α and IKK β) and one regulatory subunit (IKK γ). TNFR family members activate NF- κ B through a canonical pathway and/or an alternative noncanonical pathway.⁵ The canonical NF- κ B pathway depends on the presence of both IKK β and IKK γ and is activated by most TNF superfamily members, resulting in the coordinate expression of multiple inflammatory and innate immune genes. The noncanonical pathway depends on NIK and IKK α and is activated by a subset of TNF family members, such as B-cell-activating factor, lymphotoxin α and β heterotrimers, and the CD40 ligand. This pathway triggers the expression of genes involved in adaptive immunity and lymphoid organogenesis.⁵

Recently, an increasing number of studies have demonstrated that both TRAF2 and TRAF6 possess E3 ligase activity and that they are capable of catalyzing the formation of noncanonical K63-linked polyubiquitin chains on themselves and on their substrates. These studies have also suggested that such K63-linked ubiquitination is essential for activation of the JNK and IKK pathways in response to TNF α and interleukin (IL)-1 β stimulation.^{6–8} Ubc13/UEV1a is the only E2 ubiquitin-conjugating enzyme complex that is currently known to bind to TRAF2 and TRAF6 and to catalyze K63-linked ubiquitination of these proteins.⁶ Inhibition of Ubc13 expression by small interfering RNA (siRNA) however results in inhibition of TNF α -induced JNK activation, but not that of IKK activation.⁹ A recent study has also demonstrated that conditional ablation of Ubc13 results in considerably impaired JNK activation in response to a variety of stimuli in B cells and mouse embryonic fibroblasts (MEFs) without affecting IKK activation under the same conditions. This suggests that either K63-linked polyubiquitination of TRAF2/TRAF6 is not essential for IKK activation or IKK can also be activated by an alternative ubiquitin-independent mechanism in response to cytokine stimulation.¹⁰

TRAF2 knockout (T2 KO) MEFs are completely defective in TNF α -induced JNK activation but only partially deficient in NF- κ B activation.¹¹ Interestingly, TRAF2-deficient macrophages overproduce TNF α and nitric oxide upon TNF α stimulation. In addition, whereas T2 KO mice die prematurely, mice with double mutants for TRAF2 and either TNF α or TNFR1 survive for several months, suggesting that TRAF2 negatively regulates certain aspects of TNFR1 signaling and thus the canonical NF- κ B pathway.¹² Also, conditional KO of TRAF2 in B cells results in constitutive activation of the noncanonical NF- κ B pathway, suggesting that TRAF2 also negatively regulates this pathway.¹³

Tada *et al.* have reported that whereas TRAF5-null MEFs respond normally to TNF α -induced JNK and NF- κ B activation, TRAF2 and TRAF5 double knockout (T2/5 DKO) MEFs exhibit an almost complete loss of TNF α -induced NF- κ B activation.¹⁴ These data suggest that TRAF2 can regulate TNFR1 signaling in either a positive or a negative direction, in a manner that likely varies according to cell type.

We identified a phosphorylation site at the N-terminal region of TRAF2 by a classic phosphopeptide mapping approach.¹⁵ While carrying out functional studies investigating the role of TRAF2 phosphorylation in TNF α -induced NF- κ B activation in T2/5 DKO cells reconstituted with empty vector, wild type (WT), or phosphomutant TRAF2, we repeatedly observed that control T2/5 DKO MEFs exhibit high basal IKK activity and elevated NF- κ B target gene expression in the absence of stimulation with TNF α . Surprisingly, stimulation of these cells with TNF α , which merely activates the canonical NF- κ B pathway through the TRAF2/5–RIP1 (receptor-interacting protein 1)–IKK cascade, further increased the expression of NF- κ B target genes—to a level higher than that in TNF α -stimulated WT MEFs. To clarify these contradictory observations, we extensively analyzed the activation status of both the canonical and noncanonical NF- κ B pathways in T2 KO and T2/5 DKO MEFs. Here, we show that both the canonical and noncanonical NF- κ B pathways are constitutively activated in these cells and that the primary function of TRAF2 in TNFR1 signaling is to activate the JNK pathway while inhibiting TNF α -induced cell death by recruiting antiapoptotic proteins to the TNFR1 complex.

Results

TRAF2 negatively regulates basal NF- κ B activity

Numerous studies have shown that in HeLa cells, transient TRAF2 expression induces NF- κ B and c-Jun activation in the absence of TNF α stimulation.^{1,4} As expected, expression of TRAF2 in HeLa cells increased both basal and TNF α -induced NF- κ B and c-Jun activation (Fig. S1). Unexpectedly, we found that in T2/5 DKO MEFs, transient expression of TRAF2 significantly reduced basal NF- κ B activity, which was otherwise quite high compared with that in WT MEFs (Fig. 1a). In contrast, both basal and induced c-Jun activities were lower in T2/5 DKO MEFs than in WT MEFs, and TRAF2 expression restored TNF α -induced c-Jun activation (Fig. 1b). These data suggest that TRAF2 negatively regulates basal NF- κ B activity in unstimulated MEFs.

TRAF2 suppresses the basal activity of IKK complex

Processing of p100 to p52, a hallmark of activation of the noncanonical NF- κ B pathway, takes place

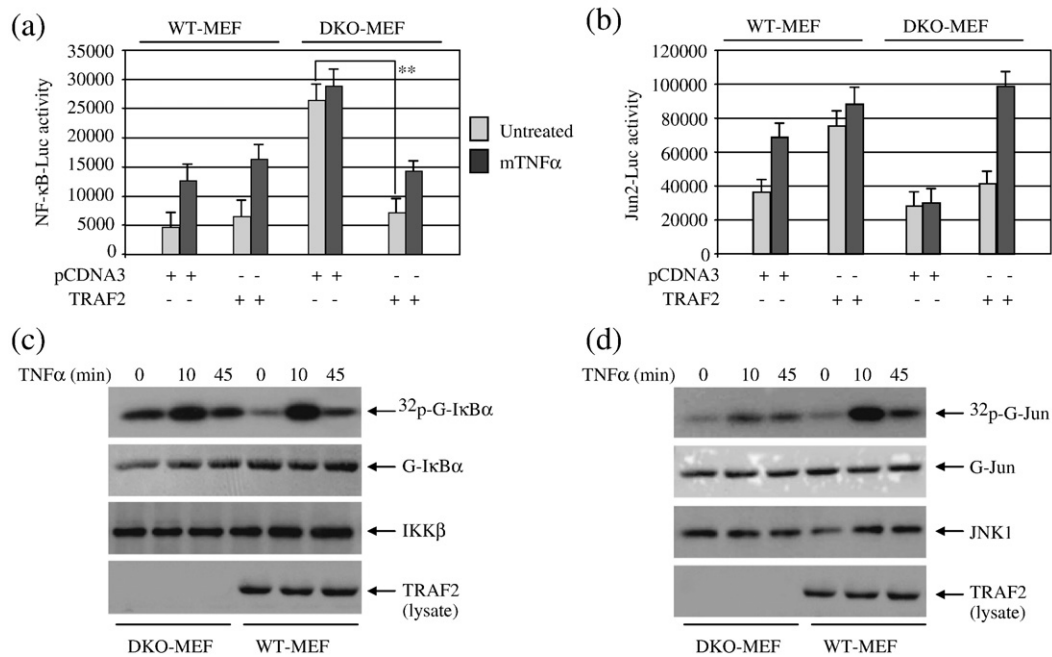


Fig. 1. T2/5 DKO MEFs exhibit elevated basal IKK and NF- κ B activities. (a and b) WT MEF and T2/5 DKO MEFs were co-transfected with either NF- κ B-Luc (a) or Jun2-Luc (b), plus pRL-TK and either pCDNA3 or TRAF2. Thirty-six hours after transfection, cells were left untreated or treated with mTNF α (5 ng/ml) for 4 h, after which the NF- κ B-Luc and Jun2-Luc activities were measured and normalized to pRL-TK activity. Data are presented as the mean \pm SD of the results from three independent experiments carried out in triplicate. $^{**}p < 0.01$. (c and d) T2/5 DKO and WT MEF cells were left untreated or treated with mTNF α (10 ng/ml) as indicated. The IKK complex and JNK1 were then immunoprecipitated with anti-IKK γ and anti-JNK1 antibodies and subjected to *in vitro* kinase assays in which GST-I κ B α ¹⁻⁵⁵ and GST-jun¹⁻⁸⁷ served as substrates, respectively. The reaction mixtures were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and exposed to X-ray film for 4–6 h (³²p-G-I κ B α and ³²p-G-jun). The same membranes were stained with Ponceau S to detect G-I κ B α and G-jun and blotted with anti-IKK β and anti-JNK1 antibodies, respectively.

constitutively in both T2 KO and T2/5 DKO MEFs.¹⁶ Therefore, it is possible that the elevated basal NF- κ B activity we detected in T2/5 DKO MEFs stems from constitutive activation of the noncanonical NF- κ B pathway. The IKK immunokinase assay [using glutathione *S*-transferase (GST)-I κ B α fusion protein as a substrate] is one of the most sensitive and reliable methods for assessing the activity of the IKK complex. To investigate the activity of the IKK complex and to rule out the possibility that an IKK α homodimer affects GST-I κ B α phosphorylation in the kinase assay, we immunoprecipitated the IKK complex with anti-IKK γ antibody and extensively washed the IKK-bound G-protein beads with lysis buffer containing 350 mM NaCl. As shown in Fig. 1c, the IKK complex was constitutively activated in T2/5 DKO MEFs and stimulation of these cells with TNF α led to a further, but weak, increase in IKK activity. In contrast, a JNK kinase assay revealed that in T2/5 DKO MEFs, basal JNK activity was low prior to stimulation with TNF α and remained low even in the presence of TNF α (Fig. 1d). These data suggest that TRAF2 suppresses the basal activity of the IKK complex in resting cells and confirm that TRAF2 is required for TNF α -induced JNK activation.

TRAF2 and TRAF5 are not essential for TNF α -induced expression of certain NF- κ B target genes

To further examine the activation status of the canonical NF- κ B pathway in TRAF2/5 DKO cells, we analyzed the expression of nine well-known NF- κ B target genes by quantitative real-time PCR. Consistent with the elevated basal NF- κ B activity we had observed in these cells, the basal expression levels of I κ B α , IP-10, RANTES, and ICAM-1 were higher in T2/5 DKO MEFs than in WT MEFs (Fig. S2). Surprisingly, TNF α stimulation further increased the expression of these genes in T2/5 DKO MEFs to levels higher than those in WT MEFs (Fig. 2a–d). On the other hand, the basal and inducible expressions of cellular inhibitor of apoptosis 1 (cIAP1) and cellular caspase-8 (FLICE)-like inhibitory protein (cFLIP) were comparable in both types of cells (Fig. 2e and f). In the cases of IL-6, Mn-SOD, and COX-2, however, the basal expression levels were lower in the T2/5 DKO MEFs than in the WT MEFs (Fig. 2g–i) and stimulation with TNF α failed to achieve the expression levels attained in WT MEFs; more specifically, the inducibility of IL-6 was almost completely impaired, and that of COX-2 and Mn-SOD was reduced (Fig. 2g–i). To confirm these

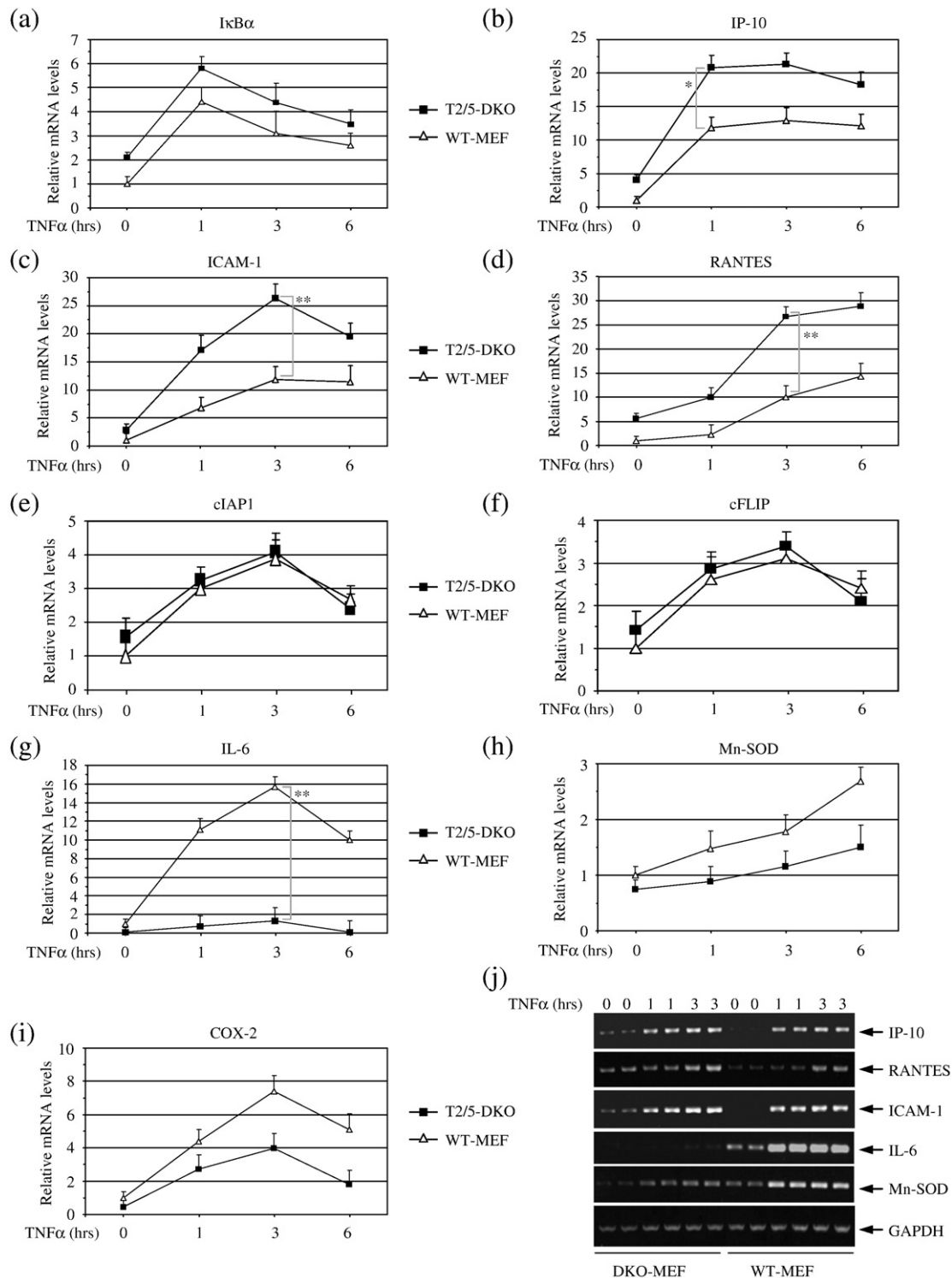


Fig. 2. In T2/5 DKO MEFs, TNF α induces the expression of certain NF- κ B target genes. (a-i) WT and T2/5 DKO MEFs were left untreated or treated with mTNF α (10 ng/ml) for 1, 3, and 6 h. Expression levels of IkB α , IP-10, RANTES, ICAM-1, cIAP1, cFLIP, IL-6, Mn-SOD, and COX-2 were then determined by real-time PCR. The relative expression level of each gene is presented as the ratio between the respective gene and the reference gene GAPDH, as the mean \pm SD from four independent experiments. * p < 0.05; ** p < 0.01. (j) WT and T2/5 DKO MEFs were left untreated or treated with mTNF α (10 ng/ml) as indicated, and the expression levels of IP-10, RANTES, ICAM-1, IL-6, COX-2, and GAPDH were determined by semiquantitative reverse transcription PCR.

results, we designed a second set of primers (Table S2) and performed conventional semiquantitative reverse transcription PCR analysis. As shown in Fig.

2j, the basal and inducible expressions of IP-10, RANTES, and ICAM-1 were indeed elevated in T2/5 DKO MEFs *versus* in WT MEFs, whereas in the case

of IL-6, inducible expression was impaired. As TNF α merely activates the canonical NF- κ B pathway, these data suggest that TRAF2 and TRAF5 are not essential for TNF α -induced expression of some NF- κ B target genes.

Restoration of TRAF2 expression leads to reduced basal IKK activity

To rule out the possibility that the differences we observed were due to an immortalization effect, we established T2/5 DKO cell lines that stably express

either empty vector or TRAF2 at a physiological level (Fig. S3). As expected, T2/5 DKO MEFs stably transfected with empty vector (pBa-C) exhibited high basal IKK activity, whereas their TRAF2-expressing counterparts (pBa-TRAF2) did not (Fig. 3a). In addition, pBa-TRAF2 cells exhibited immediate and robust JNK and IKK activation in response to TNF α stimulation (Fig. 3a and b). Consistent with a previous study,¹⁷ TNF α treatment did however induce JNK activation in pBa-C cells at a later time point after stimulation (120 min). To further rule out the possibility that the elevated IKK

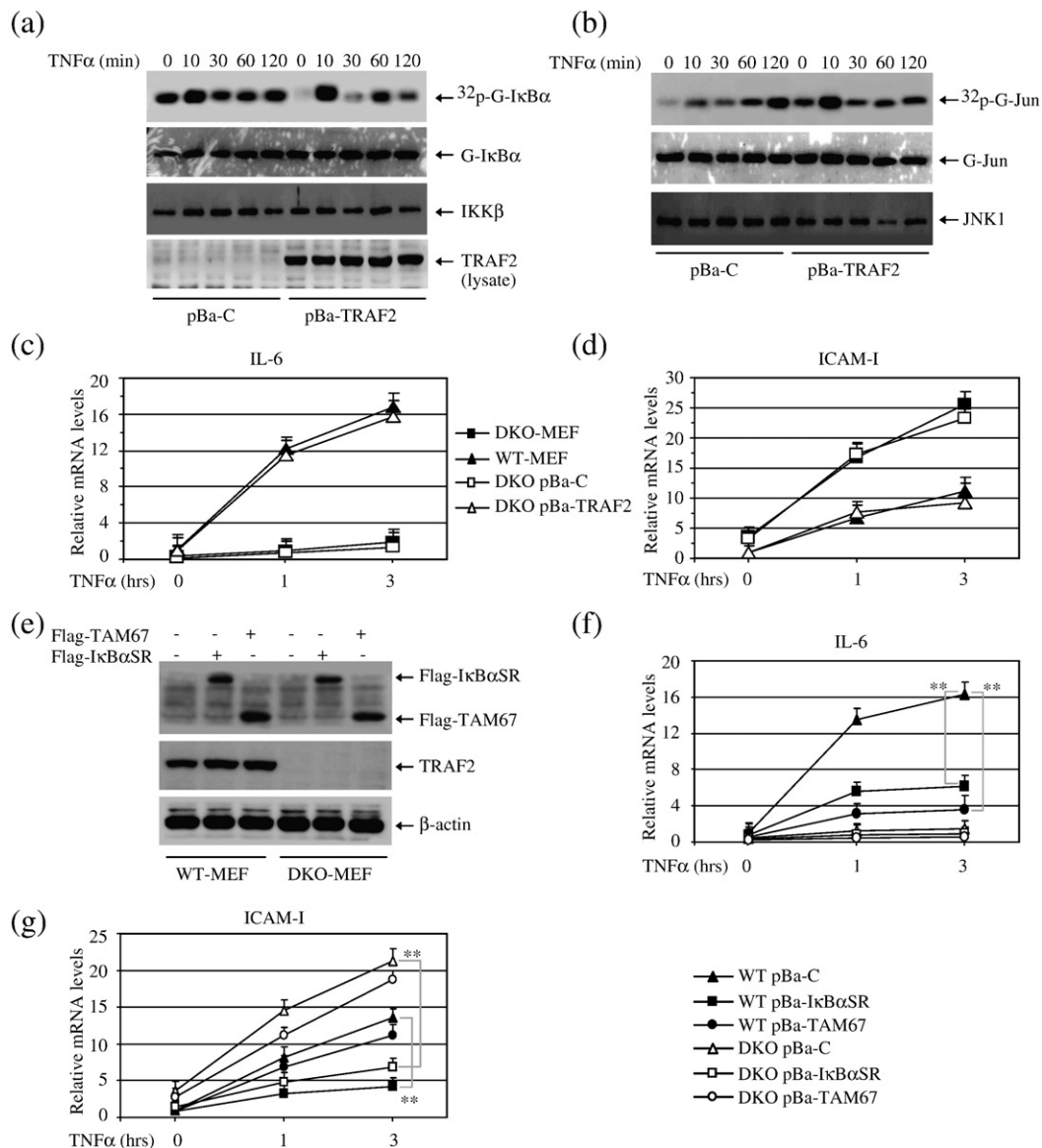


Fig. 3. In T2/5 DKO MEFs, TRAF2 expression inhibits the basal IKK activity and restores TNF α -induced JNK activation and IL-6 expression. (a and b) T2/5 DKO MEFs reconstituted with empty vector (pBa-C) or TRAF2 (pBa-TRAF2) were untreated or treated with mTNF α (10 ng/ml) as indicated. IKK and JNK kinase assays were then performed as in Fig. 1c and d. (c and d) WT, T2/5 DKO, pBa-C, and pBa-TRAF2 MEFs were untreated or treated with mTNF α (10 ng/ml) for 1 and 3 h, after which the expression of IL-6 and ICAM-1 was determined by real-time PCR, as in Fig. 2c and g. (e) WT and DKO MEFs were stably transfected with Flag-I κ B α SR or Flag-TAM67 by retroviral infection followed by puromycin selection. The expression of Flag-I κ B α SR and Flag-TAM67 was then monitored by Western blotting. (f and g) WT and DKO MEFs stably transfected with Flag-I κ B α SR or Flag-TAM67 were treated with TNF α (10 ng/ml) for 1 and 3 h, after which the expression of IL-6 and ICAM-1 was determined by real-time PCR.

activity in pBa-C cells was due to cell culture-associated artifacts, we independently established pBa-C and pBa-TRAF2 cell lines using T2/5 DKO MEFs that were at early passage numbers (lower than 6) and late passage numbers (greater than 30) and repeated the IKK immunokinase assay twice for each cell line; we obtained the same results as in the original experiments in both cases. Regardless of passage number, all pBa-C lines tested exhibited high basal IKK activity and all pBa-TRAF2 lines exhibited reduced basal IKK activity (Fig. S4). Moreover, stable expression of TRAF2 in T2/5 DKO MEFs restored TNF α -induced IL-6 expression and suppressed the elevated basal and inducible expressions of ICAM-1 to levels comparable with those in WT MEFs (Fig. 3c and d). These data demonstrated that TRAF2 expression at a physiological level is essential for the inhibition of basal IKK activity in resting cells.

Both c-Jun and NF- κ B are required for TNF α -induced IL-6 expression in MEFs

TNF α -induced IL-6 expression is completely impaired in JNK1 and JNK2 DKO MEFs.¹⁸ To confirm that impaired TNF α -induced IL-6 expression in T2/5 DKO MEFs is due to impaired JNK/c-Jun activation in these cells, we stably expressed a dominant-negative (DN) c-Jun (TAM67, having a truncated transactivation domain) or an I κ B α super-repressor (I κ B α SR, in which serine 32 and serine 36 were replaced with alanine) in WT and DKO MEFs (Fig. 3e). Interestingly, expression of either I κ B α SR or TAM67 was found to be sufficient to significantly inhibit the TNF α -induced IL-6 expression in WT MEFs (Fig. 3f). On the other hand, expression of I κ B α SR, but not that of TAM67, significantly inhibited TNF α -induced ICAM-1 expression in both WT and DKO MEFs (Fig. 3g). In fact, TNF α -induced IL-6 expression is also almost completely impaired in p65 (a subunit of NF- κ B) KO MEFs.¹⁹ This suggests that both c-Jun and NF- κ B are required for TNF α -induced IL-6 expression in MEFs.

TNF α induces I κ B α degradation and p65 nuclear translocation in T2/5 DKO MEFs

Currently, TNF α -induced NF- κ B activation is believed to be impaired in TRAF2/5 DKO cells. This conclusion is based on an assessment of I κ B α protein levels in T2/5 DKO MEFs following TNF α stimulation.¹⁴ To better assess the roles of TRAF2 and TRAF5 in TNF α -induced NF- κ B activation, we examined I κ B α degradation and p65 nuclear translocation in WT, T2 KO, and T2/5 DKO MEFs by Western blotting. In WT MEFs, TNF α stimulation caused nearly complete degradation of I κ B α within 30 min after stimulation, whereas it failed to do so in T2/5 DKO MEFs (Fig. 4a); these findings are consistent with a previous report.¹⁴ However, careful analysis of I κ B α protein levels by shorter exposure of films revealed that in T2/5 DKO MEFs, TNF α -induced I κ B α degradation is reduced

rather than completely impaired (Fig. 4a, 15-s exposure). Surprisingly, TNF α -induced I κ B α degradation was also attenuated in T2 KO MEFs. Consistent with this finding, TNF α stimulation triggered p65 translocation to the nucleus in both T2/5 DKO and T2 KO MEFs at comparable levels (Fig. 4c). However, TNF α -induced p65 nuclear translocation in these cells was reduced in comparison with that in WT MEFs (Fig. 4c). These data suggest that TNF α does in fact induce the activation of the canonical NF- κ B pathway in T2/5 DKO cells, albeit to a lesser degree than in WT MEFs.

Both the canonical and noncanonical NF- κ B pathways are constitutively activated in T2/5 DKO MEFs

To further assess the activation status of the canonical and noncanonical NF- κ B pathways, we analyzed p100 processing and I κ B α and p65 phosphorylation following the stimulation of cells with TNF α (inducer of the canonical NF- κ B pathway) or agonistic anti-LT β R antibody (inducer of the noncanonical NF- κ B pathway). As expected, in WT MEFs, anti-LT β R antibody but not TNF α induced p100 processing, whereas in T2 KO and T2/5 DKO MEFs, p100 was constitutively processed to p52 even in the absence of stimulation (Fig. 4b). In addition, a high level of processed p52 was found in the nuclear fraction of T2/5 DKO but not in that of WT MEFs under unstimulated conditions (Fig. S5). Consistently, TNF α , but not the anti-LT β R antibody, induced the phosphorylation of I κ B α and p65 in T2 KO and T2/5 DKO MEFs, albeit to a lesser degree than in WT MEFs (Fig. 4b). In light of a recent study showing that I κ B α is constitutively phosphorylated and degraded in T3 KO MEFs due to constitutive IKK activation,²⁰ we wanted to test whether I κ B α is constitutively phosphorylated and degraded in T2/5 DKO MEFs. To this end, we treated cells with the proteasome inhibitors lactacystin and MG132 for 90 min and then monitored I κ B α phosphorylation by Western blotting. As shown in Fig. 4d, when the cells were treated with proteasome inhibitors, I κ B α phosphorylation became clearly detectable in T2 KO and T2/5 DKO MEFs but not in WT MEFs, suggesting that I κ B α is constitutively phosphorylated and degraded in T2 KO and T2/5 DKO MEFs. These data suggest that both the canonical and noncanonical NF- κ B pathways are constitutively activated in T2 KO and T2/5 DKO MEFs.

TRAF2 has a nonredundant role in suppression of basal IKK activity

In both T2 KO and T2/5 DKO MEFs, I κ B α is constitutively phosphorylated and p100 is constitutively processed. This raises the question of what role TRAF5 plays in the regulation of basal and inducible IKK activities. Thus, we performed an IKK kinase assay assessing the difference in T2 KO and T2/5 DKO MEFs. Surprisingly, the IKK complex in T2 KO MEFs was also constitutively

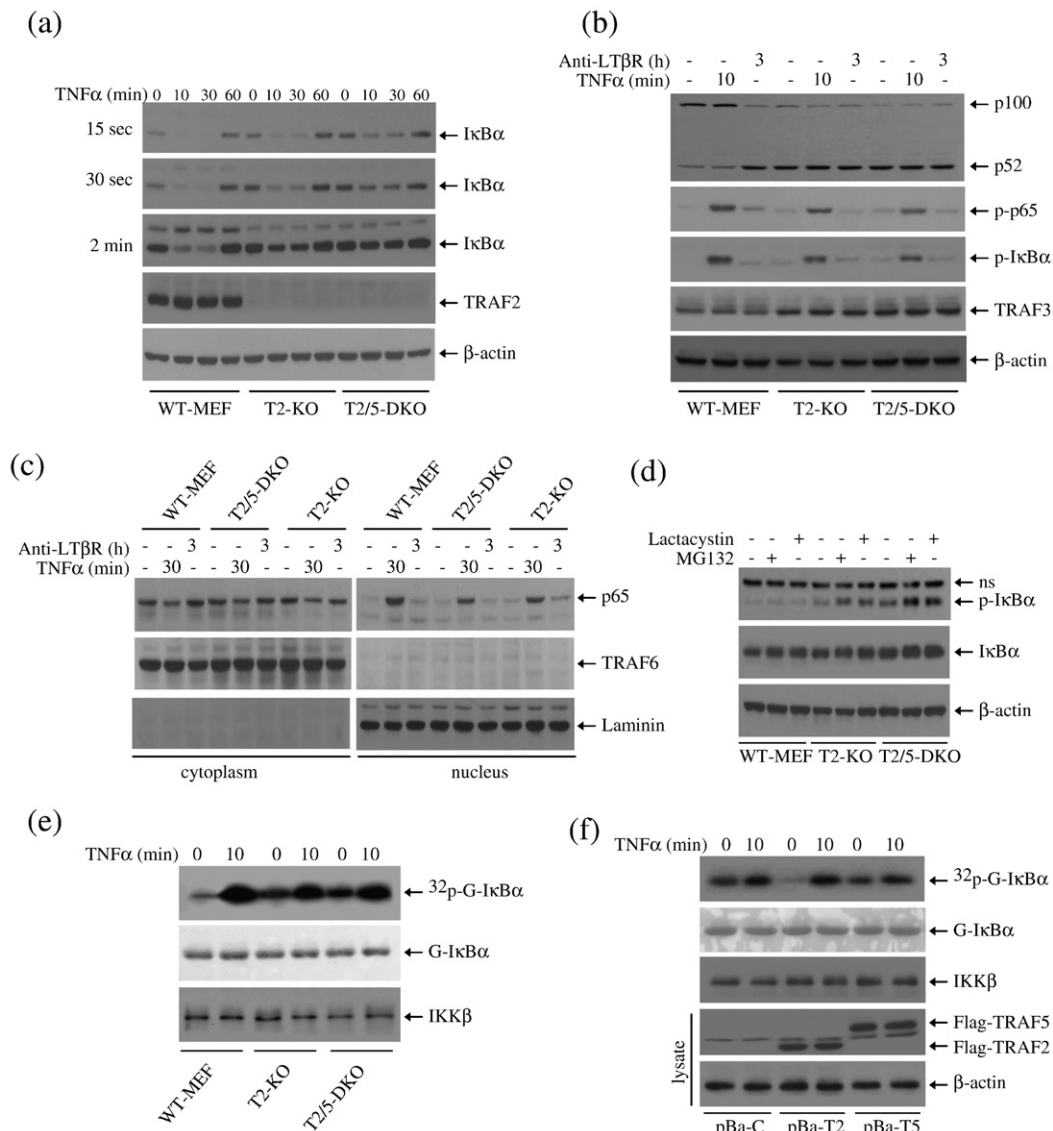


Fig. 4. In T2/5 DKO MEFs, TNF α induces I κ B α degradation and p65 nuclear translocation. (a) WT, T2 KO, and T2/5 DKO MEFs were left untreated or treated with mTNF α (10 ng/ml) as indicated, after which I κ B α degradation was detected by Western blotting. Fifteen-second, 30-s, and 2-min labels at the left side indicate the X-ray film exposure times. (b) WT, T2 KO, and T2/5 DKO MEFs were untreated or treated with mTNF α (10 ng/ml) for 10 min or with anti-LT β R antibody (0.5 μ g/ml) for 3 h, after which p100 processing, p65 and I κ B α phosphorylation, and TRAF3 expression were monitored by Western blotting. (c) WT, T2 KO, and T2/5 DKO MEFs were untreated or treated with mTNF α (10 ng/ml) for 30 min or with anti-LT β R antibody (0.5 μ g/ml) for 3 h, after which nuclear translocation of p65 was monitored by Western blotting. (d) WT, T2 KO, and T2/5 DKO MEFs were untreated or treated with lactacystin (10 μ M) or MG132 (20 μ M) for 90 min, after which I κ B α phosphorylation was monitored by Western blotting. "ns" indicates nonspecific bands. (e) WT, T2 KO, and T2/5 DKO MEFs were untreated or treated with mTNF α (10 ng/ml) for 10 min, after which IKK activation was assessed by an *in vitro* kinase assay as in Fig. 1c. (f) T2/5 DKO MEFs stably expressing empty vector (pBa-C), TRAF2 (pBa-T2), and TRAF5 (pBa-T5) were untreated or treated with mTNF α (10 ng/ml) for 10 min, after which IKK activation was assessed by an *in vitro* kinase assay as in Fig. 1c.

activated at a level comparable with that in T2/5 DKO MEFs (Fig. 4e). Consistent with this finding, stable expression of TRAF5 in T2/5 DKO MEFs failed to reduce the elevated basal IKK activity to a level similar to that seen in TRAF2-expressing cells (Fig. 4f). This suggests that TRAF2 plays a nonredundant role in the suppression of basal IKK activity in resting cells.

Expression of DN-NIK in T2/5 DKO MEFs partially restores TNF α -induced robust IKK activation

Whereas RIP1 is essential for activation of the canonical NF- κ B pathway, it is NIK that activates the noncanonical NF- κ B pathway.⁵ To examine which pathway accounts for the elevated activity of the

IKK complex, we stably expressed DN-NIK and DN-RIP1 in T2/5 DKO MEFs and then monitored p100 processing and IKK complex activity. As expected, the expression of DN-NIK partially suppressed p100 processing and basal IKK activity, whereas expression of DN-RIP1 inhibited only TNF α -induced (not basal) IKK activation (Fig. 5a and b). Unexpectedly, the expression of DN-NIK led to an increase in TNF α -induced IKK activation that was comparable with that seen in WT MEFs (Fig. 5b). This suggests that NIK is responsible for the

elevated basal IKK activity in T2/5 DKO MEFs and that this elevated basal IKK activity masks TNF α -induced immediate and robust IKK activation in T2/5 DKO MEFs.

Knockdown of NIK in T2/5 DKO MEFs restores TNF α -induced robust IKK activation

As expected, NIK is accumulated both in T2 KO and T2/5 DKO MEFs to a similar level and stable expression of TRAF2, but not that of TRAF5,

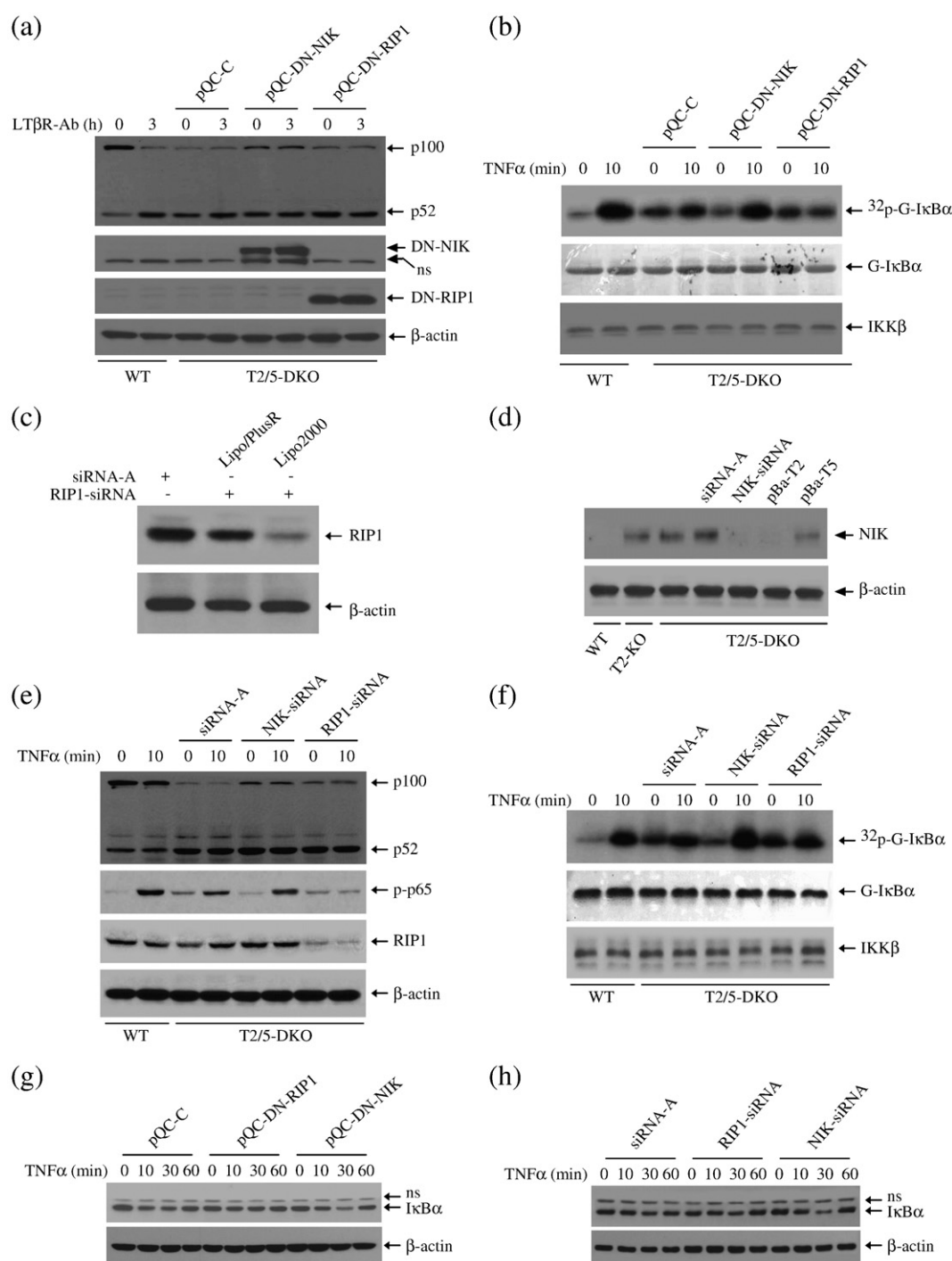


Fig. 5 (legend on next page)

suppressed NIK accumulation in T2/5 DKO MEFs (Fig. 5d). To confirm the role of NIK in constitutive activation of both NF- κ B pathways in T2/5 DKO MEFs, we knocked down NIK and RIP1 by an siRNA-mediated approach (Fig. 5c and d). Consistent with the results of the DN-NIK and DN-RIP1 expression studies, knockdown of NIK in T2/5 DKO MEFs partially suppressed p100 processing and basal IKK activity, whereas knockdown of RIP1 decreased TNF α -induced, but not basal, IKK activation (Fig. 5e and f). Moreover, knockdown of NIK in T2/5 DKO MEFs almost completely restored TNF α -induced immediate and robust IKK activation (Fig. 5f and Fig. S6). These data support the notion that elevated basal IKK activity masks TNF α -induced robust IKK activation and that TNF α -induced IKK activation is otherwise almost intact in T2/5 DKO MEFs.

Inhibition of NIK in T2/5 DKO MEFs restores TNF α -induced I κ B α degradation

Given that I κ B α itself is an NF- κ B target gene, constitutive activation of IKK may induce constitutive phosphorylation, degradation, and resynthesis of I κ B α , thereby masking TNF α -induced robust and immediate degradation of I κ B α . To test this hypothesis, we monitored I κ B α degradation following the expression of DN-NIK and DN-RIP1 or the knockdown of NIK and RIP1 in T2/5 DKO MEFs. As shown in Fig. 5g, the expression of DN-NIK increased TNF α -induced I κ B α degradation, whereas the expression of DN-RIP1 blocked this event. In line with this finding, the expression of DN-NIK in T2/5 DKO MEFs suppressed TNF α -induced ICAM-1 expression to a level comparable with that in WT MEFs, whereas expression of DN-RIP1 inhibited its inducible expression (Fig. S7a). As expected, stable expression of DN-NIK and DN-RIP1 had no effect on TNF α -induced IL-6 expression in T2/5 DKO MEFs (Fig. S7b). Notably, siRNA-mediated knockdown of NIK, but not that of RIP1, increased TNF α -induced I κ B α degradation (Fig. 5h). However, neither the expression of DN-NIK nor the knockdown of endogenous NIK completely inhibited p100 processing or restored TNF α -induced

I κ B α degradation to the level seen in WT MEFs (Fig. S8, and other data not shown). This is most likely because stably expressed DN-NIK is not sufficient to completely block endogenous NIK activity and because the efficiency of siRNA transfection is relatively low in T2/5 DKO MEFs. Nevertheless, we consistently observed that expression of DN-NIK and knockdown of endogenous NIK suppressed basal IKK activity and increased TNF α -induced IKK activation and I κ B α degradation. Collectively, these data suggest that elevated IKK activity and a high rate of constitutive I κ B α degradation and resynthesis in T2/5 DKO MEFs mask TNF α -induced robust and immediate IKK activation and I κ B α degradation. Otherwise, TNF α activation of the canonical NF- κ B pathway is normal even in the absence of both TRAF2 and TRAF5, which explains why T2/5 DKO MEFs display normal, if not elevated, expression of NF- κ B target genes in response to TNF α stimulation.

TRAF2, but not TRAF5, expression inhibits TNF α -induced cell death independent of NF- κ B activation

TNF α -induced cell death in TRAF2/5 DKO MEFs is believed to be due to impaired NF- κ B activation.¹⁷ In fact, as we have demonstrated here, this is not the case. Interestingly, we noticed that T2/5 DKO MEFs naturally immortalized by a 3T3 protocol are sensitive to TNF α -induced cell death at early passages (Fig. S9a) but become less susceptible to TNF α -induced cell death after 2–3 months of *in vitro* culturing or following stable expression of puromycin-resistant empty vector (Fig. S9a and b). Nevertheless, both T2 KO and T2/5 DKO MEFs remained sensitive to cell death induced by TNF α (5 ng/ml) in the presence of very low levels of cycloheximide (CHX; 0.2 μ g/ml), a condition that does not cause more than 10% cell death in WT MEFs (Fig. 6a and b). To examine the roles of TRAF2 and TRAF5 in TNF α -induced cell death under the same experimental conditions, we treated T2/5 DKO MEFs stably expressing TRAF2 or TRAF5 with TNF α (5 ng/ml) plus CHX (0.2 μ g/ml). As shown in Fig. 6a, the expression of TRAF2, but not that of TRAF5,

Fig. 5. In T2/5 DKO MEFs, a reduction of NIK activity increases TNF α -induced IKK activation and I κ B α degradation. (a) T2/5 DKO MEFs stably expressing empty vector (pQC-C), DN-NIK (pQC-DN-NIK), or DN-RIP1 (pQC-DN-RIP1) were untreated or treated with anti-LT β R antibody (0.5 μ g/ml) for 3 h, after which p100 processing and expression of DN-NIK and DN-RIP1 were monitored by Western blotting. (b) pQC-C, pQC-DN-NIK, and pQC-DN-RIP1 cells were untreated or treated with mTNF α (10 ng/ml) for 10 min, after which IKK activity was assessed by an *in vitro* kinase assay as in Fig. 1c. (c) T2/5 DKO MEFs were transfected with a scrambled control siRNA-A or an siRNA specific to mouse RIP1 using either Lipofectamine Plus Reagent or Lipofectamine 2000. At 68 h after transfection, the expression level of RIP1 was assessed by Western blotting. (d) T2/5 DKO MEFs were transfected with an siRNA specific to mouse NIK using Lipofectamine 2000. At 68 h after transfection, the expression levels of NIK in siRNA transfected cells, as well as in the other cell lines indicated, were assessed by Western blotting. (e) T2/5 DKO MEFs were transfected with the indicated siRNA. At 68 h after transfection, p100 processing and p65 phosphorylation were monitored by Western blotting. (f) T2/5 DKO MEFs were transfected with siRNA as indicated. At 68 h after transfection, IKK activity was assessed by an *in vitro* kinase assay as in Fig. 1c. (g) pQC-C, pQC-DN-NIK, and pQC-DN-RIP1 cells were untreated or treated with mTNF α (10 ng/ml) as indicated, after which I κ B α degradation was monitored by Western blotting. “ns” indicates nonspecific bands. (h) T2/5 DKO MEFs were transfected with the indicated siRNA using Lipofectamine 2000. At 68 h after transfection, the cells were untreated or treated with mTNF α (10 ng/ml) as indicated, after which I κ B α degradation was monitored by Western blotting.

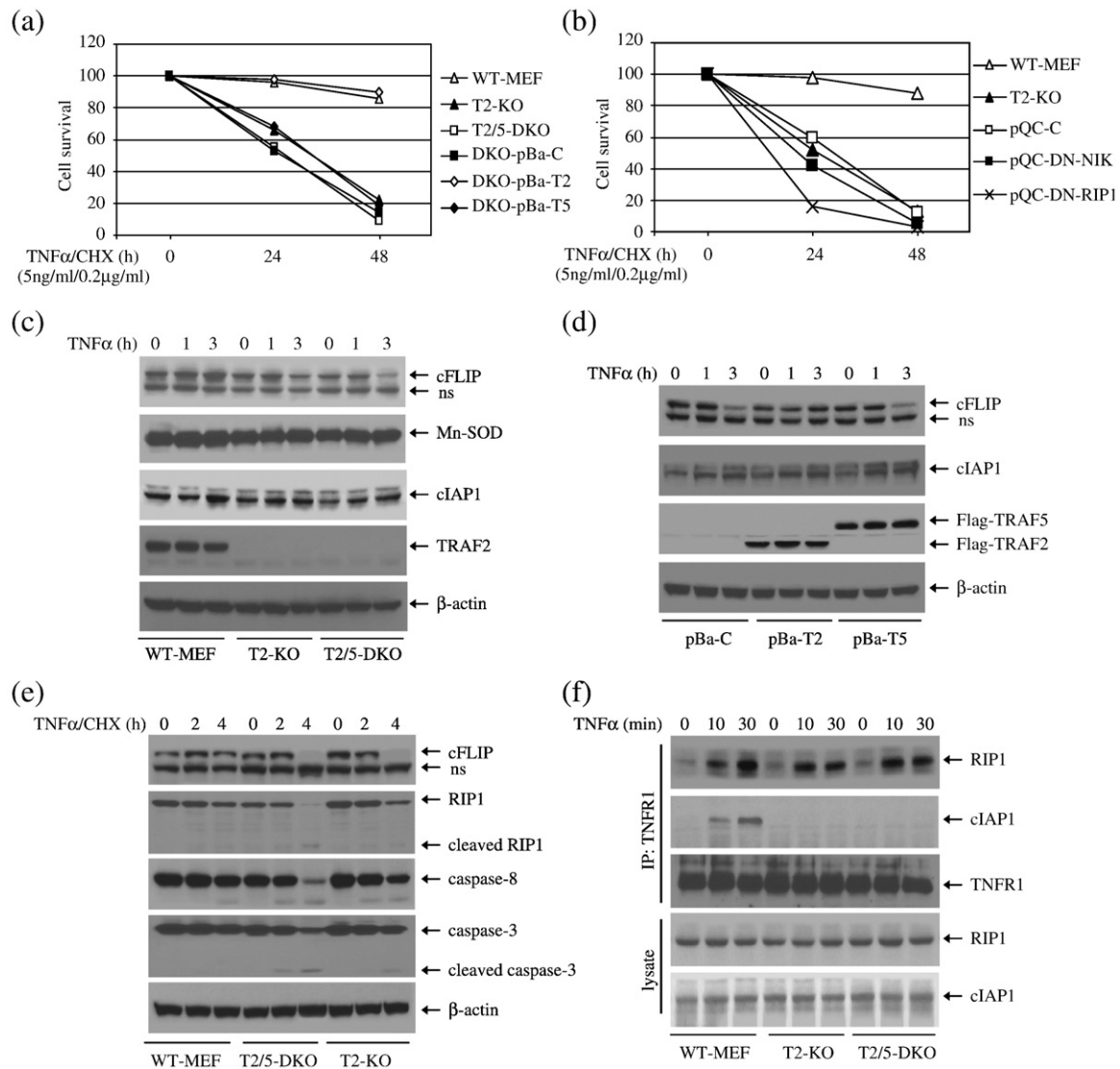


Fig. 6. TRAF2 inhibits TNF α -induced cell death by indirectly inhibiting caspase-8 activation and cFLIP degradation. (a and b) The indicated cells were untreated or co-treated with mTNF α (5 ng/ml) and CHX (0.2 μ g/ml), and 24 and 48 h after treatment, the rate of cell death was assessed via the trypan blue exclusion assay. The data shown represent the average of three experiments performed in triplicate. (c) WT, T2 KO, and T2/5 DKO MEFs were untreated or treated with mTNF α (10 ng/ml) for 1 and 3 h, after which the expression levels of cFLIP, Mn-SOD, cIAP1, and TRAF2 were monitored by Western blotting. (d) pBa-C, pBa-T2, and pBa-T5 cells were left untreated or treated with mTNF α (10 ng/ml) for 1 and 3 h, after which the expression levels of cFLIP, cIAP1, TRAF2, and TRAF5 were monitored by Western blotting. (e) WT, T2 KO, and T2/5 DKO MEFs were left untreated or treated with mTNF α (5 ng/ml) and CHX (0.2 μ g/ml) for 2 and 4 h, after which the expression and cleavage of cFLIP, RIP1, caspase-8, and caspase-3 were monitored by Western blotting. (f) WT, T2 KO, and T2/5 DKO MEFs were left untreated or treated with mTNF α (10 ng/ml) for 10 and 30 min, after which the TNFR1 complexes were immunoprecipitated with anti-TNFR1 antibody. The recruitment of RIP1 and cIAP1 to the TNFR1 complexes was then monitored by Western blotting.

inhibited TNF α -induced cell death, suggesting that TRAF2 plays a primary role in the inhibition of TNF α -induced cell death. Although expression of DN-NIK suppressed basal IKK activity and increased inducible IKK activity in T2/5 DKO MEFs, it had no effect on TNF α -induced cell death in these cells (Fig. 6b). In contrast, expression of DN-RIP1 significantly sensitized T2/5 DKO MEFs to TNF α /CHX-induced cell death.

Mn-SOD, cIAP1, and cFLIP are among the anti-apoptotic proteins that inhibit TNF α -induced cell

death by scavenging reactive oxygen species (ROS) or by blocking caspase activation.² Therefore, we analyzed the expression of these anti-apoptotic proteins by Western blotting. As shown in Fig. 6c, the basal expression levels of Mn-SOD, cIAP1, and cFLIP are comparable among WT, T2 KO, and T2/5 DKO MEFs. When each cell type was stimulated with TNF α for 1 or 3 h, cIAP1 and Mn-SOD protein levels were slightly increased in all cell types, whereas cFLIP protein levels were increased in WT-MEFs but reduced in both T2 KO and T2/5 DKO

MEFs. Given that the cFLIP mRNA level increased upon TNF α stimulation in all these cells, the decrease in cFLIP protein levels in T2 KO and T2/5 DKO MEFs must be due to post-translational cleavage and degradation.²¹ To further examine the expression of these proteins in the same genetic background, we analyzed the expression of these proteins in T2/5 DKO MEFs that stably express empty vector (pBa-C), TRAF2 (pBa-T2), or TRAF5 (pBa-T5). As expected, the cIAP1 protein level was slightly increased in all cell types, whereas the cFLIP protein level was increased in pBa-T2 cells but decreased in pBa-C and pBa-T5 cells (Fig. 6d). These findings are consistent with the results that stable expression of TRAF2, but not that of TRAF5, inhibits TNF α -induced cell death in T2/5 DKO MEFs (Fig. 6a). These data suggest that TRAF2, but not TRAF5, plays a primary role in the inhibition of TNF α -induced cell death and that the anti-apoptotic role of TRAF2 is independent of NF- κ B activation.

TRAF2 inhibits TNF α -induced cell death by recruiting anti-apoptotic proteins to the TNFR1 complex

Caspase-8 is essential for TNF α -induced cell death.^{4,22} Therefore, we examined caspase-8 activation, as well as the cleavage of its well-known substrate RIP1, by Western blotting. Indeed, treatment of cells with TNF α in the presence of 0.2 μ g/ml CHX clearly induced caspase-8 activation and led to the subsequent cleavage of RIP1 in both T2 KO and TRAF2/5 DKO MEFs but did not have a significant effect on this in WT MEFs (Fig. 6e). In addition, caspase-3 activation was also clearly detected in T2 KO and T2/5 DKO MEFs. NF- κ B-dependent expression of TRAF2, cIAP1, and cIAP2 suppresses TNF α -induced caspase-8 activation, and TRAF2 and cIAP1 are components of the

TNFR1 signaling complex.^{23,24} Therefore, we further examined the recruitment of cIAP1 and cIAP2 to TNFR1 following TNF α stimulation. As shown in Fig. 6f, cIAP1 was recruited to TNFR1 in WT MEFs but not in T2 KO and T2/5 DKO MEFs. Consistently, stable expression of TRAF2, but not that of TRAF5, in T2/5 DKO MEFs restored TNF α -induced cIAP1 recruitment to the TNFR1 complex (Fig. S10). We were not able to reproducibly detect endogenous cIAP2 expression in MEFs by Western blotting. Thus, it is not clear whether cIAP2 is also recruited to TNFR1 in a TRAF2-dependent manner. Nevertheless, these data suggest that TRAF2 inhibits caspase-8 activation by recruiting cIAP1 (and possibly also cIAP2) to the TNFR1 complex following TNF α stimulation.

TNF α -induced RIP1 ubiquitination is impaired in both T2 KO and T2/5 DKO MEFs

RIP1 is ubiquitinated immediately upon TNF α stimulation, and this ubiquitination is believed to be catalyzed by TRAF2.^{6,7,25} We found that TNF α -induced RIP1 ubiquitination is indeed reduced dramatically, but not impaired completely, in T2/KO and T2/5 DKO MEFs (Fig. 7a). As NIK knockdown restored TNF α -induced IKK activation in T2/5 DKO MEFs, we next analyzed RIP1 ubiquitination in T2/5 DKO MEFs following NIK knockdown. As shown in Fig. 7b, the knockdown of NIK neither increased nor decreased TNF α -induced RIP1 ubiquitination in T2/5 DKO MEFs. TNF α -induced recruitment of TNFR-associated death domain (TRADD) and IKK β to the TNFR1 complex also occurred equally in these cells regardless of the level of NIK protein. This suggests either that RIP1 ubiquitination has no role in TNF α -induced NF- κ B activation or that this pathway is redundant with another that does not require RIP1 ubiquitination.

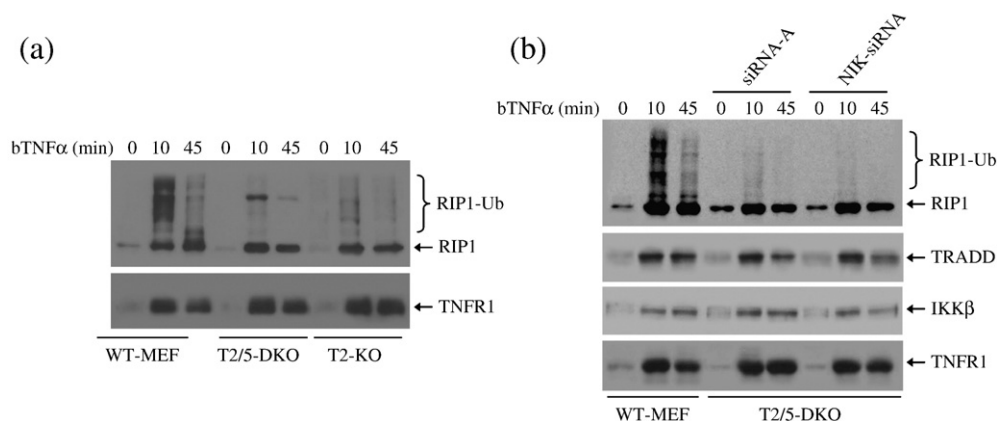


Fig. 7. TRAF2 is involved in TNF α -induced RIP1 ubiquitination *in vivo*. (a) WT, T2 KO, and T2/5 DKO MEFs were left untreated or treated with bTNF α (100 ng/ml) for 10 and 45 min, after which the TNFR1 complexes were pulled down with the aid of Streptavidin-coupled Dynabeads. RIP1 ubiquitination was then monitored by Western blotting using anti-RIP1 antibody. The same membrane was stripped and reprobed with anti-TNFR1 antibody. (b) T2/5 DKO MEFs were transfected with NIK-specific or control siRNA-A using Lipofectamine 2000. At 68 h after transfection, cells were left untreated or treated with bTNF α (100 ng/ml) for 10 and 45 min, after which the TNFR1 complexes were pulled down as described above. The ubiquitination of RIP1 and the recruitment of TRADD and IKK were then monitored by Western blotting.

Discussion

Currently, it is widely accepted that TRAF2- and TRAF5-mediated K63-linked RIP1 ubiquitination is essential for TNF α -induced NF- κ B activation.²² We report here that (i) TRAF2 suppresses basal IKK complex activity in resting cells by inhibiting NIK activity, (ii) TNF α can also activate the NF- κ B pathway in the absence of TRAF2 and TRAF5 expression and RIP1 ubiquitination, and (iii) TRAF2 inhibits TNF α -induced cell death by recruiting cIAP1 to the TNFR1 complex rather than by activating the NF- κ B pathway. In fact, our conclusions are supported directly or indirectly by the following published findings: (i) TNF α -induced RIP1 ubiquitination is impaired in T2 KO MEFs,²⁵ but TNF α -induced NF- κ B activation is not abrogated in TRAF2-deficient cells, although these cells are still sensitive to TNF α -induced cell death,^{11,12} and (ii) TRAF2 inhibits TNF α -induced cell death by recruiting cIAP1 to the TNFR1 complex.²⁶ Recent gene KO studies have revealed that in TRADD-deficient macrophages, RIP1 is still recruited to TNFR1 in response to TNF α stimulation but is not efficiently ubiquitinated due to the lack of TRAF2 recruitment to TNFR1 in these cells. Surprisingly, such RIP1 recruitment to TNFR1 in TRADD-deficient macrophages is sufficient to activate IKK in the absence of K63-linked polyubiquitination.^{27,28} Bertrand *et al.* have recently reported that cIAP1/2 constitutively target RIP1 for ubiquitination in cancer cells.²⁹ It has now been well established that TRAF2 directly associates with and recruits cIAP1/2 to the TNFR1 complex following TNF α stimulation.^{22,30–32} Thus, no matter whether TRAF2/5 or cIAP1/2 catalyze RIP1 ubiquitination, neither TRAF2/5 nor cIAP1/2 are present in the TNFR1 complex in T2/5 DKO MEFs. Therefore, the lack of RIP1 ubiquitination in T2/5 DKO MEFs following TNF α stimulation is not surprising. Collectively, all these published findings and our data presented here suggest that efficient RIP1 recruitment to TNFR1 is sufficient to activate IKK in the absence of TRAF2/5 expression and K63-linked RIP1 polyubiquitination.

Studies using a transient overexpression system have shown that TRAF2, TRAF5, and TRAF6 can positively regulate the canonical NF- κ B pathway.^{1,22} However, gene KO studies have revealed that TRAF5 deficiency has no effect on TNF α -induced JNK and IKK activation, whereas TRAF2 deficiency abolishes TNF α -induced JNK, but not NF- κ B, activation.^{11,14} The conclusion that KO of TRAF2 and TRAF5 abrogates TNF α -induced NF- κ B activation was based on an analysis of I κ B α protein level in these cells.¹⁴ In WT MEFs, TNF α -induced I κ B α phosphorylation is robust and immediate, resulting in complete degradation of I κ B α within 30 min. I κ B α protein returns to a normal level within 60 min after TNF α stimulation, because the expression of I κ B α itself is induced by NF- κ B. If, however, the IKK complex is constitutively activated, I κ B α will be constitutively phosphorylated, degraded, and

resynthesized, which will partially mask stimulation-induced immediate and complete I κ B α degradation. Our data show that this is in fact what happens in T2/5 DKO MEFs upon TNF α stimulation. Therefore, the incomplete degradation of I κ B α in T2/5 DKO MEFs following TNF α exposure is not caused by impaired IKK activation but by the constitutive degradation and resynthesis of I κ B α prior to stimulation. The IKK immunokinase assay we have used is one of the most sensitive and reliable methods for assessing the activation of the canonical NF- κ B pathway, and it clearly demonstrates that basal IKK activity is elevated in T2 KO and T2/5 DKO MEFs and that stable expression of TRAF2, but not that of TRAF5, suppresses basal IKK activity in T2/5 DKO MEFs. In addition, we demonstrate the dependence of this elevated basal IKK activity on NIK, by showing that either the expression of DN-NIK or the knockdown of NIK in T2/5 DKO MEFs reduces the elevated basal IKK activity and increases TNF α -induced immediate activation of IKK (Fig. 5b and f). The fact that treatment of T2 KO and T2/5 DKO MEFs with proteasome inhibitors makes I κ B α phosphorylation detectable in these cells but that the same does not hold true in WT MEFs lends further support to the notion that IKK is constitutively activated and I κ B α is constitutively phosphorylated and degraded in T2 KO and T2/5 DKO MEFs (Fig. 4d). Moreover, our demonstration that the expression of DN-RIP1 in T2/5 DKO MEFs blocks TNF α -induced I κ B α degradation and the expression of NF- κ B target genes suggests that TNF α -induced expression of NF- κ B target genes in T2/5 DKO MEFs depends on the RIP1-mediated canonical NF- κ B pathway. Therefore, we conclude that TRAF2 and TRAF5 are not essential for TNF α -induced activation of the canonical NF- κ B pathway.

Our analysis of NF- κ B-dependent gene expression by real-time PCR revealed that cIAP1 and cFLIP are normally expressed in T2/5 DKO MEFs, whereas the basal and inducible expressions of I κ B α , IP-10, ICAM-I, and RANTES were significantly increased in T2/5 DKO MEFs compared with those in WT MEFs. Expression of cIAP1 and cFLIP is induced primarily by the canonical NF- κ B pathway, whereas the expression of I κ B α , IP-10, ICAM-I, and RANTES is regulated by both the canonical and noncanonical NF- κ B pathways.^{22,24,33,34} Thus, the two pathways synergistically induce the expression of I κ B α , IP-10, ICAM-I, and RANTES in T2/5 DKO MEFs in response to TNF α stimulation, thereby resulting in significantly elevated expression of these genes in T2/5 DKO MEFs. In contrast, IL-6 induction by TNF α was almost completely impaired in T2/5 DKO cells, and stable expression of TRAF2 fully restored TNF α -induced IL-6 expression (Fig. 3c). Likewise, TNF α -induced JNK activation was impaired in T2/5 DKO MEFs and restored by TRAF2 expression. These data suggest that TNF α -induced IL-6 expression is also controlled by c-Jun activity. At least it is clear that NF- κ B alone is not sufficient to transactivate IL-6 expression in MEFs.

The efficient induction of IL-6 depends on both c-Jun and NF- κ B activities, as inhibition of either pathway almost completely abolishes TNF α -induced IL-6 expression (Fig. 3f and g). This is supported by the fact that TNF α -induced IL-6 expression is impaired in both JNK1/2 DKO and p65 KO MEFs.^{18,19} Many TNF α -inducible genes have both NF- κ B and c-Jun binding sites.⁴ Therefore, efficient up-regulation of some immediate genes, such as IL-6, in response to TNF α might be dependent on both NF- κ B and c-Jun activities, while the inducible expression of other genes, such as ICAM-1, might be regulated primarily by NF- κ B activity. Based on our data, we now believe that in TRAF2-deficient macrophages, the basal NF- κ B activity is elevated and TNF α -induced NF- κ B activation is not impaired. This explains why TRAF2-deficient macrophages overproduce TNF α and nitric oxide in response to TNF α stimulation.¹²

A recent report has shown that in TRAF2-deficient B cells, CD40 fails to effectively activate the canonical NF- κ B pathway.¹³ This conclusion was also based on an analysis of I κ B α phosphorylation and degradation following the treatment of cells with anti-CD40 antibody. TNFR1 can recruit RIP1 independent of TRAF2 expression, and both TRAF2 and RIP1 can independently recruit IKK to the TNFR1 complex.^{4,22} On the other hand, CD40 and TNFR2 directly associate with TRAF2 but not with RIP1. Therefore, it is possible that in the absence of TRAF2/5, TNFR1 is still able to recruit RIP1 and IKK and can thereby activate the canonical NF- κ B pathway. However, in the absence of TRAF2, CD40 may not be able to recruit IKK and activate NF- κ B.

Activation of the canonical NF- κ B pathway is robust and transient, whereas activation of the noncanonical NF- κ B pathway is slow and prolonged. The canonical NF- κ B pathway is subject to strong negative feedback regulation, which involves the recruitment of TRAF1, A20, and cIAP1/2 to the TNFR1 complex, dephosphorylation of IKK at its T-loop, cleavage and degradation of p65, rapid resynthesis of I κ B α , and so on.^{5,22} Therefore, it is possible that the negative regulators of the canonical NF- κ B pathway are also activated constitutively, at least at some level, in T2/5 DKO MEFs. This would partially explain why p52, but not p65, accumulates in the nuclei of T2/5 DKO MEFs (Fig. S5), even though the IKK complex is constitutively activated in these cells. Notably, although the IKK complex is constitutively activated in T2/5 DKO MEFs, it is not activated maximally. Thus, TNF α stimulation results in a further increase in IKK activity, which leads to inducible expression of NF- κ B-dependent genes, such as RANTES and ICAM-1. The constitutive activation of the IKK complex in T2/5 DKO MEFs is, at least partially, due to the accumulation and/or activation of NIK. Thus, NIK knockdown down-regulates the activities of both positive and negative regulators, thereby rendering the cells more responsive to TNF α stimulation.

In T2/5 DKO MEFs, TNF α triggers the cleavage and degradation of cFLIP and ectopic expression of cFLIP inhibits TNF α -induced cell death,²¹ suggest-

ing that cFLIP degradation is a critical event that leads to cell death in T2/5 DKO MEFs. cFLIP is an enzymatically inactive homolog of caspase-8 and is cleaved by caspase-8.³⁵ Most recently, two studies have clearly demonstrated that small-molecule IAP antagonists induce cIAP1/2 degradation and TNF α -dependent apoptosis, as well as NF- κ B activation,^{30,31} suggesting that NF- κ B activation is not sufficient to inhibit TNF α -induced cell death in the absence of cIAP1/2. As TRAF2 does not directly inhibit caspase-8 activity, activation of caspase-8 in T2/5 DKO MEFs is most likely due to the impaired recruitment of anti-apoptotic proteins, such as cIAP1, to the TNFR1 complex. However, such caspase-8 activation is not sufficient to trigger apoptotic cell death, as the majority of T2/5 DKO MEFs undergo necrotic cell death upon TNF α treatment.¹⁷ cFLIP suppresses the prolonged phase of JNK activation and ROS accumulation induced by TNF α .²¹ cFLIP also inhibits TNF α -induced cell death by directly associating with and inhibiting the full activation of caspase-8.³⁵ Therefore, in T2 KO and T2/5 DKO MEFs, caspase-8-mediated cleavage and proteasome-dependent degradation of cFLIP result in prolonged activation of JNK and the accumulation ROS, events that culminate in necrotic and apoptotic cell death. Notably, we repeatedly observed that although TNF α -induced cFLIP degradation was comparable in the T2 KO and T2/5 DKO MEFs, RIP1 cleavage and caspase-3 activation were more prominent in T2/5 DKO MEFs (Fig. 6e). Moreover, these DKO MEFs were more susceptible to TNF α -induced cell death than the T2 KO MEFs. This suggests that although TRAF5 does not recruit cIAP1 to TNFR1, it plays a certain role in inhibiting TNF α -induced cell death. However, the anti-apoptotic function of TRAF5 is marginal compared with that of TRAF2. IKK β ^{-/-} and p65^{-/-} cells are also sensitive to TNF α -induced cell death.²² Therefore, based on our data and on findings that have been published by others, we propose that two events are required to inhibit TNF α -induced cell death: (i) NF- κ B-dependent expression of anti-apoptotic proteins, such as cIAP1/2 and cFLIP, and (ii) TRAF2-mediated recruitment of anti-apoptotic proteins to the TNFR1 signaling complex. Ablation of either event sensitizes cells to TNF α -induced cell death.

Materials and Methods

Cell lines, plasmids, and reagents

HeLa, WT MEF, T2 KO MEF, and T2/5 DKO MEF cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum and antibiotics. Antibodies and reagents were purchased as follows: anti-TRAF2, anti-JNK, anti-IKK γ , anti-IKK β , anti-cIAP1, anti-Mn-SOD, and anti-NIK antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phospho-JNK antibody from Promega; anti-cFLIP Ab from Alexis (San Diego, CA); anti-I κ B α , anti-caspase-8, and anti-caspase-2

antibodies from Cell Signaling (Danvers, MA); mouse TNF α (mTNF α) and human TNF α from Roche (Indianapolis, IN); anti-Flag Ab from Sigma Chemicals (St. Louis, MO); and cocktail inhibitors of proteases and phosphatases from Pierce (Rockford, IL). siRNAs for mouse NIK, RIP1, and control siRNA-A (with scrambled sequences that will not lead to the specific degradation of any known cellular mRNA) were purchased from Santa Cruz Biotechnology. Constructs encoding 2 \times NF- κ B-Luc and Jun2-Luc reporter genes and Flag-TRAF2 have been previously described.⁹ pCDNA3-TRAF5 and PEAK12-RIP1 plasmids were generously provided by Dr. Adrian Ting (Mount Sinai Medical Center, New York, NY), and the DN pCDNA3-NIK-KK429/430AA plasmid was provided by Dr. Gail Bishop (University of Iowa). The retroviral plasmids pBabe-Flag-TRAF2, pBabe-Flag-TRAF5, pQCXIH-DN-NIK, and pQCXIH-DN-RIP1 (encoding the C-terminal 529–671 aa region) were generated by subcloning the TRAF2, TRAF5, DN-NIK, and RIP1-529–671 cDNAs into the pBabe-puro or pQCXIH-hygro plasmid.

Luciferase reporter gene assays

Cells cultured in six-well plates were transfected with an NF- κ B or a c-Jun firefly luciferase reporter plasmid (NF- κ B-Luc or Jun2-Luc; 0.2 μ g) together with a Flag-TRAF2 (0.2 μ g) and a Renilla luciferase reporter plasmid (pRL-TK; 0.01 μ g) using Lipofectamine 2000 reagents. Thirty-six hours after transfection, test cells were treated with human TNF α (10 ng/ml) or mTNF α (5 ng/ml), and protein samples were prepared at 6 h (HeLa) or 4 h (MEFs) after treatment. The firefly and Renilla luciferase activities were then measured by using the Dual-Luciferase Assay System according to the manufacturer's instructions (Promega).

JNK and IKK immunokinase assays

MEF cells were treated with mTNF α (10 ng/ml), and protein samples were extracted using TNE lysis buffer (20 mM Hepes, pH 7.4, 350 mM NaCl, 1% Triton X-100, 1 mM DTT, 1 mM ethylenediaminetetraacetic acid, 20% glycerol, and a cocktail of protease and phosphatase inhibitors). Endogenous JNK1 or IKK complex was immunoprecipitated using anti-JNK1 or anti-IKK γ antibody and then subjected to *in vitro* kinase assays, in which GST-Jun^{1–87} (for JNK) or GST-I κ B α ^{1–55} (for IKK) served as a substrate, as described previously.⁹

Preparation of retroviral supernatants and infection of T2/5 DKO MEFs

293T cells at 60%–70% confluence were co-transfected with 2 μ g of pMD.OGP (encoding gag-pol), 2 μ g of pMD.G (encoding vesicular stomatitis virus G protein), and 2 μ g of pBabe-Flag-TRAF2, -TRAF5, pQCXIH-DN-NIK, or pQCXIH-DN-RIP1 by the standard calcium phosphate precipitation method. Forty-eight hours after transfection, the viral supernatant was collected and filtered through a 0.45- μ m filter. TRAF2 retroviral supernatant was diluted one-, two-, three-, and fourfold with 10% fetal bovine serum/Dulbecco's modified Eagle's medium and then immediately used for the infection of T2/5 DKO MEFs in the presence of 4 μ g/ml polybrene for 6 h. Forty-eight hours after infection, cells were selected with puromycin (2.0 μ g/ml) for 6 days, and resistant cells were pooled together and frozen. Cells that stably express Flag-TRAF2 at the physiological level (Fig. S3; threefold) were used for

the functional experiments within 1 month of establishment. TRAF5 retroviral supernatant was diluted threefold before being used for the infection of T2/5 DKO MEFs. DN-NIK and DN-RIP1 retroviral supernatants were not diluted and were used immediately for the infection of T2/5 DKO MEFs.

Real-time PCR

MEF cells were treated with mTNF α (10 ng/ml), and total RNA was prepared using an RNeasy Mini Kit (Qiagen). Five micrograms of total RNA was treated with RQ1 RNase-free DNase for 30 min at 37 °C and then reverse transcribed using an oligo-dT primer. The resulting cDNA was subjected to quantitative real-time PCR using a Power SYBR Green AB Master Mix and an ABI Prism 7700 Sequence Detector (Applied Biosystems). Mouse GAPDH-specific primers were used to generate an internal control, and the average threshold cycle (C_T) for samples in triplicate was used in the subsequent calculations. Relative expression levels of NF- κ B target genes were calculated as the ratio with respect to the GAPDH expression level. The mean \pm SE of four independent experiments was considered to be statistically significant at $p < 0.05$. Real-time PCR products were also separated on agarose gel to confirm the presence of single bands (Fig. S11). All primer pair sets were designed to flank an intron (Table S1).

RIP1 ubiquitination

TNFR1 complex was immunoprecipitated using biotinylated mTNF α (bTNF α) in combination with Streptavidin-coupled Dynabeads (Invitrogen). Recombinant mTNF α was biotinylated using Sulfo-NHS-LC-Biotin (Pierce) at 1 mg/ml for 1 h according to the manufacturer's instructions. Unincorporated biotin was removed from bTNF α by buffer exchange into phosphate-buffered saline on PD-10 columns (Amersham). The biological activity of bTNF α was determined by its apoptosis-inducing capacity and found to be comparable with nonbiotinylated mTNF α . MEFs (cells in four 100 mm plates each at 90–95% confluency per treatment) were treated with bTNF α for 10 and 45 min, and then cells were washed twice with ice-cold phosphate-buffered saline and lysed in TNE lysis buffer (containing 0.5% Chaps and 5 mM N-ethyl maleimide) on ice for 30 min followed by centrifugation at 13,000g for 20 min at 4 °C. The TNFR1 complex was then precipitated using 30 μ l of Streptavidin-coupled Dynabeads at 4 °C for 4 h. Precipitates were washed four times with the same lysis buffer containing 2 mM N-ethyl maleimide. RIP1 recruitment to TNFR1 and its ubiquitination were then monitored by Western blotting.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jmb.2009.04.054](https://doi.org/10.1016/j.jmb.2009.04.054)

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