Published in final edited form as:
Mol Cell. 2009 January 16; 33(1): 30–42. doi:10.1016/j.molcel.2008.11.023.

PKC phosphorylation of TRAF2 mediates IKKα/β recruitment and K63-linked polyubiquitination

Shitao Li[1,2], **Lingyan Wang**[1,2], and **Martin E. Dorf**[1,*]

1 *Department of Pathology, Harvard Medical School, Boston, MA 02115 USA*

Summary

Tumor necrosis factor (TNF) receptor-associated factor 2 (TRAF2) is a key mediator in TNF signaling. Previous studies suggested that TRAF2 functions as an adaptor in the NF-κB and AP-1 pathways. However, the precise molecular mechanisms by which TRAF2 relays signals are unknown. We previously reported that TRAF2 is phosphorylated following TNF stimulation and now identify the PKC kinases responsible for phosphorylation. Phosphorylated TRAF2 facilitates recruitment of IKKα and IKKβ to the TNF receptor. Phosphorylation also determines K63-linked polyubiquitination of TRAF2 at lysine 31. TRAF2 K63-linked ubiquitination contributes to associations with TAB2/3 and activation of the downstream IKK and JNK kinases. The combined data reveal that phosphorylation of TRAF2 plays a critical role in TNF signaling by directing the IKK complex to the membrane, promoting TRAF2 K63-linked ubiquitination, and positioning the IKKα and IKKβ chains with the TAK1/TAB kinase.

Introduction

The evolutionarily conserved transcription factor nuclear factor κB (NF-κB) plays a pivotal role in development, inflammation, immune responses, and cell survival (Gilmore, 2006). Stimulation of the tumor necrosis factor receptor I (TNFR1) with the proinflammatory cytokine TNFα is a prototypic model of NF-κB activation. The TNF receptors lack intrinsic enzymatic activity; thus signaling is achieved by recruitment of intracellular adaptors including TNFR1-Associated Death Domain (TRADD), TNF receptor associated factor 2 (TRAF2), and receptor interacting protein 1 (RIP1) (Hayden and Ghosh, 2008). Following TNFα treatment the intracellular adaptors recruit and activate the IκB-kinase (IKK) complex. The IKK complex is composed of two catalytic subunits, IKKα and IKKβ, and a regulatory chain called IKKγ/NEMO. IKK phosphorylation of IκB proteins leads to their K48-linked polyubiquitination and subsequent degradation allowing the freed NF-κB molecules to translocate to the nucleus and regulate the transcription of target genes.

Like most TRAFs, TRAF2 has distinct domains that are involved in interactions with other proteins and signaling functions. The two TRAF subdomains (TRAF-N and TRAF-C) independently mediate self-association, interaction with TNF receptors, and aggregation with other TRAF molecules while the N-terminal RING finger and two adjacent zinc fingers are required for NF-κB activation (Rothe et al., 1995). Although it is clear that TRAF2 plays an

* Contact: dorf@hms.harvard.edu; phone 617-432-1978; FAX 617-432-2789.
[2]These authors contributed equally to this work.

important role in TNF signaling, cells from TRAF2-deficient mice showed only modest impairment in TNFα-induced NF-κB activation (Yeh et al., 1997), suggesting that alternative signaling pathways may compensate for TRAF2 deficiency. Cells from TRAF2 and TRAF5 double knockout mice demonstrate severely impaired TNFα-induced NF-κB activation, implying that TRAF5 may compensate for TRAF2 deficiency (Tada et al., 2001).

Phosphorylation and ubiquitination are two major post-translational modifications in NF-κB pathway. Phosphorylation has been shown to regulate various components including IκB and NF-κB (Hayden and Ghosh, 2004). In recent years the role of proteasome-independent K63-linked polyubiquitination has also been documented for various components in the NF-κB signaling pathway. One of the best characterized examples is the signal-induced K63-linked polyubiquitination of TRAF6 which serves as a scaffold to facilitate the interaction with the TAK1 complex required for IKK phosphorylation and activation (Adhikari et al., 2007).

Several post-translational modifications of TRAF2 are also involved in TNFα signaling. Upon TNFα stimulation, TRAF2 undergoes K48 and K63-linked ubiquitination (Habelhah et al., 2004; Shi and Kehrl, 2003; Wu et al., 2005). We previously found that TRAF2 is phosphorylated following TNFα stimulation and phosphorylation is needed for NF-κB activity (Li et al., 2006). However, the mechanisms underlying TRAF2 phosphorylation and ubiquitination, and how these post-translational modifications mediate downstream events remain unknown. In this report, we identify the PKC kinases responsible for TRAF2 phosphorylation and demonstrate phosphorylation of TRAF2 at Thr^{117} promotes recruitment of the catalytic IKKα and IKKβ chains. In addition, phosphorylation determines K63-linked polyubiquitination of TRAF2. K63-linked polyubiquitination at lysine 31 contributes to TRAF2 associations with the TAK1 binding TAB2/3 adaptors and subsequent activation of the downstream IKK and JNK kinases.

Results

TRAF2 phosphorylation is induced by TNF and required for NF-κB activation

To examine the role of TRAF2 phosphorylation we infected *traf2*-/- mouse embryonic fibroblasts (MEFs) with retrovirus expressing either TRAF2 or the TRAF2 phosphorylation deficient mutant, TRAF2(T117A). Reconstituted MEFs expressed levels of TRAF2 protein comparable to endogenous TRAF2 (Supplementary Figure 1A). Antibodies prepared against a phospho-TRAF2 peptide detected phosphorylated TRAF2 only after TNF stimulation in MEFs reconstituted with wild type TRAF2 but not with T117A mutant (Figure 1A).

Since NF-κB signaling depends on activation of the IKK complex (Delhase et al., 1999), we examined the effect of TRAF2 phosphorylation on IKK activation. TRAF2 knockout cells and stable cell lines expressing wild-type or mutant TRAF2 were stimulated with TNFα, then the IKK complex was immunoprecipitated and assayed for IKK kinase activity. In wild-type MEFs IKK activity was detected 5 min after TNFα stimulation, but the appearance of kinase activity was delayed in TRAF2 knockout cells (Figure 1B). Importantly, wild type TRAF2, but not the T117A point mutation was able to rescue IKK activation in reconstituted *traf2*-/- cells (Figure 1B). Since TRAF5 can compensate for TRAF2 we suppressed TRAF5 mRNA by siRNA (Supplementary Figure 1B). After TRAF5 was silenced with siRNA TNF-induced IKK activation was found in wild type and TRAF2 reconstituted cells but abolished in *traf2*-/- cells and TRAF2(T117A) reconstituted cells (Figure 1C and Supplementary Figure 1C). Mutation of the Thr^{117} residue reduced the ability of ectopically expressed TRAF2 to activate NF-κB reporter activity (Supplementary Figure 1D). We further examined the effect of TRAF2 phosphorylation on two NF-κB-regulated genes, A20 and IL-6. mRNA expression of A20 and IL-6 was induced in wild type and TRAF2 reconstituted cells but blocked in *traf2*-/- cells and TRAF2(T117A) reconstituted cells (Figure 1D). Similar results were also found in cells treated

with TRAF5 RNAi (Supplementary Figure 1E). Furthermore, TNF-induced IκBα phosphorylation and degradation were inhibited in *traf2*-/- cells and TRAF2(T117A) reconstituted cells treated with TRAF5 RNAi (Figure 1E). These results indicate that TRAF2 phosphorylation at residue Thr^{117} is required for activation of IKK kinase and NF-κB activity in response to TNFα.

PKC phosphorylates TRAF2 at Thr^{117} site and promotes IKK activation

Ectopic expression of several kinases involved in NF-κB signaling including RIP1, IKK, MEKK3, NIK and TAK1 failed to phosphorylate TRAF2 (data not shown). Furthermore, TRAF2 phosphorylation at Thr^{117} was independent of IKKγ and RIP1 expression as demonstrated by phosphorylation of endogenous TRAF2 in IKKγ and RIP1 deficient cells (Supplementary Figure 2A and 2B). Based on predictions from the Scansite 2.0 program (Obenauer et al., 2003) and a report that PKCε participated in NF-κB signaling (Castrillo et al., 2001) we suspected PKCδ/ε involvement in the phosphorylation of TRAF2 at Thr^{117}. Overexpression of PKCδ or PKCε promoted TRAF2 Thr^{117} phosphorylation. In contrast, a dominant negative (DN) PKCε and PKCζ failed to phosphorylate TRAF2 (Figure 2A). *In vitro*, PKCδ and PKCε, but not PKCε, phosphorylated GST fused to a Thr^{117} motif (Figure 2B). The additional phosphorylated band in Figure 2B represents an established PKC site in GST [(Rodriguez et al., 2005) and data not shown]. PKCδ is also capable of phosphorylating full-length TRAF2 *in vitro* (Figure 2C). We next tested the effect of a PKC inhibitor on TRAF2 phosphorylation. Thirty minutes pretreatment with chelerythrine chloride blocked TNF-induced TRAF2 phosphorylation (Supplementary Figure 3A). Although TRAF2 phosphorylation was partially reduced and delayed in PKCε deficient MEFs (Figure 2D), TNF-induced TRAF2 phosphorylation was nearly abolished in PKCε deficient MEFs transfected with PKCδ siRNA oligos (Figure 2D and Supplementary Figure 3B) or a dominant negative PKCδ (data not shown). The combined results indicate that PKCδ and PCKε are involved in TRAF2 phosphorylation.

Both PKCδ and PKCε synergized with TRAF2 to enhance NF-κB reporter activity (Supplementary Figure 3C). Conversely, IKK kinase activity and IκBα phosphorylation were reduced and delayed in PKCε deficient cells transfected with PKCδ siRNA oligos (Figure 2D and Supplementary Figure 3D). Furthermore, mRNA expression of A20 and IL-6 were suppressed in PKCε deficient cells with PKCδ RNAi (Figure 2E). PKC phosphorylation of TRAF2 therefore promotes IKK and NF-κB activation.

Phosphorylated TRAF2 recruits IKKα and IKKβ

Previous studies reported that TRAF2 interacted with IKKα and IKKβ (Devin et al., 2000; Devin et al., 2001). We confirmed that endogenous TRAF2 coimmunoprecipitated with endogenous IKKα/β in response to TNFα stimulation (Figure 3A). To determine which domain of TRAF2 was involved we generated expression constructs for truncated TRAF2 proteins. Similar to a previous report (Devin et al., 2001) deletion of both the RING and zinc finger domains of TRAF2 abolished the association with IKKβ, whereas TRAF2 truncations carrying deletions in the RING or TRAF-C domains were still able to bind IKKβ although with reduced binding ability (Supplementary Figure 3E).

Since the Thr^{117} site is located in the first zinc finger of TRAF2, we suspected that Thr^{117} phosphorylation might be required for recruitment of IKKα/β. Indeed TRAF2 coimmunoprecipitated with IKKα and IKKβ, more strongly than with mutant TRAF2(T117A) (Figure 3B). Furthermore, TNFα stimulation promoted TRAF2 associations with IKKα and IKKβ (Figure 3B). In reciprocal coimmunoprecipitations IKKα and IKKβ associated with phosphorylated, but not the non-phosphorylatable form of TRAF2 (Supplementary Figure 3F and 3G). We also tested *traf2*-/- stable cell lines expressing either TRAF2 or the TRAF2

(T117A) mutant and found associations between TRAF2 and endogenous IKKα/β depended upon TNFα–induced Thr^{117} phosphorylation (Figure 3C).

To view TRAF2 associations with IKKβ in cells we first examined IKK and TRAF2 distribution in *traf2*-/- MEFs. Endogenous IKKα/β and TRAF2 were evenly distributed throughout the cell (Supplementary Figure 4A and data not shown). Ectopically expressed IKK was evenly distributed throughout the cell while TRAF2 or TRAF2(T117A) showed distinct aggregates in the cytoplasm of most cells when expressed in *traf2*-/- MEFs alone (Supplementary Figure 4B and 4C). Overexpression of TRAF2 resulted in the movement of endogenous IKKα/β and ectopically expressed IKKβ into TRAF2 aggregates (Supplementary Figure 4B and 4D). These TRAF2-IKKβ aggregates were observed in 50% of cells after TNFα treatment while there was little IKKβ aggregate formation (less than 10% of cells) with mutant TRAF2(T117A) (Supplementary Figure 4D and data not shown). We further used fluorescence resonance energy transfer (FRET) to examine the *in vivo* association between TRAF2 with IKKα/β. Strong FRET signals were detected in cells coexpressing CFP-IKKβ or CFP-IKKα with YFP-TRAF2 but not with YFP-TRAF2(T117A) (Figure 3D and Supplementary Figure 4E-4G).

Since phosphorylation of TRAF2 at Thr^{117} is required for IKKα and IKKβ recruitment *in vivo*, we next tested whether PKC phosphorylation of TRAF2 enhanced the ability to bind IKK. Indeed, overexpression of either PKCδ or PKCε enhanced the ability of TRAF2 to pull down IKKα and IKKβ in 293T cells (Supplementary Figure 3H and 3I). We also reconstituted FLAG-TRAF2 phosphorylation *in vitro* by addition of recombinant PKCδ, and then performed GST pull-down or immunoprecipitation with anti-FLAG antibody. TRAF2 phosphorylated by PKC exhibited strong IKKβ-binding capability while non-phosphorylated TRAF2 and TRAF2 (T117A) failed to pull down IKK (Figure 3E). Conversely, suppression of PKCδ expression in PKCε deficient cells inhibited the association of TRAF2 with IKKα and IKKβ (Figure 3F). Collectively the data indicate that phosphorylated Thr^{117} is required for recruitment of IKKα and IKKβ.

TNFR1 mediates TRAF2 phosphorylation and recruitment of the IKK complex

To examine the role of TNF receptors we first examined endogenous TRAF2 phosphorylation in TNFR1 knockout and wild type astrocytes. After TNFα stimulation TRAF2 phosphorylation was observed in wild type but not in *tnfr1*-/- astrocytes (Figure 4A). We also examined TRAF2 phosphorylation in *tnfr1*-/-; *tnfr2*-/- double knockout (DKO) MEFs transfected with hTNFR1 or hTNFR2. Consistently, only TNFR1 restored TNFα induced endogenous TRAF2 phosphorylation in DKO cells (Figure 4B).

A previous study found that TNFα stimulation resulted in IKK recruitment to TNFR1 and this association was mediated by TRAF2 (Devin et al., 2001). To determine if phosphorylation of TRAF2 is required for recruitment of the IKK complex to the receptor, we stimulated the various stable MEF cell lines with mTNFα and then isolated the TNFR1 complex using anti-TNFR1 antibody. As shown in Figures 4C and 4D, TNFα stimulation led to the recruitment of IKKα/β and IKKγ to TNFR1 in wild type and *traf2*−/− MEFs reconstituted with hTRAF2. However, neither IKKα/β nor IKKγ were recruited to TNFR1 in *traf2*−/− or TRAF2(T117A) reconstituted cells. Significantly, engagement of the TNF receptor recruited TRADD and RIP1 to TNFR1 in *traf2*-/- and both reconstituted MEFs. Importantly, PKCδ and PKCε were also recruited to the TNFR1 complex upon TNF stimulation in wild type, *traf2*−/−, and reconstituted MEFs (Figure 4C and 4D) suggesting PKC phosphorylation of TRAF2 occurs at the engaged TNFR1 complex. Mutant TRAF2 remained capable of binding to the TNFR1 complex in TRAF2(T117A) reconstituted cells (Figure 4D), indicating that mutation of Thr^{117} does not alter TRAF2 recruitment to the engaged receptor complex.

Phosphorylation determines TRAF2 K63-linked ubiquitination at lysine 31

To examine the effect of TRAF2 phosphorylation on RIP1 ubiquitination, RIP1 was cotransfected with either TRAF2 or TRAF2(T117A) into 293T cells. Both TRAF2 and the TRAF2(T117A) mutant promoted signal-induced RIP1 ubiquitination (Supplementary Figure 2C), indicating RIP1 ubiquitination is independent of TRAF2 phosphorylation.

Like RIP1, TRAF2 becomes polyubiquitinated following TNFα stimulation (Habelhah et al., 2004; Shi and Kehrl, 2003). To directly observe TRAF2 ubiquitination in cell lysates, we found an antibody specific for human TRAF2 that recognized ubiquitinated hTRAF2 (Supplementary Figure 5A and 5B). Using this antibody we found TNF induced rapid TRAF2 ubiquitination in 293T cells expressing hTRAF2, but ubiquitination was dramatically reduced in cells expressing hTRAF2(T117A) (Supplementary Figure 5C). Furthermore, ubiquitination was induced following TNFα stimulation of TRAF2 reconstituted *traf2*-/- MEFs, but was not detectable in T117A reconstituted *traf2*-/- MEFs (Figure 5A). These results indicate that TRAF2 polyubiquitination is phosphorylation dependent.

To determine the type of ubiquitin linked chains on phosphorylated TRAF2, we transfected FLAG-hTRAF2 or hTRAF2(T117A) into 293T cells with HA-tagged ubiquitin mutants containing only one lysine at either position 48 (K48) or 63 (K63). We also treated cells with the proteasome inhibitor MG132 to prevent degradation of K48-linked TRAF2. TRAF2 was more heavily coupled to K63 ubiquitin while the T117A mutant was primarily conjugated with K48 ubiquitin (Supplementary Figure 5D). To avoid the interference of endogenous ubiquitin, we immunoprecipitated TRAF2 with anti-FLAG antibody and blotted with anti-HA antibody to examine the conjugation of exogenously expressed ubiquitins with TRAF2. TRAF2(T117A) did not conjugate with ubiquitin mutants containing lysine at only position 63, but robustly coupled with K48 only ubiquitin mutants in cells treated with MG132 (Figure 5B). Conversely, TRAF2 conjugated with the K63 only ubiquitin construct and weakly conjugated with K48 only ubiquitin in MG132 treated cells (Figure 5B). The UbcH13/Uev1A complex has been identified as an E2 ubiquitin-conjugating enzyme for TRAF2 K63-linked ubiquitination (Deng et al., 2000). Ectopic expression of UbcH13 promoted polyubiquitination of wild type but not the TRAF2(T117A) mutant (Figure 5C).

To identify the acceptor site(s) for ubiquitin conjugation we tested the ability of mutants at conserved lysine residues to undergo polyubiquitination following TNFα stimulation (Supplementary Figure 5E). Lysine point mutations at K31 and K115 reduced TNF-induced TRAF2 polyubiquitination (Supplementary Figure 5F). We also individually introduced the TRAF2 lysine mutations into *traf2*-/- MEFs by retroviral infection and established stable cell lines. Unlike the mapping of lysine mutation by overexpression, only K31R point mutation abolished TNFα-induced polyubiquitination of TRAF2, whereas the K115R mutation had little effect and K119R potentiated polyubiquitination (Figure 5D). Furthermore, UbcH13 did not promote ubiquitination of the K31R mutant (Figure 5E). To determine if Lys^{31} was a K63-linked ubiquitin acceptor site we transfected FLAG-hTRAF2 or hTRAF2(K31R) into 293T cells along with the HA-tagged ubiquitin K63 mutant. K63-linked polyubiquitination was observed with wild type TRAF2 but not with the mutant following TNF stimulation (Figure 5F). These results indicate that Lys^{31} is the primary ubiquitin acceptor site of TRAF2 following TNFα stimulation.

Since TNF also induces TRAF2 degradation, we next examined which lysine(s) may function as acceptor sites for K48-linked ubiquitination. Because TRAF2 degradation occurs 2–6 h after TNF stimulation (Wu et al., 2005), it is difficult to detect TRAF2 ubiquitination directly in cell lysates. Thus, we coexpressed the HA-tagged ubiquitin K48 only mutant with various FLAG-tagged TRAF2 lysine mutants in 293T cells. After immunoprecipitation with anti-FLAG antibody, multiple lysine point mutations including K31R reduced TRAF2 K48-linked

polyubiquitination (Supplementary Figure 5G). These data suggest that K48-linked ubiquitin chains can potentially be assembled on multiple lysine residues in TRAF2, including Lys^{31}.

TAB2/3 binding to K63-linked polyubiquitin of TRAF2 is required for IKK and JNK activation

TAB2 and TAB3 are known to associate with TRAF2 (Hong et al., 2007; Ishitani et al., 2003). The homologous TAB2 and TAB3 molecules contain two ubiquitin binding domains (UBD), the CUE and zinc finger regions (Kanayama et al., 2004). The zinc fingers of TAB2 are required for TRAF2 association (Supplementary Figure 6A). We found the N-terminal 99 residues containing the RING domain of TRAF2 was also indispensable for TAB3 interaction (Supplementary Figure 6B). TAB2 and TAB3 can bind to the K63-linked polyubiquitin chains of RIP1, TRAF6, IKKγ, and MALT1 (Kanayama et al., 2004; Oeckinghaus et al., 2007). Therefore, we examined the effect of TRAF2 ubiquitination on recruitment of TAB2/3. When overexpressed in 293T cells TRAF2 coimmunoprecipitated with TAB2 and TAB3, while the TRAF2(T117A) and TRAF2(K31R) mutants failed to bind TAB2 or TAB3 (Figure 6A and 6B; Supplementary Figure 6C and 6D). Furthermore, TNFα stimulation promoted the association of TRAF2 with TAB2 and TAB3 (Figure 6A and 6B; Supplementary Figure 6C and 6D). We further tested *traf2*-/- stable cell lines expressing either TRAF2 or TRAF2(K31R). The associations between TRAF2 and endogenous TAB2 were dependent upon TNFα–induced polyubiquitination at Lys^{31} (Figure 6C).

Furthermore, TRAF2(K31R) is phosphorylated and capable of binding to IKKβ (Figure 6D). Analysis of the TNFR1 complex confirmed that TRAF2 and IKKβ were recruited to TNFR1 following TNF stimulation, however, the K31R mutant failed to recruit either TAB2 or TAK1 to the TNFR1 complex (Figure 6E). These data suggest TRAF2 ubiquitination plays a key role in recruitment of the TAK1/TAB kinase complex to TNFR1.

Since TRAF2 is required for IKK and JNK activation (Baud et al., 1999) we examined the effect of TRAF2 polyubiquitination on NF-κB and JNK activity. K31R mutation demonstrated markedly impaired NF-κB reporter activity while K115R mutation showed slightly reduced activity and other lysine mutations had activity comparable to wild type TRAF2 (Supplementary Figure 6E and 6F). K31R mutation also blocked endogenous A20 and IL-6 mRNA expression (Figure 7A) and IκBα phosphorylation (Figure 1E). Accordingly, K31R point mutation abolished the ability of TRAF2 to rescue IKK kinase activity in reconstituted *traf2*-/- cells and knockout cells treated with TRAF5 RNAi (Figure 7B). Similarly, K31R mutation impaired and delayed JNK kinase activity (Figure 7C) and JNK1/2 phosphorylation in TRAF2 reconstituted knockout cells (Figure 7D). Consistent to a previous report (Devin et al., 2003), TRAF2 deletion partially inhibited TNF-induced p38 and ERK activation (Figure 7D) but restoration of p38 and ERK phosphorylation did not depend upon Lys^{31} polyubiquitination or Thr^{117} phosphorylation (Figure 7D). The combined data demonstrate that ubiquitination at Lys^{31} of TRAF2 is required for TNF-induced IKK and JNK activation.

It has been shown that *traf2*-/- cells are sensitive to TNFα-induced cell death due to the delay of NF-κB activation and defective JNK activation (Yeh et al., 1997). To assess the effect of TRAF2 phosphorylation on TNF-induced cell death, we treated wild type MEFs and *traf2*-/- stable cell lines with cycloheximide (CHX) or TNF, or both. As reported before (Yeh et al., 1997), TRAF2 deficient MEFs are sensitive to the combination of TNF and CHX. Similarly, the viability of TRAF2(T117A) and TRAF2(K31R) reconstituted cells was reduced by 40%. In contrast, 5-10% of TRAF2 and TRAF2(K119R) reconstituted cells were dead after the treatment with TNF and CHX (Figure 7E). Thus, the absence of TRAF2 polyubiquitination sensitizes cells to TNF-induced cell death.

Discussion

Upon ligand binding TNFR1 recruits several signaling intermediates, including TRADD, TRAF2, and RIP1 to membrane microdomains prior to recruiting the IKK complex (Hayden and Ghosh, 2008). Recruitment and activation of the IKK complex is required for NF-κB activation. It has been suggested that TRAF2 and RIP1 account for IKK recruitment based on the direct interaction of TRAF2 with IKKα and IKKβ (Devin et al., 2001), and RIP1 with IKKγ (Zhang et al., 2000). A recent model proposed a detailed mechanism for IKK recruitment in which K63-linked polyubiquitinated RIP1 was a target of the ubiquitin binding protein IKKγ which draws the remaining members of the IKK complex to the TNFR1 receptor (Ea et al., 2006; Wu et al., 2006). But the mechanism by which TRAF2 recruited IKKα and IKKβ was unknown. Our study demonstrates that TNFα stimulation of TNFR1 induces PKC-mediated phosphorylation of TRAF2 at Thr^{117} which promotes association with IKKα/β resulting in recruitment of IKK to the membrane.

PKC is also involved in T cell receptor (TCR) and B cell receptor (BCR)-mediated NF-κB signaling. Engagement of TCR or BCR leads to recruitment and activation of PKCθ and PKCβ, respectively (Hacker and Karin, 2006). However, the mechanism by which TNF signaling activates PKCδ and PKCε is still elusive. Early reports identified phosphatidylcholine-specific phospholipase C (PC-PLC) as a signaling enzyme activated through TNF receptors (Machleidt et al., 1996; Schutze et al., 1992). Stimulation of PC-PLC activity degrades phosphatidylcholine into diacylglycerol, the physiological activator of PKCδ/ε. PKCδ associates with the TNFR1 complex after TNF stimulation (Kilpatrick et al., 2006) and PKCε are enriched in lipid rafts where the engaged TNFR1 complex resides (Dykstra et al., 2003). One potential mechanism for PKC mediated phosphorylation of TRAF2 is that after ligand binding TNFR1 oligomers relocate to lipid rafts where associations with adaptor molecules including TRAF2 are enhanced (Legler et al., 2003), activated PKCε may be prepositioned in these membrane microdomains awaiting their substrates.

In addition to phosphorylation, ubiquitination is another major post-translational modification of TRAF2. Crosstalk between phosphorylation and ubiquitination has been found in many proteins (Gao and Karin, 2005; Hunter, 2007). Phosphorylation can regulate ubiquitination by modulation of E3 ligase activity or facilitating E3 ligase recognition (Hunter, 2007). Like most TRAFs, TRAF2 has a RING domain which may function as an E3 ligase. TRAF2 itself and RIP1 are two possible TRAF2 substrates. However, *in vitro* autoubiquitination assays showed very little or no ubiquitination of TRAF2 (Li and Dorf, unpublished data; B. Darnay, personal communication). RIP1 ubiquitination is reportedly dependent on TRAF2 expression (Lee et al., 2004), but direct evidence demonstrating that TRAF2 acts as an E3 ligase to direct RIP1 ubiquitination is missing. Our data suggest that recruitment of RIP1 to the TNFR1 complex and RIP1 ubiquitination are independent of Thr^{117} phosphorylation of TRAF2 (Figure 4D and Supplementary Figure 2C). We also demonstrate that TRAF2 K63-linked ubiquitination is induced by TNFα and is coordinated by TRAF2 phosphorylation. Phosphorylation may provide a molecular context for selective targeting of the Lys^{31} site for K63-linked ubiquitination. Phosphorylation may also alter TRAF2 conformation to promote recognition of the UbcH13/Uev1A complex or to recruit another E3 ligase.

In the NF-κB system K63-linked ubiquitination provides various targeted proteins with an ability to engage in new protein interactions with molecules containing ubiquitin binding domains (UBD) (Hicke et al., 2005). In TNF signaling, UBDs are found in IKKγ and the TAK1 adaptor proteins TAB2 and TAB3. We now demonstrate that the K63 polyubiquitin chain conjugated to Lys^{31} of TRAF2 recruits TAB2/3 bridging the TAK1 complex to IKKα/β on a TNFR1 membrane platform (Figure 6E). However, K63-linked polyubiquitination is dispensable for TRAF2 recruitment of IKKα/β. This conclusion is based on the finding that

some IKK binding involves nonubiquitinated TRAF2 (Figure 6D); *in vitro* IKKβ is able to pull down purified nonubiquitinated, but phosphorylated TRAF2 protein (Figure 3E); and TRAF2 (K31R) recruits IKKβ (Figure 6E). Thus, our data demonstrate that phosphorylation is mainly responsible for TRAF2 recruitment of IKKα and IKKβ. Yet, TRAF2 K63-linked ubiquitin may still enhance TRAF2-IKK associations through promotion and stabilization of TRAF2 oligomerization (Shi and Kehrl, 2003). It was suggested that TRAF2 oligomerization is sufficient to activate IKK (Baud et al., 1999).

Multiple interactions between polyubiquitinated proteins and UBD containing proteins are required for NF-κB signaling. In the TNF induced-NF-κB pathway, K63-linked ubiquitination has been noted for TRAF2 (Habelhah et al., 2004; Shi and Kehrl, 2003), RIP1 (Ea et al., 2006), IKKγ (Tang et al., 2003), and TAB3 (Ishitani et al., 2003). Following TNF stimulation polyubiquitin-UBD interactions have been found between RIP1 and IKKγ (Ea et al., 2006; Wu et al., 2006), RIP1 and TAB2/3 (Kanayama et al., 2004), IKKγ and TAB2/3 (Kanayama et al., 2004), plus the interactions between TRAF2 and TAB2/3 (Figure 6A-C, S6C, S6D). Similarly in the IL-1 pathway associations between ubiquitin and UBDs have been reported between TRAF6 and TAB2/3 (Kanayama et al., 2004), IRAK1 and TAB2/3 (Windheim et al., 2008), IRAK1 and IKKγ (Conze et al., 2008; Windheim et al., 2008), IKKγ and TAB2/3 (Kanayama et al., 2004). In the TCR signaling pathway, polyubiquitin-UBD interactions have been found between Bcl10 and IKKγ (Wu and Ashwell, 2008), Malt1 and IKKγ (Oeckinghaus et al., 2007), Malt1 and TAB2/3 (Oeckinghaus et al., 2007), and IKKγ with TAB2/3 (Kanayama et al., 2004).

Both protein-linked phosphate and ubiquitin are recognized by modular domains, which mediate inducible protein-protein associations. However, the interactions between ubiquitin and UBDs are low affinity (Hicke et al., 2005) and the recognitions of ubiquitin by UBDs do not appear to be influenced by the primary sequence embedding the ubiquitinated lysine (Hunter, 2007). To overcome the low affinity and specificity between ubiquitinated proteins and UBDs, one likely possibility is that multiple ubiquitin-UBD interactions would establish a stable interaction network to provide increased affinity and specificity. As discussed above, a striking similarity in various NF-κB pathways is the requirement for multiple ubiquitin-UBD interactions. Multiple polyubiquitin-UBD interactions may anchor the association of the TAK1 and IKK complexes with the TNFR1 signalosome. Multiple ubiquitin-UBD interactions could focus and confine ubiquitinated proteins and their UBD containing partners to the TNFR1 complex avoiding nonspecific binding with other polyubiquitinated proteins. Ubiquitin-UBD interaction networks also provide many targets for regulation because disruption of any interaction might lead to network disassembly. It is well known that TNF-induced NF-κB signaling is rapid and transient. Termination of NF-κB signaling is dependent on disruption of the TNFR1 complex. Phosphatase and deubiquitinase (DUB) enzymes have been shown to down-regulate NF-κB signaling (Li and Lin, 2008). We identified a PP2A holoenzyme that rapidly regulates TNF-induced NF-κB activity by dephosphorylation of TRAF2 at Thr^{117} (Li et al., 2006), leading to TRAF2 dissociation with IKK α/β. Similarly, DUBs like A20 and CYLD target K63-linked ubiquitin of RIP1, TRAF2 and IKKγ to disassemble the polyubiquitin chain network and terminate NF-κB signaling.

Like TRAF2, K63-ubiquitinated RIP1 is also capable of recruiting the IKK and TAK1 complexes by interaction with IKKγ and TAB2/3, respectively (Kanayama et al., 2004; Ea et al., 2006). But the absence of RIP1 abolishes association of the IKK complex with TNFR1 (Ea et al., 2006). However, in both RIP1 and IKKγ deficient cells TRAF2 phosphorylation was intact and TRAF2 ubiquitination was still observed (Supplementary Figure 2A and 2B), indicating that these TRAF2 modifications are insufficient for recruiting the IKK complex to TNFR1. This may be due to technical considerations as the indirect interaction of TNFR1 with IKK may be more sensitive to detergent and susceptible to disruption by the strong wash

conditions used for receptor pull-down experiments. To obtain a clean background, in our study we used a strong lysis buffer (containing 1% Triton X-100) for immunoprecipitation of the TNFR1 complex and used stringent wash conditions (3 washes with lysis buffer). The absence of TRAF2 also abolishes IKK recruitment to TNFR1 (Devin et al., 2001), perhaps due to participation of TRAF2 in RIP1 K63-linked polyubiquitination (Lee et al., 2004). But our favored explanation is that the presence of both TRAF2 and RIP1 is required for the recruitment of IKK to TNFR1. Because the IKK complex is large (700–900 kDa) and the association with TNFR1 is indirect, maintenance of a specific stable TNFR1 signalosome may require multiple weak interactions with the IKK and TAK1 complexes as discussed above. TRAF2 or RIP1 alone may provide relatively weak associations with the IKK and TAK1 complexes but the synergistic effects may secure these interactions. Deletion of either TRAF2 or RIP1 would therefore severely affect the stability of the TNFR1 signalosome.

The combined data lead to a model of TNF signaling (Figure 7F) in which PKC phosphorylation of TRAF2 mediates recruitment of the IKK catalytic subunits, IKKα and IKKβ. Subsequently, phosphorylation determines TRAF2 K63-linked polyubiquitination leading to recruitment of the TAB/TAK1 complex. Meanwhile, RIP1, IKKγ, and TABs also undergo K63-linked polyubiquitination. The critical role of K63 ubiquitinated RIP1 as a target of the UBD containing proteins IKKγ and TAB2/3 has been well established and demonstrates the multiple requirements for assembly of a functional signalosome. The juxtaposition of the IKK and TAK1 kinase complexes on TRAF2 and RIP1 may facilitate IKK phosphorylation and activation. The polyubiquitin chains of TRAF2, RIP1 and other proteins foster colocalization with UBD containing molecules and creation of the supramolecular complex including TRAF2, RIP1, TAB2/3, TAK1, IKKα/β and IKKγ onto the ligated TNFR1 receptor. The K63-linked polyubiquitin network may further serve as a scaffold to stabilize the supramolecular complex and sustain TNF signals.

Experimental Procedures

Immunoblotting and immunoprecipitation

Western blot was performed as previously described (Li et al., 2006). For TNFR1 immunoprecipitation, cells were lysed in IP buffer containing 50 mM HEPES (pH 7.4), 1% Triton X-100, 150 mM NaCl, 10% glycerol, 10 mM NaF, 1 mM Na_3VO_4, 0.5 mM DTT, and a cocktail of protease and phosphatase inhibitors (Pierce Biotechnology). For ubiquitin immunoprecipitation, cells were lysed in the RIPA buffer (Sigma) with *N*-ethylmaleimide (Calbiochem), protease inhibitor cocktail (Roche) and phosphatase inhibitors (Pierce). Other protein immunoprecipitations were performed with IP lysis buffer without 10% glycerol. The cell lysates were incubated with the indicated antibody and mixed end-over-end at 4°C overnight. The beads were then 3X washed (5 min each time) with the same buffer used for cell lysis.

In vitro kinase assay

Kinase assays for IKK (Cell Signaling) and JNK (Promega) were performed according to the manufacturer's protocols. *In vitro* PKC phosphorylation was performed with lipid activator and Mg/ATP cocktail (Upstate) and PKC (Calbiochem) using the manufacturer's protocol.

Luciferase reporter assay, cell transfection and infection

Transfection of plasmids was performed using Polyfect (Qiagen) and Lipofectamine 2000 (Invitrogen) according to the manufacturers' protocol. The NF-κB luciferase activity assay was previously described (Di et al., 2008).

For retroviral infection, PKC, TRAF5, TRAF2, or their mutant constructs were cloned into pLHCX and pLPCX vectors (Clontech Laboratories). Virus packaging and infection were performed according to the manufacturer's protocol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by NIH grant NS057162 plus NMSS grant RG3871A4. We thank Michael Berman for making constructs and Dr. Bryant Darnay for *in vitro* ubiquitination assays and discussion. We also thank Drs T. Mak, M. Schmidt-Supprian, B. Seed, and R. Messing for their generous gifts of TRAF2, IKKγ, RIP1, and PKCε deficient MEFs, respectively, and Drs. A. Toker, E. Kieff, S-C Sun, K. Matsumoto, R. Siegel, and G. Hotamisligil for donation of PKCζ, IKKα-Myc, HA-ubiquitin, TAB2, TAB3, HA-hTNFR1, and hTNFR2 constructs.

References

Adhikari A, Xu M, Chen ZJ. Ubiquitin-mediated activation of TAK1 and IKK. Oncogene 2007;26:3214–3226. [PubMed: 17496917]

Baud V, Liu ZG, Bennett B, Suzuki N, Xia Y, Karin M. Signaling by proinflammatory cytokines: oligomerization of TRAF2 and TRAF6 is sufficient for JNK and IKK activation and target gene induction via an amino-terminal effector domain. Genes Dev 1999;13:1297–1308. [PubMed: 10346818]

Castrillo A, Pennington DJ, Otto F, Parker PJ, Owen MJ, Bosca L. Protein kinase Cepsilon is required for macrophage activation and defense against bacterial infection. J Exp Med 2001;194:1231–1242. [PubMed: 11696589]

Conze DB, Wu CJ, Thomas JA, Landstrom A, Ashwell JD. Lys (K)63-linked polyubiquitination of IRAK-1 is required for IL-1 receptor- and Toll-like receptor-mediated NF-{kappa}B activation. Mol Cell Biol. 2008

Delhase M, Hayakawa M, Chen Y, Karin M. Positive and negative regulation of IkappaB kinase activity through IKKbeta subunit phosphorylation. Science 1999;284:309–313. [PubMed: 10195894]

Deng L, Wang C, Spencer E, Yang L, Braun A, You J, Slaughter C, Pickart C, Chen ZJ. Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell 2000;103:351–361. [PubMed: 11057907]

Devin A, Cook A, Lin Y, Rodriguez Y, Kelliher M, Liu Z. The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation. Immunity 2000;12:419–429. [PubMed: 10795740]

Devin A, Lin Y, Liu ZG. The role of the death-domain kinase RIP in tumour-necrosis-factor-induced activation of mitogen-activated protein kinases. EMBO Rep 2003;4:623–627. [PubMed: 12776182]

Devin A, Lin Y, Yamaoka S, Li Z, Karin M, Liu Z. The alpha and beta subunits of IkappaB kinase (IKK) mediate TRAF2-dependent IKK recruitment to tumor necrosis factor (TNF) receptor 1 in response to TNF. Mol Cell Biol 2001;21:3986–3994. [PubMed: 11359906]

Di Y, Li S, Wang L, Zhang Y, Dorf ME. Homeostatic interactions between MEKK3 and TAK1 involved in NF-kappaB signaling. Cell Signal 2008;20:705–713. [PubMed: 18206350]

Dykstra M, Cherukuri A, Sohn HW, Tzeng SJ, Pierce SK. Location is everything: lipid rafts and immune cell signaling. Annu Rev Immunol 2003;21:457–481. [PubMed: 12615889]

Ea CK, Deng L, Xia ZP, Pineda G, Chen ZJ. Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. Mol Cell 2006;22:245–257. [PubMed: 16603398]

Gao M, Karin M. Regulating the regulators: control of protein ubiquitination and ubiquitin-like modifications by extracellular stimuli. Mol Cell 2005;19:581–593. [PubMed: 16137616]

Gilmore TD. Introduction to NF-kappaB: players, pathways, perspectives. Oncogene 2006;25:6680–6684. [PubMed: 17072321]

Habelhah H, Takahashi S, Cho SG, Kadoya T, Watanabe T, Ronai Z. Ubiquitination and translocation of TRAF2 is required for activation of JNK but not of p38 or NF-kappaB. Embo J 2004;23:322–332. [PubMed: 14713952]

Hacker H, Karin M. Regulation and function of IKK and IKK-related kinases. Sci STKE 2006;2006:re13. [PubMed: 17047224]

Hayden MS, Ghosh S. Signaling to NF-kappaB. Genes Dev 2004;18:2195–2224. [PubMed: 15371334]

Hayden MS, Ghosh S. Shared principles in NF-kappaB signaling. Cell 2008;132:344–362. [PubMed: 18267068]

Hicke L, Schubert HL, Hill CP. Ubiquitin-binding domains. Nat Rev Mol Cell Biol 2005;6:610–621. [PubMed: 16064137]

Hong S, Lim S, Li AG, Lee C, Lee YS, Lee EK, Park SH, Wang XJ, Kim SJ. Smad7 binds to the adaptors TAB2 and TAB3 to block recruitment of the kinase TAK1 to the adaptor TRAF2. Nat Immunol 2007;8:504–513. [PubMed: 17384642]

Hunter T. The age of crosstalk: phosphorylation, ubiquitination, and beyond. Mol Cell 2007;28:730–738. [PubMed: 18082598]

Ishitani T, Takaesu G, Ninomiya-Tsuji J, Shibuya H, Gaynor RB, Matsumoto K. Role of the TAB2-related protein TAB3 in IL-1 and TNF signaling. EMBO J 2003;22:6277–6288. [PubMed: 14633987]

Kanayama A, Seth RB, Sun L, Ea CK, Hong M, Shaito A, Chiu YH, Deng L, Chen ZJ. TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains. Mol Cell 2004;15:535–548. [PubMed: 15327770]

Kilpatrick LE, Sun S, Mackie D, Baik F, Li H, Korchak HM. Regulation of TNF mediated antiapoptotic signaling in human neutrophils: role of delta-PKC and ERK1/2. J Leukoc Biol 2006;80:1512–1521. [PubMed: 17138860]

Lee TH, Shank J, Cusson N, Kelliher MA. The kinase activity of Rip1 is not required for tumor necrosis factor-alpha-induced IkappaB kinase or p38 MAP kinase activation or for the ubiquitination of Rip1 by Traf2. J Biol Chem 2004;279:33185–33191. [PubMed: 15175328]

Legler DF, Micheau O, Doucey MA, Tschopp J, Bron C. Recruitment of TNF receptor 1 to lipid rafts is essential for TNFalpha-mediated NF-kappaB activation. Immunity 2003;18:655–664. [PubMed: 12753742]

Li H, Lin X. Positive and negative signaling components involved in TNFalpha-induced NF-kappaB activation. Cytokine 2008;41:1–8. [PubMed: 18068998]

Li S, Wang L, Berman MA, Zhang Y, Dorf ME. RNAi screen in mouse astrocytes identifies phosphatases that regulate NF-kappaB signaling. Mol Cell 2006;24:497–509. [PubMed: 17188031]

Machleidt T, Kramer B, Adam D, Neumann B, Schutze S, Wiegmann K, Kronke M. Function of the p55 tumor necrosis factor receptor “death domain” mediated by phosphatidylcholine-specific phospholipase C. J Exp Med 1996;184:725–733. [PubMed: 8760826]

Obenauer JC, Cantley LC, Yaffe MB. Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. Nucleic Acids Res 2003;31:3635–3641. [PubMed: 12824383]

Oeckinghaus A, Wegener E, Welteke V, Ferch U, Arslan SC, Ruland J, Scheidereit C, Krappmann D. Malt1 ubiquitination triggers NF-kappaB signaling upon T-cell activation. EMBO J 2007;26:4634–4645. [PubMed: 17948050]

Rodriguez P, Mitton B, Kranias EG. Phosphorylation of glutathione-S-transferase by protein kinase C-alpha implications for affinity-tag purification. Biotechnol Lett 2005;27:1869–1873. [PubMed: 16328982]

Rothe M, Sarma V, Dixit VM, Goeddel DV. TRAF2-mediated activation of NF-kappa B by TNF receptor 2 and CD40. Science 1995;269:1424–1427. [PubMed: 7544915]

Schutze S, Potthoff K, Machleidt T, Berkovic D, Wiegmann K, Kronke M. TNF activates NF-kappa B by phosphatidylcholine-specific phospholipase C-induced “acidic” sphingomyelin breakdown. Cell 1992;71:765–776. [PubMed: 1330325]

Shi CS, Kehrl JH. Tumor necrosis factor (TNF)-induced germinal center kinase-related (GCKR) and stress-activated protein kinase (SAPK) activation depends upon the E2/E3 complex Ubc13-Uev1A/TNF receptor-associated factor 2 (TRAF2). J Biol Chem 2003;278:15429–15434. [PubMed: 12591926]

Tada K, Okazaki T, Sakon S, Kobarai T, Kurosawa K, Yamaoka S, Hashimoto H, Mak TW, Yagita H, Okumura K, et al. Critical roles of TRAF2 and TRAF5 in tumor necrosis factor-induced NF-kappa B activation and protection from cell death. J Biol Chem 2001;276:36530–36534. [PubMed: 11479302]

Tang ED, Wang CY, Xiong Y, Guan KL. A role for NF-kappaB essential modifier/IkappaB kinase-gamma (NEMO/IKKgamma) ubiquitination in the activation of the IkappaB kinase complex by tumor necrosis factor-alpha. J Biol Chem 2003;278:37297–37305. [PubMed: 12867425]

Windheim M, Stafford M, Peggie M, Cohen P. Interleukin-1 (IL-1) induces the Lys63-linked polyubiquitination of IL-1 receptor-associated kinase 1 to facilitate NEMO binding and the activation of IkappaBalpha kinase. Mol Cell Biol 2008;28:1783–1791. [PubMed: 18180283]

Wu CJ, Ashwell JD. NEMO recognition of ubiquitinated Bcl10 is required for T cell receptor-mediated NF-kappaB activation. Proc Natl Acad Sci U S A 2008;105:3023–3028. [PubMed: 18287044]

Wu CJ, Conze DB, Li T, Srinivasula SM, Ashwell JD. Sensing of Lys 63-linked polyubiquitination by NEMO is a key event in NF-kappaB activation [corrected]. Nat Cell Biol 2006;8:398–406. [PubMed: 16547522]

Wu CJ, Conze DB, Li X, Ying SX, Hanover JA, Ashwell JD. TNF-alpha induced c-IAP1/TRAF2 complex translocation to a Ubc6-containing compartment and TRAF2 ubiquitination. Embo J 2005;24:1886–1898. [PubMed: 15861135]

Yeh WC, Shahinian A, Speiser D, Kraunus J, Billia F, Wakeham A, de la Pompa JL, Ferrick D, Hum B, Iscove N, et al. Early lethality, functional NF-kappaB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. Immunity 1997;7:715–725. [PubMed: 9390694]

Zhang SQ, Kovalenko A, Cantarella G, Wallach D. Recruitment of the IKK signalosome to the p55 TNF receptor: RIP and A20 bind to NEMO (IKKgamma) upon receptor stimulation. Immunity 2000;12:301–311. [PubMed: 10755617]

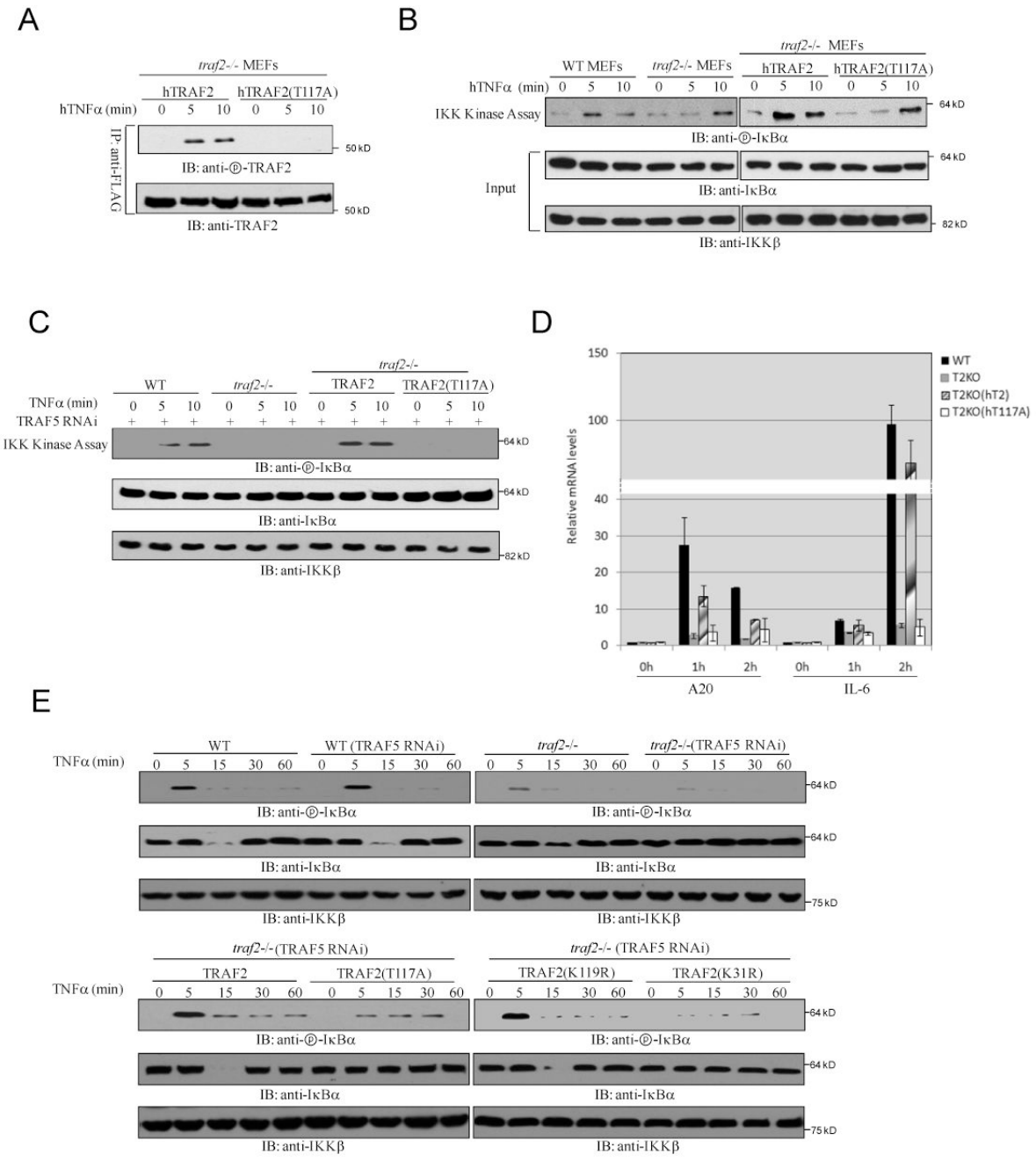

Figure 1. TRAF2 phosphorylation is induced by TNF and required for IKK activation

(**A**) TRAF2 knockout (KO) MEFs were infected with retrovirus expressing FLAG-tagged human TRAF2 or a point mutant TRAF2(T117A). After puromycin selection the two stable cell lines, TRAF2$^{-/-}$(hTRAF2) and TRAF2$^{-/-}$(hT117A) were stimulated with 20 ng/ml human TNFα (hTNFα) for the designated times. Immunoblots (IB) were probed with antibodies specific for phospho-peptide or TRAF2 protein. (**B**) TNFα-induced IKKβ kinase activity in wild type and *traf2*-/- stable cell lines. Cells were treated with hTNFα for the indicated times then cell extracts were immunoprecipitated with anti-IKKβ antibody for analysis of kinase activity using GST-IκBα as substrate. (**C**) TNFα-induced IKKβ kinase activity in wild type and *traf2*-/- stable cell lines transfected with TRAF5 siRNA oligo. (**D**) Relative mRNA levels

of A20 and IL-6 in wild type and *traf2*-/- stable cell lines. After TNF stimulation for the designated times, cells were collected for mRNA extraction and reverse transcription. cDNAs were then subjected to real-time PCR and the relative mRNA levels were calculated. (**E**) IκBα phosphorylation and degradation in wild type and *traf2*-/- stable cell lines with or without TRAF5 RNAi.

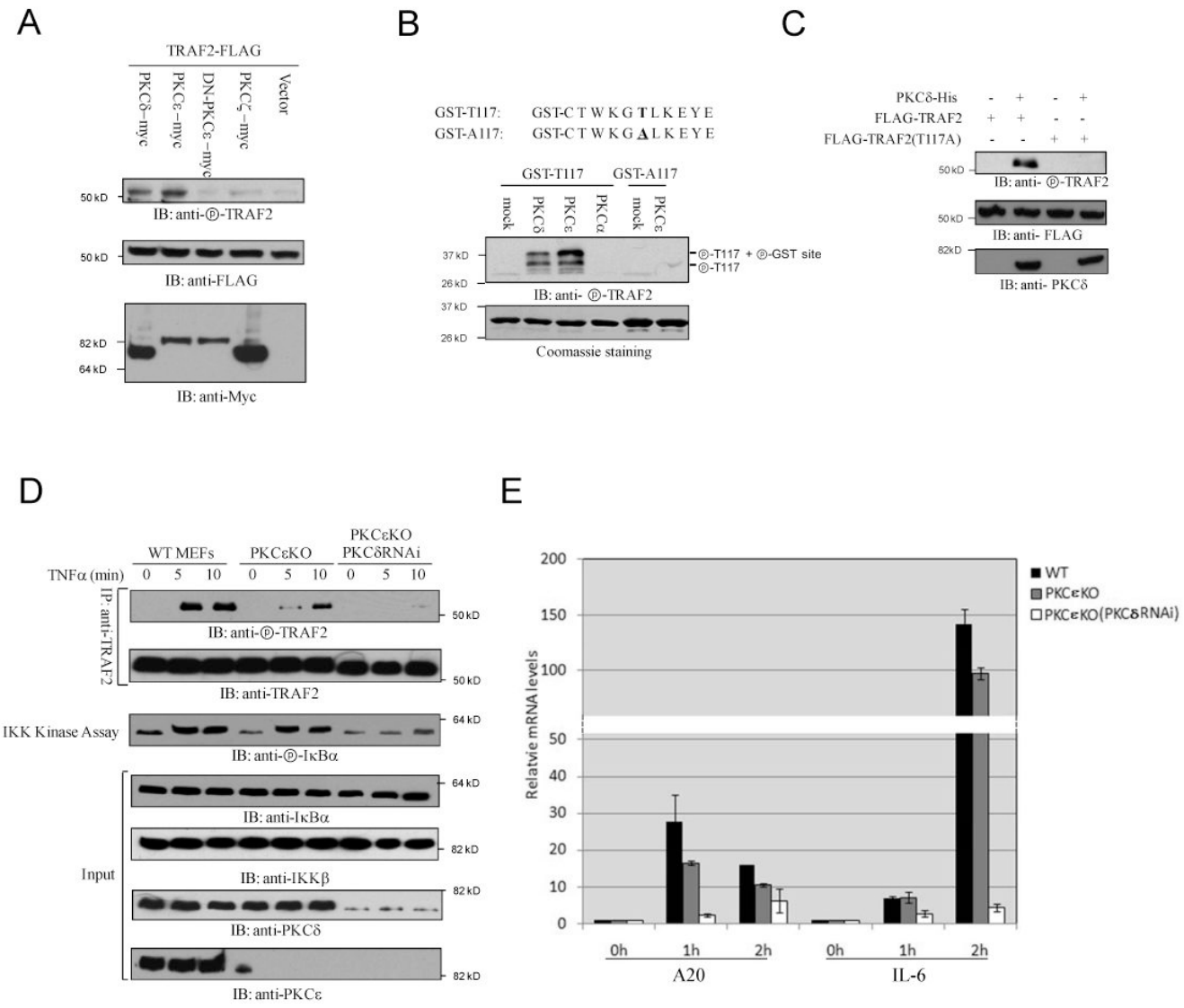

Figure 2. PKC controls TRAF2 Thr^{117} phosphorylation and promotes NF-κB activation

(**A**) FLAG-TRAF2 was cotransfected with various PKC constructs into 293T cells; after 48 h cell lysates were collected for Western Blot and probed with the indicated antibodies. Double mutation PKCε(K437W/T566A) is a dominant negative of PKCε (DN-PKCε). (**B**) *In vitro* PKC phosphorylation of the indicated GST tagged Thr^{117} peptide. Purified GST tagged T117 or A117 peptides were incubated with recombinant PKCα, PKCδ, or PKCε in PKC kinase assay buffer for 30 min at 30°C. The reaction buffer was then boiled for Western Blot and probed with anti-phospho-TRAF2 (Thr^{117}) antibody. Coomassie staining was shown as the peptide loading control. The upper band detected by anti-phospho-TRAF2 antibody was due to Thr^{117} phosphorylation plus phosphorylation of a site in GST. (**C**) Purified FLAG tagged TRAF2 was incubated *in vitro* with recombinant PKCδ, in PKC kinase assay buffer for 30 min at 30 °**C**. (**D**) TRAF2 phosphorylation and IKKβ kinase activity in wild type, PKCε deficient cells and PKCε deficient cells transfected with PKCδ siRNA oligo. Forty-eight hours after transfection cells were stimulated with 20 ng/ml mouse TNFα for the designated times. Cell lysates were immunoprecipitated with anti-TRAF2 or IKKβ antibody to examine TRAF2

phosphorylation and IKK kinase activity. (**E**) TNF-induced mRNA expression of A20 and IL-6 in wild type, PKCε deficient cells and PKCε deficient cells with PKCδ RNAi.

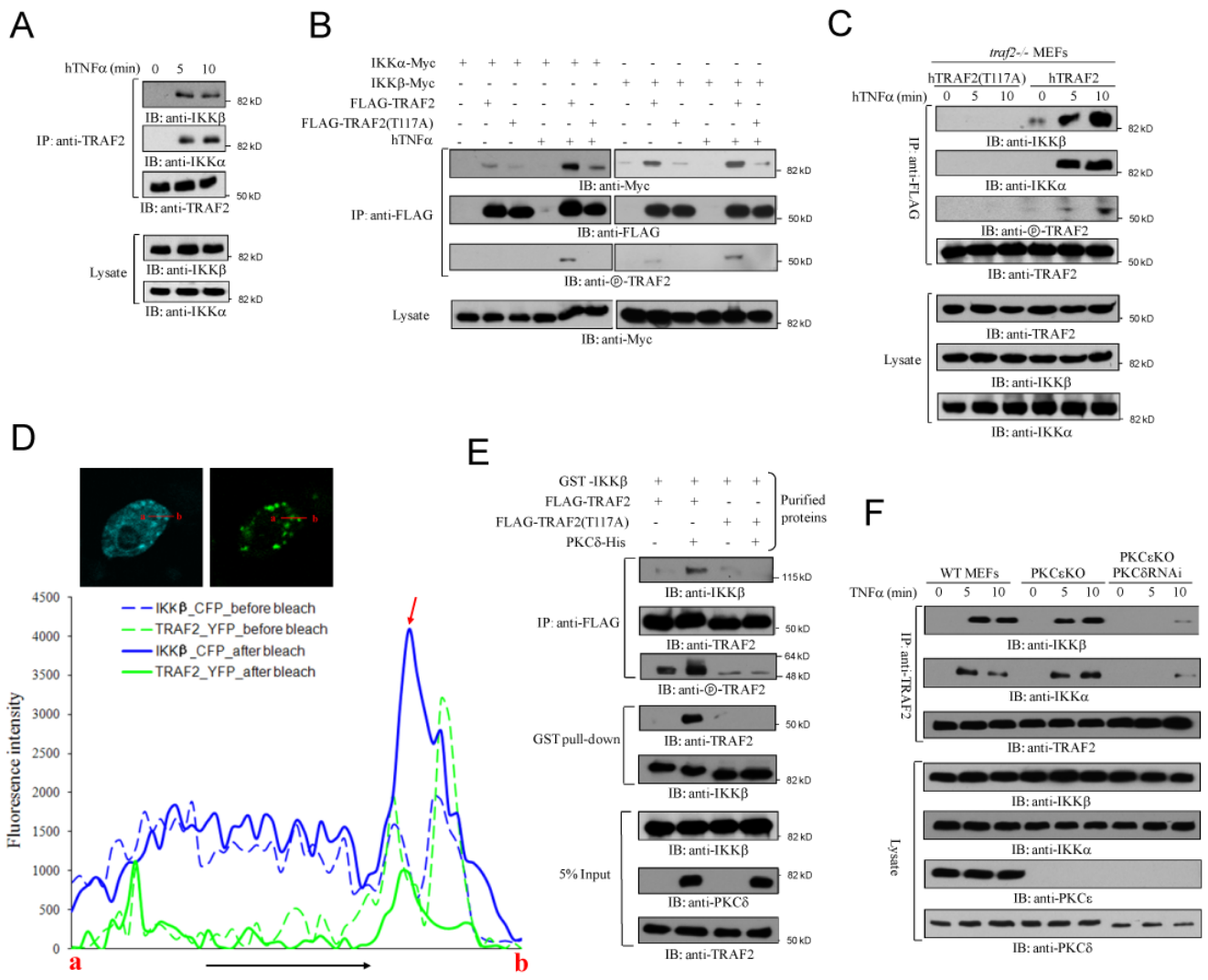

Figure 3. Thr^{117} phosphorylation controls TRAF2 interactions with IKKα and IKKβ

(**A**) After TNF treatment, 293T cell lysates were immunoprecipitated with anti-TRAF2 antibody. (**B**) Myc-tagged IKKα or IKKβ was cotransfected with FLAG-tagged TRAF2 or TRAF2(T117A) into 293T cells. Cells were treated with media or 20 ng/ml hTNFα for 10 min before harvest and immunoprecipitation with anti-FLAG antibody. (**C**) Stable *traf2*-/-(hTRAF2) and *traf2*-/-(hT117A) cells were treated with TNFα (20 ng/ml) for the indicated times before harvest and cell lysates were immunoprecipitated with anti-FLAG antibody. (**D**) Thr^{117} phosphorylation is required for fluorescence resonance energy transfer (FRET) between TRAF2 and IKKβ. The insert includes a red line (ab) to indicate the line of photobleaching. Average CFP fluorescence intensities were measured before and after photobleaching. The arrow highlights the dramatic increase in intensity of CFP tagged IKKβ after bleaching YFP tagged TRAF2. (**E**) Purified FLAG-TRAF2 or TRAF2(T117A) was phosphorylated *in vitro* with recombinant PKCδ, and then precipitated with GST-IKKβ by GST pull-down or anti-FLAG antibody. (**F**) Wild type, PKCε deficient cells, and PKCε deficient cells with PKCδ RNAi were treated with TNFα for the indicated times. Cell extracts were then immunoprecipitated with anti-TRAF2 antibody.

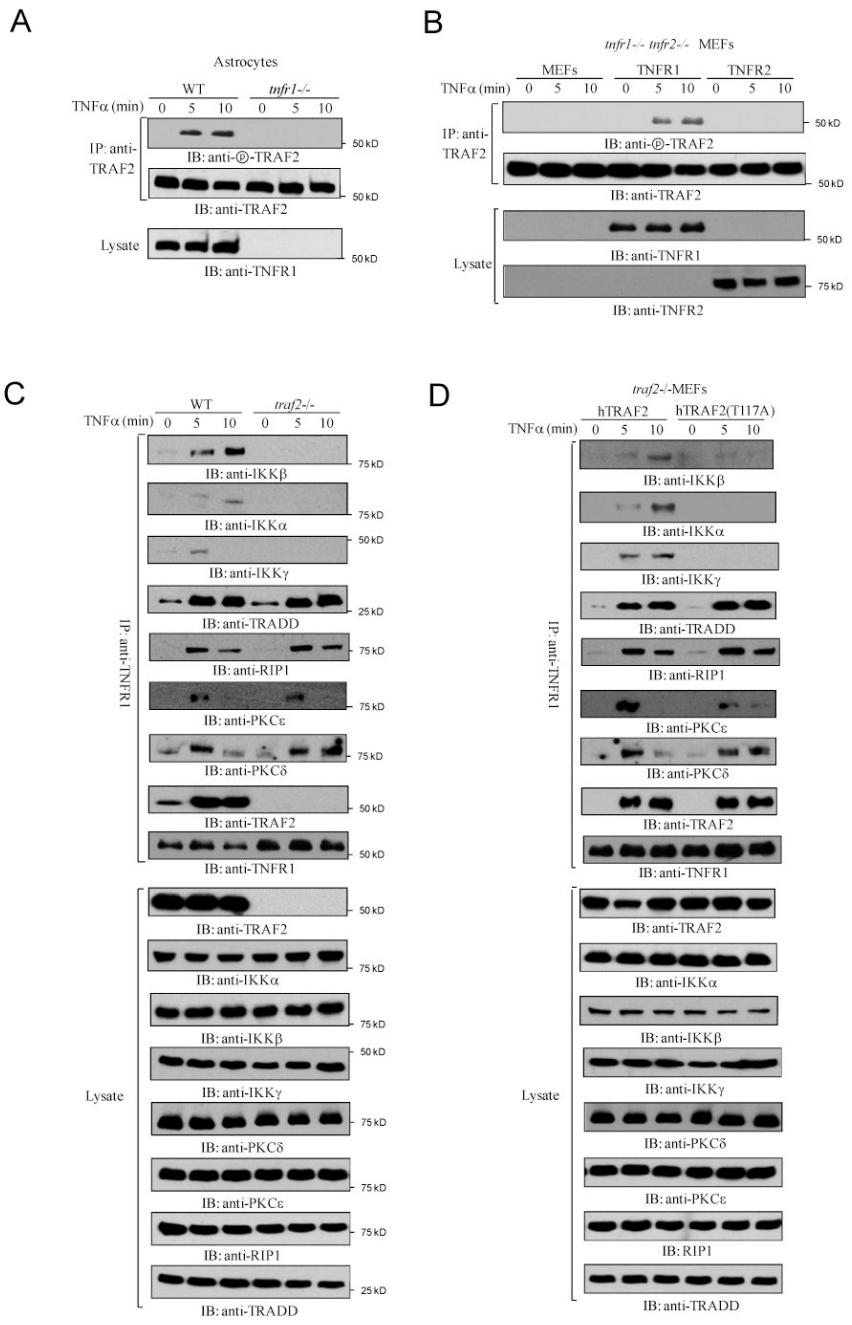

Figure 4. TNF signaling through TNFR1 mediates endogenous TRAF2 phosphorylation and recruitment of IKK complex

(**A**) Immortalized wild-type and TNFR1 deficient astrocytes were stimulated with 20 ng/ml mTNFα for the indicated times. Cell extracts were then immunoprecipitated with anti-TRAF2 antibody. (**B**) Immortalized TNFR1 and TNFR2 double deficient MEFs were transfected with hTNFR1 or hTNFR2 and stimulated with 20 ng/ml mTNFα for the indicated times. Cell extracts were then immunoprecipitated with anti-TRAF2 antibody. (**C-D**) Indicated MEFs or reconstituted cells were stimulated with 20 ng/ml mTNFα for the designated times. Cells were harvested and immunoprecipitated with anti-mTNFR1 antibody.

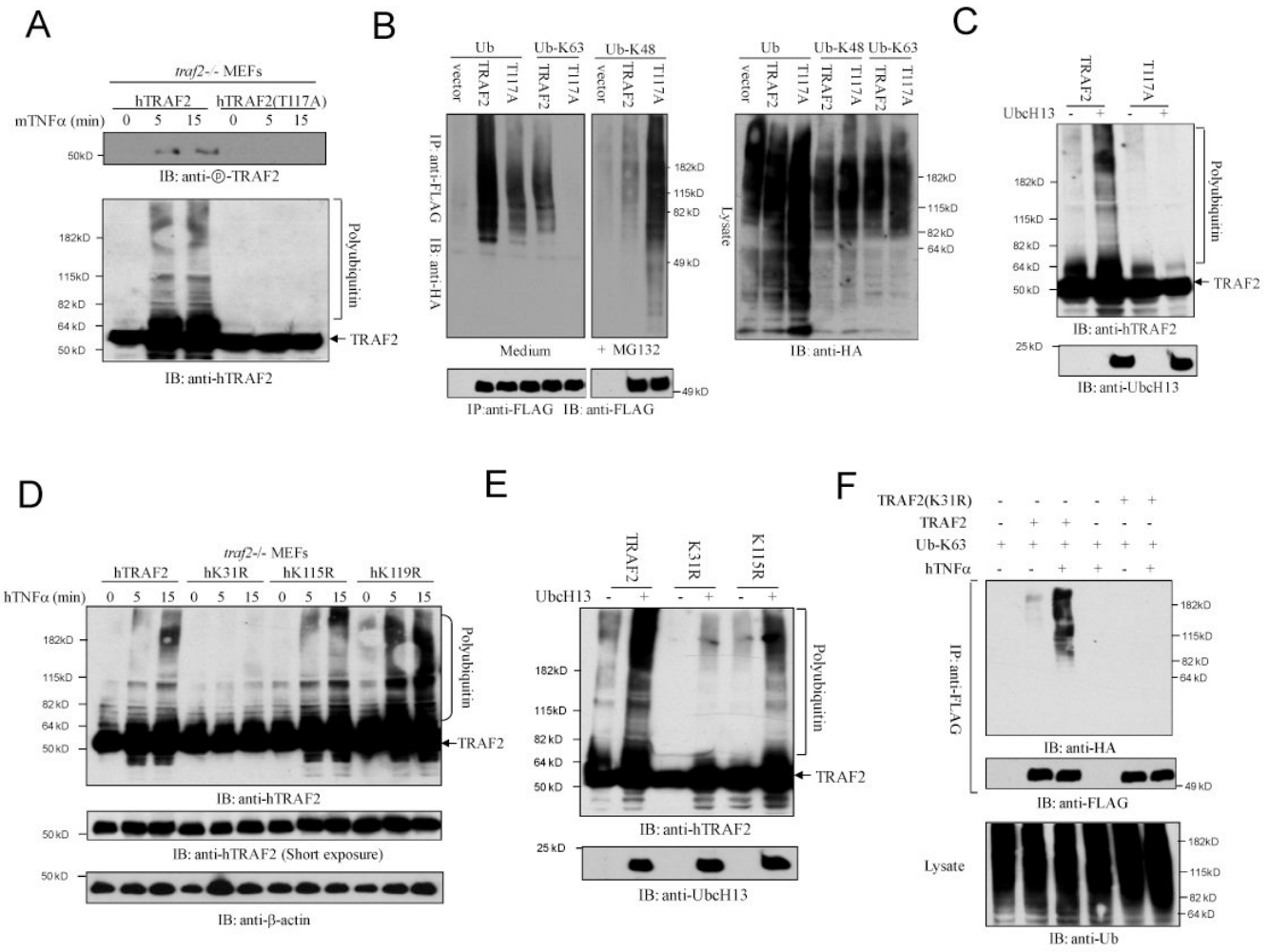

Figure 5. Phosphorylation controls K63-linked ubiquitination of TRAF2 at lysine 31

(**A**) Stably reconstituted *traf2*$^{-/-}$(hTRAF2) and *traf2*$^{-/-}$(hT117A) cells were stimulated with 20 ng/ml TNFα for the designated times. Immunoblots were probed with antibodies specific for TRAF2 phospho-peptide or human TRAF2. (**B**) FLAG-TRAF2 or TRAF2(T117A) constructs were transfected into 293T cells with HA-Ub or its mutants containing only one lysine at either K48 or K63. Ub-K48 expressing cells were also treated with the proteasome inhibitor MG132 to prevent the degradation of K48-linked TRAF2. The cell lysates were immunoprecipitated with anti-FLAG antibody. (**C**) FLAG-TRAF2 or TRAF2(T117A) constructs were transfected into 293T cells with or without UbcH13. (**D**) TRAF2 knockout MEFs were infected with retrovirus expressing FLAG-tagged human TRAF2 or lysine point mutants TRAF2(K31R), TRAF2(K115R) and TRAF2(K119R). After puromycin selection these stable cell lines were stimulated with 20 ng/ml TNFα for the designated times. (**E**) FLAG-TRAF2, TRAF2(K31R) or TRAF2(K115R) constructs were transfected into 293T cells with UbcH13. (**F**) FLAG-TRAF2 or TRAF2(K31R) constructs were transfected into 293T cells with HA-Ub K63 only. After TNF stimulation, cells were harvested for IP with anti-FLAG antibody.

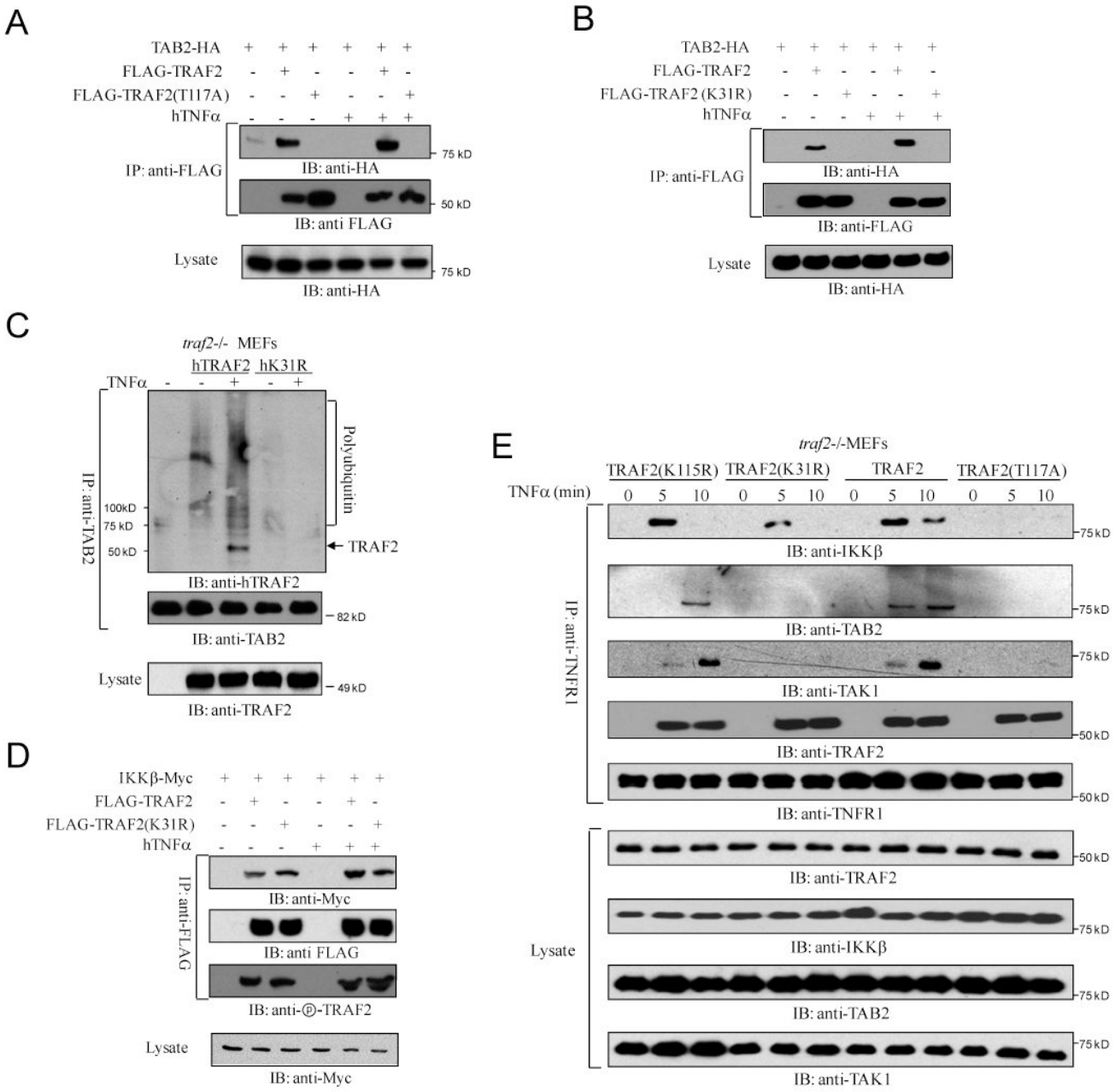

Figure 6. K63-linked ubiquitination of TRAF2 mediates the association with TAB2/3

(**A-B**) HA-tagged TAB2 was cotransfected with FLAG-tagged TRAF2, TRAF2(T117A) or TRAF2(K31R) into 293T cells. Indicated groups were treated with 20 ng/ml hTNFα for 10 min before harvest and immunoprecipitated with anti-FLAG antibody. (**C**) After TNF stimulation reconstituted *traf2*$^{-/-}$(hTRAF2) vs. *traf2*$^{-/-}$(hK31R) MEFs were immunoprecipitated with anti-TAB2 antibody. (**D**) Epitope-tagged IKKβ and TRAF2 or TRAF2(K31R) were cotransfected into 293T cells. After TNF treatment TRAF2 was immunoprecipitated and blots were probed with the indicated antibodies. (**E**) Indicated reconstituted *traf2*-/- cells were stimulated with 20 ng/ml mTNFα for the designated times. Cells were harvested and immunoprecipitated with anti-mTNFR1 antibody.

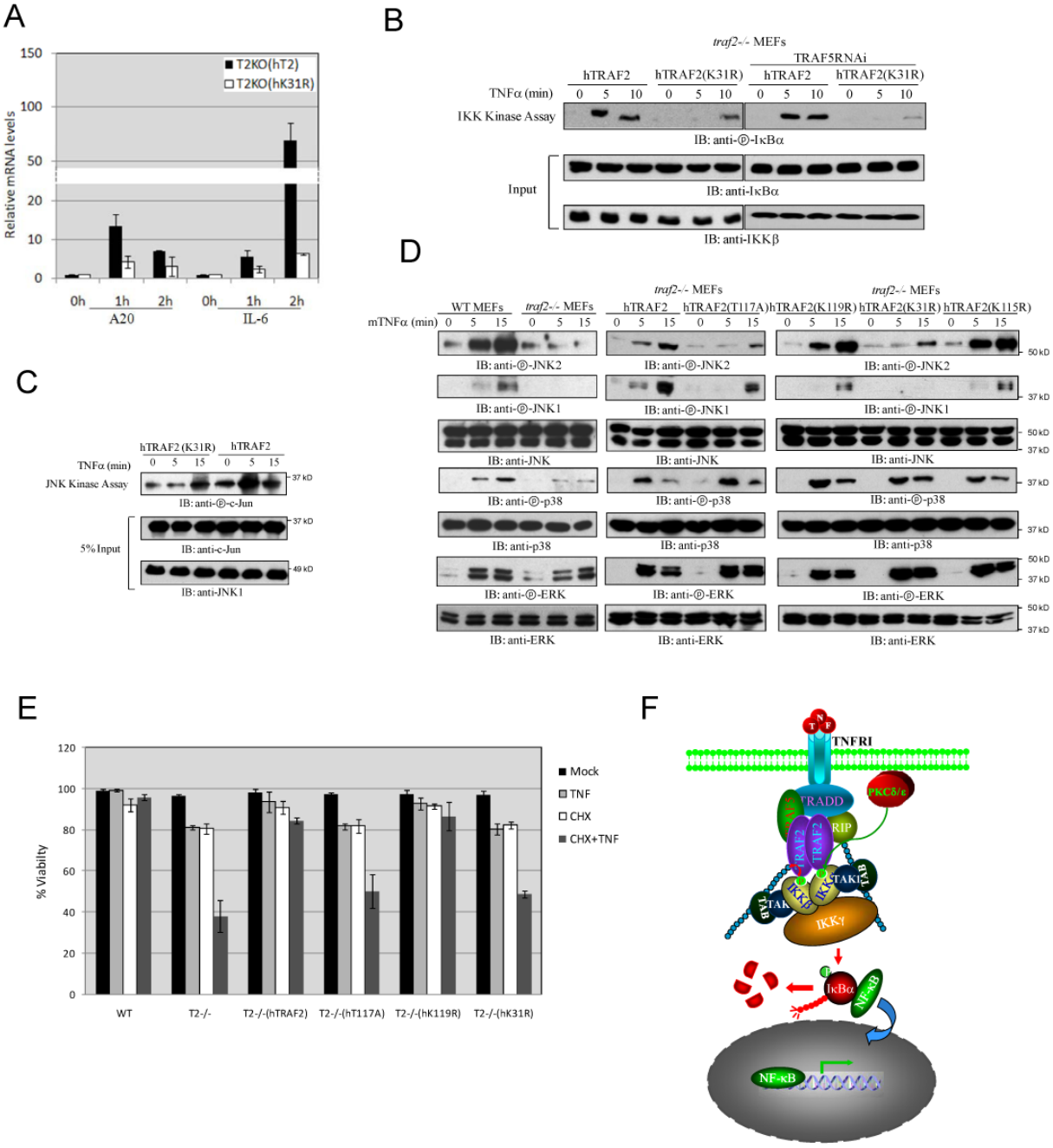

Figure 7. Ubiquitination of TRAF2 regulates IKK and JNK activity

(**A**) TNF-induced mRNA expression of A20 and IL-6 in stably reconstituted *traf2*$^{-/-}$(hTRAF2) vs. *traf2*$^{-/-}$(hK31R) MEFs. (**B**) TNFα-induced IKKβ kinase activity in stably reconstituted *traf2*$^{-/-}$(hTRAF2) vs. *traf2*$^{-/-}$(hK31R) MEFs or in the same cell lines with TRAF5 RNAi. (**C**) TNFα-induced JNK kinase activity in stably reconstituted *traf2*$^{-/-}$(hTRAF2) vs. *traf2*$^{-/-}$(hK31R) MEFs. (**D**) TNF-induced p38, ERK and JNK activation in wild type, *traf2*$^{-/-}$, and MEFs stably reconstituted with the indicated mutants. Cells were treated with 20 ng/ml hTNFα as designated. Immunoblots were probed as indicated. (**E**) MTT assay with wild type MEFs and various *traf2*-/- stable cell lines treated for 24h with cycloheximide (0.25 μg/

ml; CHX), or TNFα (10 ng/ml), or both. The percent live cells ±SD is indicated. (**F**) A model of TNF-induced IKK activation.