ARTICLE

Journal of Cellular Biochemistry 110:763–771 (2010)

Journal of Cellular Biochemistry

TRAF6 Is Autoinhibited by an Intramolecular Interaction Which Is Counteracted by *Trans*–Ubiquitination

Kent Z.Q. Wang,[1] Deborah L. Galson,[2] and Philip E. Auron[1*]

[1] *Department of Biological Sciences, Duquesne University, Pittsburgh, Pennsylvania 15282*

[2] *Department of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15240*

ABSTRACT

The tumor necrosis factor (TNF) receptor associated factor (TRAF) class of intracellular signal transducers is responsible for mediating many of the activation events initiated by TNF receptor (TNFR) and Toll-like/Interleukin-1, -17, and -18 receptor (TIR) families. Investigation of the mechanism by which TRAF6 is activated has demonstrated that two critical domains of the molecule required for activation and downstream signaling are involved in an interaction which renders the molecule inactive and structurally closed, as well as incapable of auto-ubiquitination. Contrary to its assumed role as a direct mediator of protein–protein interaction, TRAF auto-ubiquitination is a means of sustaining an open conformation active in downstream signaling. Furthermore, the inferred *cis*-function of TRAF auto-ubiquitination is now demonstrated to act in *trans* and requires both the RING-Zinc (RZ) fingers region and coiled-coil domain. We also observed that both the RZ fingers region and the MATH domain are targets for ubiquitination. Although TRAF6 ubiquitination has emerged as a hallmark of activation, *trans*-ubiquitination induced by two TRAF6 muteins is insufficient for NF-κB activation. J. Cell. Biochem. 110: 763–771, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: TRAF6; E3 UBIQUITIN LIGASE; UBIQUITINATION; NF-κB

Tumor necrosis factor (TNF) receptor associated factor (TRAF) family proteins are ubiquitin E3 ligases that promote TNF receptor (TNFR) and TIR signaling events, resulting in the induction of numerous genes [Chung et al., 2002; Aggarwal, 2003; Wu and Arron, 2003]. One of the most widely studied TRAF-regulated transcription factors is NF-κB, which is activated by IκB kinase (IKK) modification and subsequent proteasomal degradation of the inhibitory IκB protein [Xia and Chen, 2005]. Such activation is central to a large variety of immune and inflammatory processes relevant to normal homeostasis and disease. TRAF6 is one of the most distinct members of its family, being uniquely able to transduce signals by all TIRs as well as a subclass of TNFR that includes CD40, RANK, and NGFR. These receptors are pivotal in innate and adaptive immunity, bone resorption, and nerve cell growth [Lomaga et al., 1999; Wu and Arron, 2003; Frossard et al., 2004; Wooten et al., 2005]. The exact mechanism by which these ligand bound receptors activate TRAF is unclear. It is known that these receptors can, both directly and indirectly, activate TRAF6 via specific binding of proteins containing TRAF Interacting Motif (TIM) peptides to a groove on the carboxyl-terminal MATH domain. This, in turn, leads to K63-linked auto-ubiquitination and NF-κB activation [Ye et al., 2002]. It has also been suggested that K63-linked polyubiquitin chains anchored to TRAF6 are specific signals for downstream interaction with the IKK system [Wang et al., 2001]. However, this concept has recently been disputed by studies demonstrating that auto-ubiquitination is dispensable for NF-κB activation [Walsh et al., 2008]. Furthermore, unlike some signaling molecules, wtTRAF overexpression acts as a dominant-positive via an unknown mechanism [Dadgostar and Cheng, 2000].

In this study we investigated the molecular mechanism that regulates TRAF6 auto-ubiquitination and its role in TRAF6 activation. Our results implicate autoinhibition of TRAF6 by intramolecular interaction between the RING-Zinc (RZ) region and MATH domain. These modules of TRAF6 are required, respectively, for association with upstream TIM activators [Wu and Arron, 2003] and downstream signaling [Baud et al., 1999]. TRAF6 polyubiquitination requires both the RZ and coiled-coil and is mediated in *trans*. Furthermore, this process disrupts the intramolecular interaction between these two modules, sustaining TRAF6 in an open and active state.

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Bayer School of Natural and Environmental Sciences at Duquesne University; Grant sponsor: NIH; Grant number: AR057310.

*Correspondence to: Philip E. Auron, PhD, Department of Biological Sciences, Duqesne University, 600 Forbes Ave., Pittsburgh, PA 15282. E-mail: auronp@duq.edu

Received 16 November 2009; Accepted 18 February 2010 • DOI 10.1002/jcb.22589 • © 2010 Wiley-Liss, Inc.
Published online 1 April 2010 in Wiley InterScience (www.interscience.wiley.com).

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

ANTI-FLAG® M2 Affinity Gel and ANTI-FLAG® M2 were purchased from Sigma–Aldrich. Ubiquitin antibody (P4D1) and TRAF6 (H-274) antibodies were purchased from Santa Cruz Biotechnology. FuGENE HD transfection reagent and green fluorescent protein (GFP) antibody were purchased from Roche Applied Science. Luciferase assay system was purchased from Promega.

CELL LINES

HEK293 (293) and HEK293T (293T) cells were from American Type Culture Collection and were cultured in EMEM medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mg/ml L-glutamine. HEK293T constitutively express SV40 T antigen which results in amplification of SV40 plasmids containing the SV40 replication origin.

REPORTERS AND EXPRESSION PLASMIDS

The NF-κB luciferase reporter has been described previously [Yoshida et al., 2004]. The HcRed1-κB reporter was constructed by replacing the luciferase coding sequence in the NF-κB/pGL2 used for the luciferase assays with HcRed1 cDNA derived from HcRed1-Nuc (BD Biosciences) inserted into the *Hin*dIII and *Mfl*MI restriction sites in the NF-κB/pGL2 vector. The human wild-type TRAF6 and mutant coding sequences were engineered in both untagged pcDNA3.1(+) and dual-tagged pFLAG CMV 5a-YFP expression vectors in the *Eco*RI and *Eco*RV sites by PCR subcloning strategies (Fig. 1A). The sequences coding for: TRAF6(1–273) is designated as RZ; TRAF6(1–358) as RZcc; TRAF6(346–522) as MATH; TRAF6(125–522) as ΔR; TRAF6(156–522) as ΔRZ1; TRAF6(274–522) as ΔRZ; and TRAF6(274–345) as Δcc. TRAF6(K124R) designated as K124R and TRAF6(I98L, D100G) as mR were engineered by a QuickChange II XL site-directed mutagenesis kit (Stratagene) with wild-type TRAF6pcDNA3.1 and TRAF6pFLAG CMV 5a-YFP as templates. The pFLAG CMV 5a-YFP control vector was generated by inserting an EYFP coding sequence into the *Eco*RV and *Bam*HI sites of pFLAG CMV 5a plasmid (Sigma–Aldrich).

CELL TRANSFECTION

Reporters or expression plasmids were transfected into 293 or 293T cells with FuGENE HD transfection reagent in a ratio of 1:3, according to the manufacturer's procedures.

LUCIFERASE BULK-CELL ASSAY

A luciferase reporter (NF-κBpGL2) was co-transfected with indicated expression plasmids into 293 cells. After 24 h, the transfected cells were lysed and analyzed for luciferase activity analysis using a Veritas luminometer (Turnaer BioSystems, Sunnyvale, CA) according to the manufacturer's procedures.

ANALYSIS OF NF-κB ACTIVATION WITH LIVE CELL IMAGING

The HcRed1-κB reporter was co-transfected with indicated TRAF6 mutant constructs using the transfection procedures described above. Fluorescence was visualized in living cells using a Zeiss AxioObserver Z1 inverted microscope equipped with a Yokagawa QLC100 spinning disk confocal head, a high-speed −50°C ultra-cooled Hamamatsu C9100-02 Electron Multiplier CCD camera and a Melles Griot Kr/Ar Laser. Images were collected using the Hammamatsu-Compix Simple PCI software suite.

IMMUNOPRECIPITATION AND WESTERN BLOT

After indicated treatments, cell lysate supernatant (200 μl/10^6 cells) was incubated with a specific antibody and protein A/G-conjugated agarose slurry (Santa Cruz Biotechnology). Adsorbed proteins were eluted with 1× SDS–PAGE sample buffer, loaded into a pre-cast 4–12% gradient SDS–polyacylamide gel (Lonza). Resolved proteins were transferred onto a PVDF membrane, incubated with indicated antibodies and visualized by chemiluminescence.

RESULTS

CORRELATION OF SEQUESTOSOMAL LOCALIZATION, UBIQUITINATION, AND NF-κB ACTIVITY OF TRAF6 AND DERIVED MUTEINS

We previously reported that ectopically expressed wtTRAF6-YFP is mainly localized to the cytoplasm of transfected 293 cells as small speckles and large punctate sequestosomes [Wang et al., 2006]. This is similar to what has been reported by others for endogenous TRAF6 in IL-1-treated HepG2 cells [Sanz et al., 2000]. Sequestosomes appear as punctate spots in the cytoplasm, which harbor sequestosome 1/P62 protein. Ectopically expressed TRAF6 co-localizes with P62 in sequestosomes (Fig. S1). They have also been reported to be sites where ubiquitinated TRAF6 co-localizes with proteasomes [Seibenhener et al., 2004]. In order to determine whether any of the known TRAF6 structural domains (Fig. 1A,B) dictate specific subcellular localization that correlate with activity, we have expressed various dual YFP and FLAG-tagged muteins either alone or along with a constitutively expressed nuclear-localizing HcRed1 marker and examined TRAF6 subcellular localization along with ubiquitination (Fig. 1C–E) and NF-κB (Fig. 2A) activities. Figure 1E also presents confocal sections of 293 cells ectopically expressing wtTRAF6 and muteins along with a summary of their auto-ubiquitination status and NF-κB activities. In addition to the quantitative luciferase-based NF-κB activity (Fig. 2A), we have also designed a fluorescent assay for determination of NF-κB activity in single living cells. This assay utilizes the co-transfection of an NF-κB-driven reporter expressing a nuclear-localizing HcRed1 marker instead of luciferase. This approach permits analysis of individual cell NF-κB activity to be correlated with subcellular localization of the tagged TRAF6 activators. As shown in Figure 1F, there is strong correlation between the HcRed1-κB nuclear signal and NF-κB luciferase activity (Figs. 1F and 2A), when this reporter is co-expressed along with either untagged or YFP-FLAG-tagged TRAF6 and muteins.

Consistent with our previous report [Wang et al., 2006], wtTRAF6-YFP shows cytoplasmic localization consisting of a large number of small cytoplasmic structures (speckles) and a lesser number of more discrete larger puncta (sequestosomes). Although TRAF6 was observed in most cells to be exclusively cytoplasmic, ~5% of the cells also revealed nuclear localization (not shown),

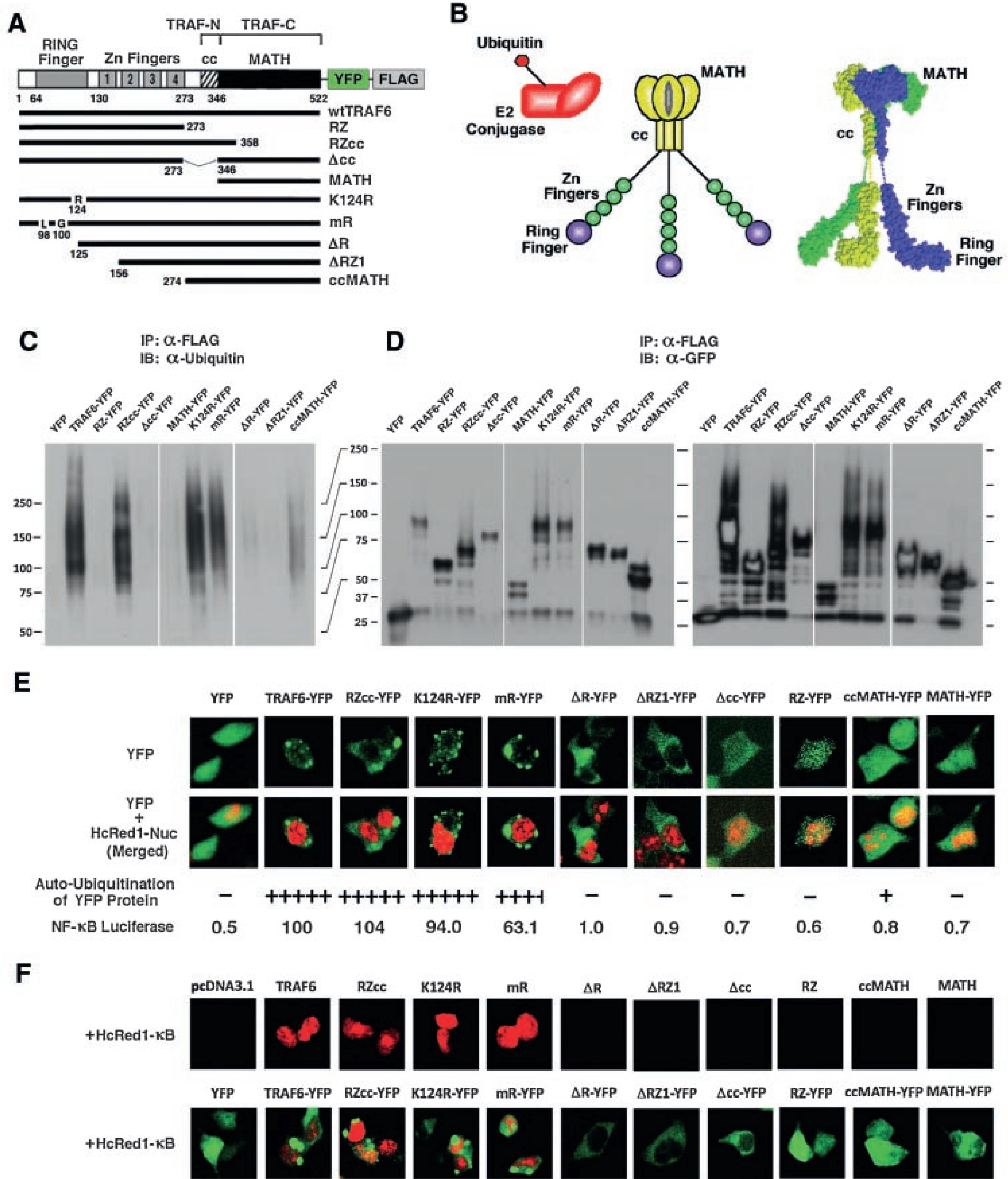

Fig. 1. Subcellular localization patterns and activities for expressed TRAF6 and derived muteins. A: Schematic diagram of TRAF6 structural domains showing the nature of the mutated proteins (muteins) described in this study. These proteins were expressed both with and without a C-terminal dual YFP-FLAG tag. B: Cartoon representation of the proposed "Open" structure of TRAF6 with labeled structural domains, simulated from various X-ray structural studies referenced in text. Also shown is a representation of the E2 conjugase with covalently attached ubiquitin (Red). C: Each indicated expression plasmid (1 μg/well) was transiently transfected into 293T cells that were pre-seeded in six-well tissue culture plates. Twenty-four hours after transfection, transfected cells were lysed and immunoprecipitated with anti-FLAG antibody. Immunoprecipitated proteins containing either wtTRAF6 or derived muteins were analyzed by Western blot for polyubiquitination. D: Immunoprecipitated proteins from (C) were immune-blotted with anti-GFP antibody. A short exposure (left) displays relative amount of expressed proteins, whereas a long exposure (right) confirms heterogeneous molecular sizes of ubiquitinated proteins. E: Subcellular localization presented as confocal microscopic sections of 293 cells ectopically expressing YFP-FLAG-tagged wtTRA6 and muteins along with summary results for ubiquitination and NF-κB activities. The upper row presents only the YFP channel pseudocolored green. The lower row presents a two-channel merged image of the YFP channel along with that of an HcRed1 constitutive nuclear-localizing protein marker (HcRed1 Nuc). F: Single-cell NF-κB activity measured using a novel live-cell reporter vector (HcRed1-κB). HcRed1-κB expression following co-expression with un-tagged (top) and YFP-tagged (bottom) wtTRAF6 and TRAF6 muteins.

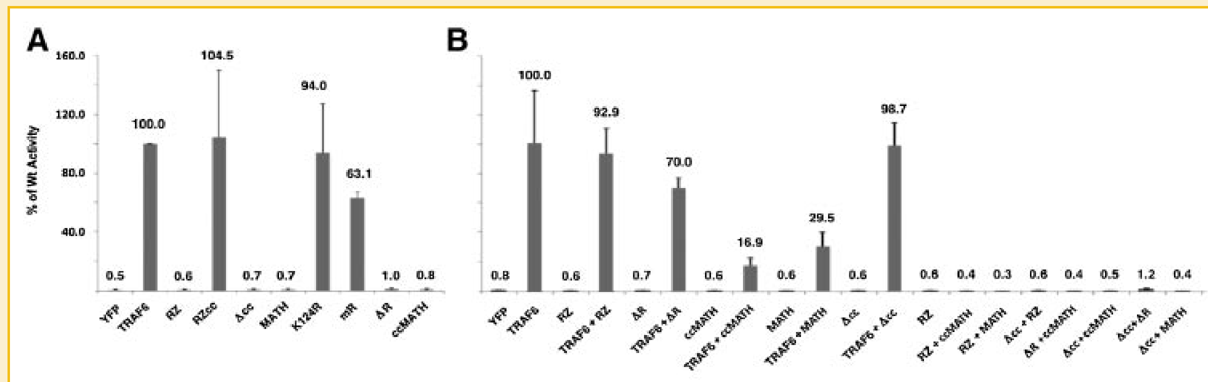

Fig. 2. NF-κB luciferase activity from transfected 293 cells Each indicated expression plasmid (50 ng/well) was co-transfected with 50 ng of NF-κB luciferase reporter into 293T cells that were pre-seeded in 24-well tissue culture plates. Transfected cells were lysed and subject for luciferase assay 24 h post-transfection. A: NF-κB luciferase activity from ectopically expressed wtTRAF6 and derived muteins. B: NF-κB luciferase activity from wtTRAF6 co-expressed with inactive muteins as well as from pairs of inactive muteins co-expressed in the absence of wtTRAF6. Results shown in (A) and (B) are averages of three independent experiments.

supporting a recent report of TRAF6 nuclear function [Pham et al., 2008]. Ectopic expression of wtTRAF6 also revealed a high level of both auto-ubiquitination and NF-κB activities (Figs. 1C–F and 2A). Lysine 124 of TRAF6 has been reported to be critical for ubiquitination and activity [Lamothe et al., 2007], yet K124R-YFP unexpectedly exhibited a subcellular localization pattern, ubiquitination, and NF-κB activities similar to that of the wild-type (Fig. 1C–F) [Walsh et al., 2008]. Furthermore, in contrast to a report that residues 98–100 are important for auto-ubiquitination [Markin et al., 2008], mR-YFP encoding two substitutions, I98L and D100G, unexpectedly generated wild-type cellular localization along with moderate ubiquitination and NF-κB activities (Fig. 1C–F). Therefore, residues 98–100 of TRAF6 within the RING domain may not be directly involved in E2 binding. This result is consistent with a recent X-ray structure which places these residues adjacent to, but not in contact with, the Ubc13 E2 conjugase [Yin et al., 2009]. The observation that RZcc is capable of ubiquitination and NF-κB activation (Fig. 1C–F) supports previous reports that the RZ fingers plus the coiled-coil domain, in the absence of the MATH domain, are collectively sufficient for auto-ubiquitination and downstream signaling [Deng et al., 2000; Yang et al., 2004]. A summary of statistical results related to the correlation of sequestosome formation with the ubiquitination of various TRAF6 proteins and their NF-KB activity is available in the additional supporting information (Fig, S2 and Table S1).

TRAF6 UBIQUITINATION IS MEDIATED IN *TRANS* BY ITS RING-ZINC AND COILED-COIL DOMAINS

In contrast to the wtTRAF6 and muteins described above, the collection of six RING and coiled-coil domain deletion muteins (ΔR-, ΔRZ1-, Δcc-, RZ-, ccMATH-, and MATH-YFP) demonstrated substantial loss of sequestosomes and ubiquitination (Fig. 1C,D). Surprisingly, when these proteins were co-expressed with unlabeled wtTRAF6, sequestosome formation was restored for ΔR-, ΔRZ1-, and Δcc-YFP muteins, while RZ-, ccMATH-, and MATH-YFP displayed nuclear localization (Fig. 3A). Strikingly, wtTRAF6 co-expression rescued ubiquitination for all of these muteins (Fig. 3B). A duplicate membrane blotted with anti-GFP antibody shows molecular size agreement with the lowest size of each heterogeneous polyubiqutinated mutein, demonstrating target specificity. This result suggests that lysine residues located at both the N- and C-termini of TRAF6 are targets of ubiquitination, and that this process occurs in *trans*.

In order to further explore the mechanism of such *trans*-ubiquitination, untagged versions of Δcc and RZ were individually co-expressed with one of each of the RZ-, ΔR-, ccMATH-, or MATH-YFP muteins. Our data reveal that ubiquitination only occurs when one mutein contains the RZ and the other, the coiled-coil domain (Fig. 3B). We also observed changes in subcellular localization for YFP-tagged muteins induced by trans-ubiquitination. For example, when ccMATH-YFP was co-expressed with either un-tagged RZ or Δcc (Fig. 3C), as compared to that of ccMATH-YFP expressed alone (Fig. 1E), and when ΔR-YFP was co-expressed with un-tagged RZ (Fig. 3C), as compared to that of ΔR-YFP expressed alone (Fig. 1E). This supports a mechanism in which RZ collaborates with the coiled-coil domain in the recruitment of the TRAF-specific Ubc13/Uev1A ubiquitin E2 conjugase.

TRANS-UBIQUITINATION IS INSUFFICIENT FOR ACTIVATION OF TRAF6 INACTIVE MUTEINS

Attempting to evaluate whether *trans*-ubiquitination can rescue NF-κB activity of inactive muteins, due to the observation of correlation between ubiquitination and activity of some active TRAF6 proteins, we co-expressed different combinations of inactive muteins in 293 cells and monitored their activities with an NF-κB luciferase reporter. As expected, combination of two incapable of *trans*-ubiquitination muteins (RZ + MATH, ΔR + ccMATH, and Δcc + MATH) did not yield significant NF-κB activity. However, none of the *trans*-ubiquitination-capable pairings (RZ + ccMATH, Δcc + ccMATH, and Δcc + ΔR) rescued NF-κB activity (Figs. 3B,C and 2B). Therefore, ubiquitination appears to be necessary, but insufficient, for NF-κB activation, suggesting a disconnection between ubiquitination and downstream signaling.

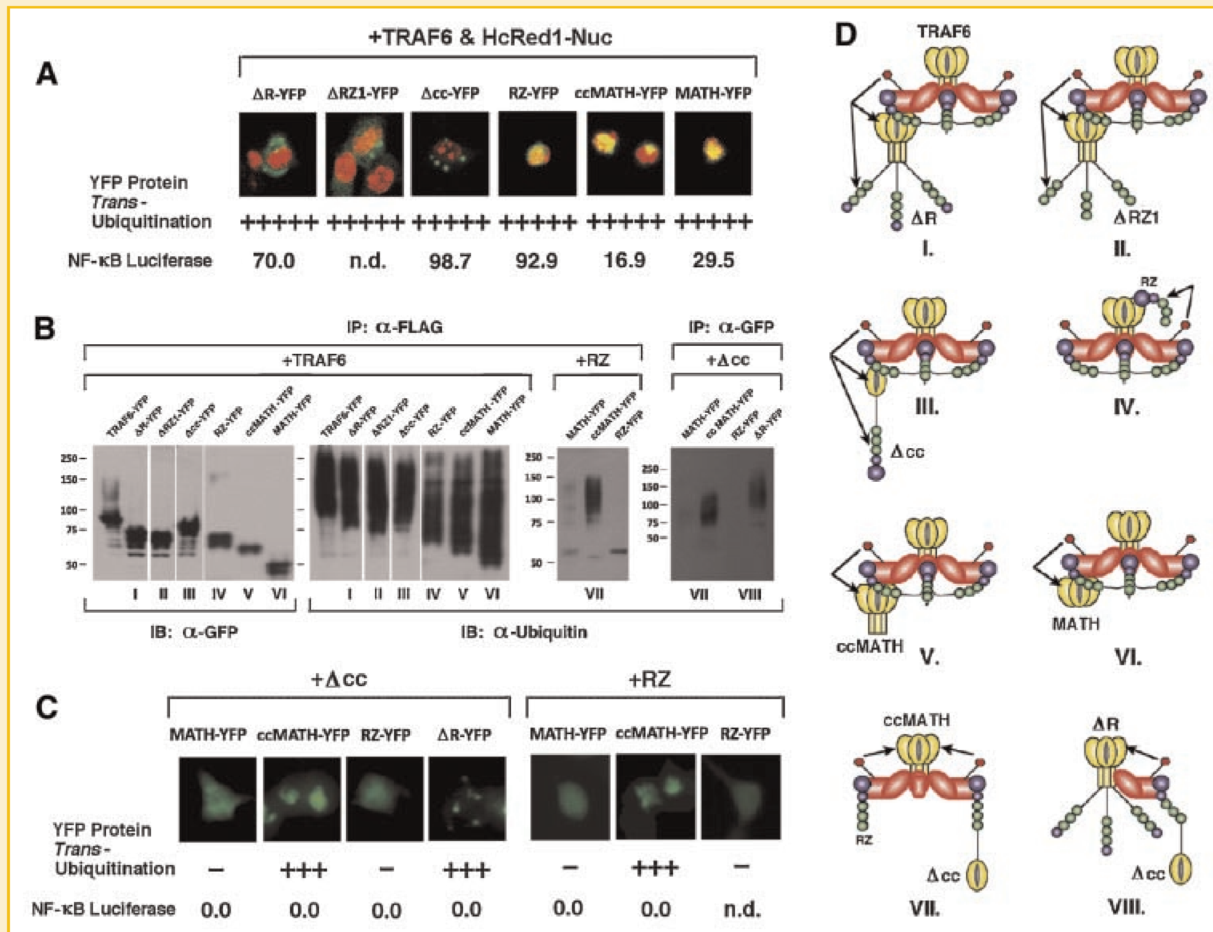

Fig. 3. *Trans*–ubiquitination of inactive TRAF6 muteins. Each indicated expression plasmid (1 μg/well) was transiently transfected into 293T cells that were pre-seeded in six-well tissue culture plates. All analyses were performed 24 h post-transfection. A: Besides indicated plasmids, the HcRed1-Nuc (1 μg) vector was also co-transfected into cells as a nuclear location marker. Confocal microscopic sections and summary results for ubiquitination and NF-κB luciferase activities that ectopically co-expressed un-tagged wtTRAF6 along with various inactive muteins, as indicated. B: Western blots showing *Trans*–ubiquitination of wtTRAF6 derived muteins. C: Wide-field fluorescent images along with summary results for ubiquitination and NF-κB luciferase assays that ectopically co-expressed un-tagged Δcc, or RZ along with various inactive muteins, as indicated. D: Cartoons suggesting likely interactions responsible for results presented in A and B, as designated by Roman numerals. It should be noted that wtTRAF6 may also form heterotrimers with some muteins.

MOLECULAR INTERACTION BETWEEN THE RZ REGION AND MATH DOMAIN

In order to further pursue the concept of *trans*-ubiquitination, wtTRAF6 was co-expressed along with several inactive muteins. TRAF6 activation of NF-κB was significantly inhibited by MATH- and ccMATH-YFP, but not significantly by ΔR- Δcc-, and RZ-YFP (Fig. 2B). This is suggestive of an effect that is specific to the MATH domain. The dominant-negative effect of ccMATH has been reported in many studies of IL-1/LPS signaling, and was described as a competitor for TRAF6 recruitment by upstream activators [Darnay et al., 1999]. However, our study is independent of upstream activators, suggesting a distinct mechanism. Therefore, we hypothesized the existence of a potential molecular interaction between the MATH domain and critical functional domains of TRAF6. Interaction between the MATH domains may be unlikely, since a crystal structure of the TRAF6 MATH domain has been shown to be a monomer [Ye et al., 2002]. One such functional target may be the RZ region, which functions in activating downstream pathways [Baud et al., 1999]. Such reasoning is also supported by NF-κB luciferase reporter assay, in which Δcc showed minimal inhibition on TRAF6 activation of NF-κB, compared to that of the MATH and ccMATH muteins (Fig. 2B). Co-immunoprecipitation of lysates derived from ectopically expressed proteins using an anti-FLAG antibody specific for the YFP-FLAG dual-tagged proteins followed by immuno-blotting with a TRAF6 anti-RZ antibody, demonstrates that wtTRAF6 is co-immunoprecipitated with both the individual RZ region and MATH domain (Fig. 4A, lanes 3 and 4). In addition, untagged ectopically expressed RZ mutein was co-immunoprecipitated with FLAG-tagged proteins with an efficiency that decreased in the order TRAF6-YFP > ccMATH-YFP > MATH-YFP > RZ-YFP (Fig. 4A, lanes 4–11). This demonstrates that expression of either MATH or ccMATH interferes with co-transfected TRAF6 downstream signaling by interacting with and blocking the required RZ, as would happen via interdomain interaction in the intact TRAF6 before activation.

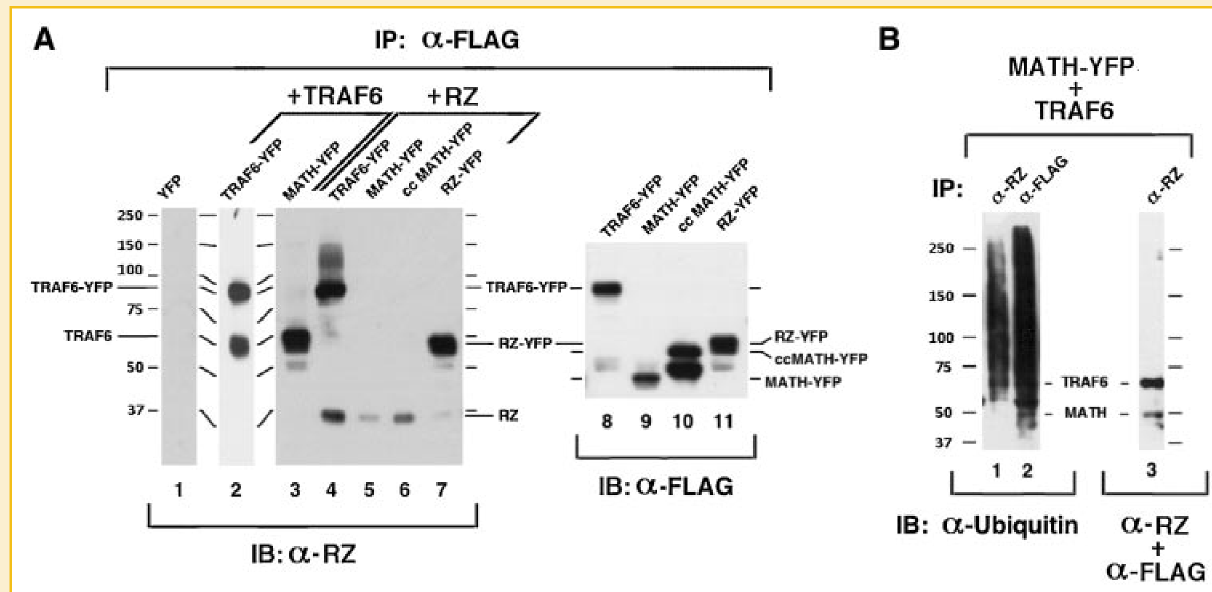

Fig. 4. Interaction between N- and C-termini of TRAF6. Each indicated expression plasmid (1 μg/well) was transiently transfected into 293T cells that were pre-seeded in six-well tissue culture plates. All analyses were performed 24 h post-transfection. A: Left panel (lanes 1–7), untagged wtTRAF6 and RZ proteins were co-expressed with various dual-tagged proteins in 293T cells and analyzed for physical interaction by co-immunoprecipitation and Western blotting, as indicated. Right panel (lanes 8–11), Western blot of tagged proteins after immunoprecipitation. B: Co-expressed untagged wtTRAF6 and dual-tagged MATH proteins were immunoprecipitated with either anti-RZ or anti-FLAG. Interaction and trans-ubiquitination were analyzed with indicated antibodies.

UBIQUITINATION INTERFERES WITH THE RZ-MATH INTERACTION

In order to investigate whether ubiquitination is involved in regulating the RZ-MATH domain interaction, untagged wtTRAF6 was co-expressed together with the dual-tagged MATH mutein. Figure 4B (lanes 1 and 2) shows that anti-RZ and -FLAG antibodies each pull down, from equal amounts of cell lysate, a distinct population of polyubiquitinated proteins in which the lowest molecular size of each is similar to that of the non-ubiquitinated forms of either TRAF6 or MATH-YFP (Fig 4B, lane 3). The absence of the polyubiquitinated MATH domain in the anti-RZ pull-down (Fig. 4B, lane 1) suggests that polyubiquitinated TRAF6 and the polyubiquitinated MATH domain do not bind efficiently. Since MATH and RZ are targeted for ubiquitination (Fig. 3B), TRAF6 polyubiquitination may provide steric bulk that disrupts the interaction between MATH and RZ. Similar results were also observed for co-expression of wtTRAF6 (62 kDa) with dual-tagged wtTRAF6 or muteins (TRAF6-, ΔR-, ΔRZ1-, and Δcc-YFP-FLAG are 90, 75, 71, and 81 kDa, respectively) (Fig. 3B, lanes I–III of 1st and 2nd panels), suggesting disruption of TRAF6 multimers upon polyubiquitination. Recalling the high correlation between ubiquitination and NF-κB activity for TRAF6 and several active muteins (Fig. 1), we propose here that polyubiquitination initially maintains an "open" active TRAF6 conformation.

EXCESSIVE UBIQUITINATION OF TRAF6 MAY BE A "DOUBLE-EDGED SWORD" IN NF-κB ACTIVATION

Since TRAF6-containing sequestosomes appear after activation, we investigated whether their formation increases in parallel with activity. To test this, we titrated the TRAF6-YFP expression vector into cells along with constant amounts of either the HcRed1-κB or luciferase reporters. The micrographs reveal an initial increase in sequestosomes correlating with increased expression of TRAF6 and NF-κB activity, followed by a dramatic decrease in activity while sequestosomes continue to significantly increase in size (Fig. 5A,B). Examination of YFP and brightfield overlays, aimed at correlating TRAF6 intracellular localization with cell morphology, reveals that decreased NF-κB activity in the presence of super-sized sequestosomes does not appear to be associated either with a generalized mechanism of protein overexpression or cell death, since expression of high levels of either YFP or RZ-YFP (neither of which are capable of auto-ubiquitination) does not result in sequestosome formation, a decrease in cell number, or obvious changes in cell morphology (Fig. 5C). Since sequestosomes have been characterized as sites of high ubiquitination and proteasome localization, this observation implies that the proteasome plays a critical role in regulating TRAF6-dependent NF-κB activity. This is consistent with reports of TRAF6 recycling by deubiquitination [Jensen and Whitehead, 2003; Trompouki et al., 2003], which may occur in the sequestosome. Since we previously reported that an IRAK2 mutein can induce TRAF6 localization to super-sized sequestosomes in parallel with a large decrease in NF-κB activity [Wang et al., 2006], we hypothesize that overexpression of TRAF6 results in an increase in NF-κB activity up to the point where the recycling capability of the sequestosome is exceeded. At that point, recycling ceases and activity is lost due to the sequestration of inactive TRAF6.

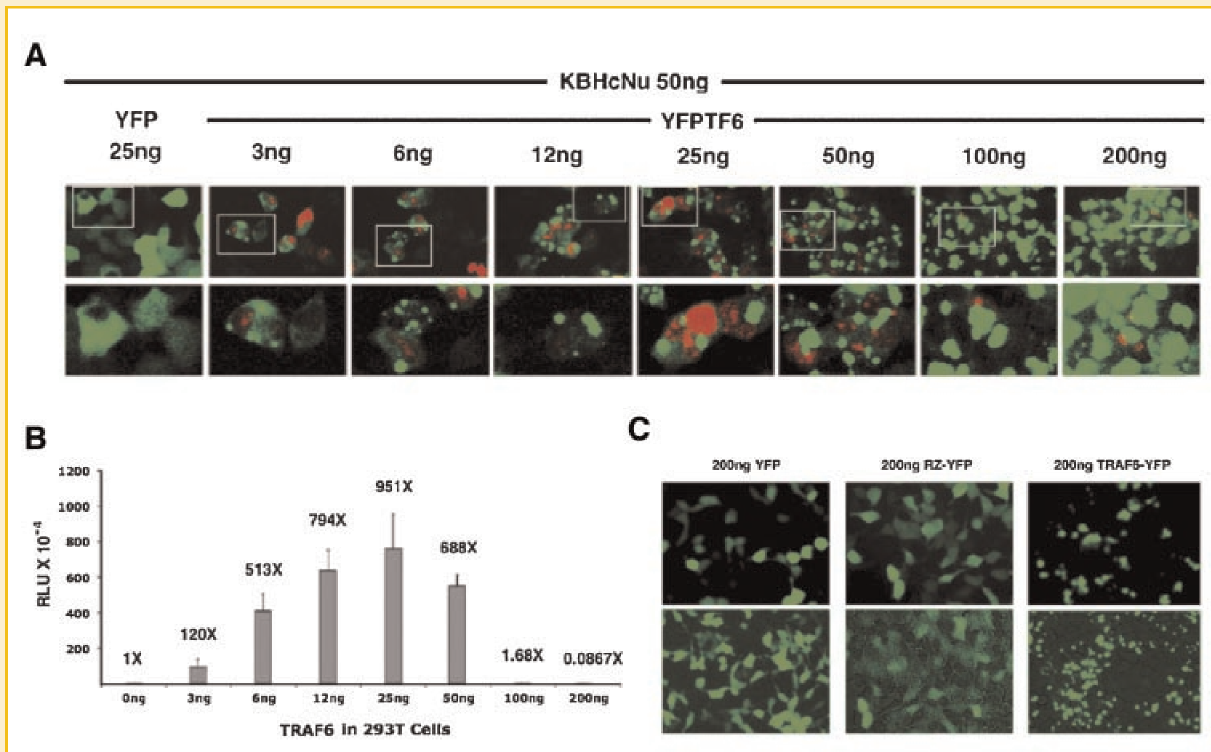

Fig. 5. Ectopic expression of TRAF6 in 293T cells transfected with varying amounts of wtTRAF6 expression plasmid. TRAF6 expression plasmid (as indicated) co-transfected with a constant amount (50 ng) of either the HcRed1-κB live-cell (A) or luciferase NF-κB (B) activity reporters into each well of 293T cells cultured in a 96-well plate. The upper row in (A) shows a field of cells in which a boxed area is magnified in the lower row. C: Transfection of a large amount (200 ng) of each of three expression vectors, as indicated, into each well of 293T cells cultured in a 96-well plate. Upper row shows fluorescent signal. Lower row superimposes a fluorescent signal upon the bright-field image of the cells.

DISCUSSION

Previous independent reports demonstrated that Ubc13 E2 ubiquitin conjugase can interact with both the RING and first Zinc finger [Yin et al., 2009] as well as the coiled-coil domain [Yang et al., 2004] of TRAF6. However, these reports did not provide evidence defining whether these two different interaction sites can function independently. Our results demonstrate that significant polyubiquitination of TRAF6 requires cooperation of both the RZ and coiled-coil. In other words, the RZ region alone, although possessing a classical E2 RING finger binding motif found in many E3 ubiquitin ligases, in our studies is insufficient to support *trans*-ubiquitination (Fig. 1C, 3rd lane). Consequently, for TRAF6, the E2 binding motif may be bipartite, consisting of both RZ and the coiled-coil, both of which may be important for function.

We demonstrate here for the first time that the RZ and MATH domain of TRAF6 are both ubiquitinated and that a molecular interaction exists between these two regions. Furthermore, this interaction is shown to be counteracted by activation-dependent *trans*-ubiquitination. Although polyubiquitination can be mediated by the RZ and coiled-coil either intra- or intermolecularly, the NF-κB activity of TRAF6 requires intramolecular collaboration between these regions (RZcc). This is supported by our observation that intermolecular interaction between wtTRAF6 and a mutein is disrupted upon *trans*-ubiquitination (Fig. 3B, lanes I–III). A similar consequence should exist for the co-expression of two muteins capable of *trans*-ubiquitination (i.e., RZ and ccMATH), which results in loss of E3 ligase function. This implicates ubiquitination of downstream molecules (e.g., TAK1, TABs, and/or NEMO) by TRAF6, but not the K63-linked polyubiquitin chains anchored to TRAF6, as important for NF-κB activation. This argument is supported by our data showing that *trans*-ubiquitination mediated by two TRAF6 muteins is insufficient for NF-κB activity (Figs. 2B and 3C). This is also consistent with a report that lysine-deficient TRAF6 demonstrates little or no significant loss of NF-κB activity [Walsh et al., 2008]. Our result may contradict a recent report that unanchored polyubiquitin chains (i.e., not attached to any protein) synthesized by TRAF6 can activate TAK1 or NEMO in the NF-κB pathway [Xia et al., 2009]. However, this might relate to the unknown nature of the polyubiquitin linkages, and their potential-specific roles in activity.

Taken together, our results revealing both intramolecular interaction between the RZ region and MATH domain along with *trans*-ubiquitination suggest that TRAF6 is maintained in an inactive "closed" state by intramolecular interaction, similar to that manifested by numerous kinases and other signaling molecules [Huse and Kuriyan, 2002]. The combined coiled-coil and MATH domains (ccMATH) of TRAF2 and TRAF3 display a similar trimeric structure. However, the crystallized MATH domain of TRAF6 is a monomer, suggesting that the coiled-coil domain may be required for trimerization and function of TRAF proteins. Although there is

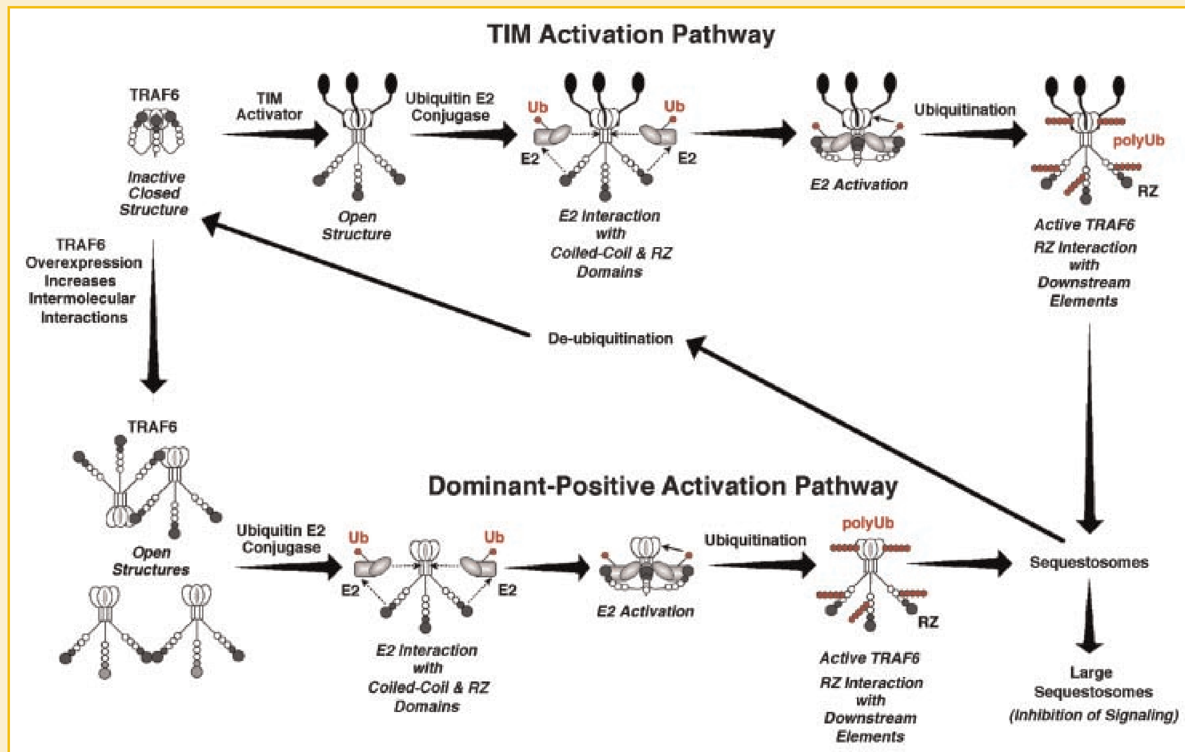

Fig. 6. Model of TRAF6 regulation. Model proposing the normal (TIM) and ectopic expression (dominant-positive) pathways for TRAF6 activation and the role played by ubiquitination to maintain an active, *open*, structure capable of interacting with downstream kinases. Also suggested is the post-activation pathway that results in the formation of large sequestosomes and inactivation, which may be related to proteasome activity. In the TIM activating pathway, the TIM binding motif on an activator interacts with the TIM on TRAF6, and other portions of the complete TIM activator (displayed as a blob sitting on a TIM in the graphs) is likely required for generating an open TRAF6 structure. The ectopic expression (dominant-positive) pathway shows increased intermolecular interaction of TRAF6, which enforces its "open" conformation.

no report showing any full-length TRAF crystal structure, we modeled a full-length TRAF6 structure based on the known X-ray structure data [Ye et al., 1999, 2002; Ni et al., 2000, 2004; Park et al., 2000]. This suggests an extended trimer, primarily self-associated via the coiled-coil domain. In this model, the RZ regions extend away from the coiled-coil with the potential for a flexible region between the two (Fig. 1B). The receptor-dependent activation event for TRAF6 induced by ligand-dependent interaction of receptor-proximal TIM-bearing proteins with the TIM-recognition groove of the MATH domain [Ye et al., 2002] may result in the disruption of this interaction and conversion to an "open" state. This would expose the RZ and coiled-coil permitting the association with E2 conjugase that results in *trans*-ubiquitination. Ubiquitination may serve as a means of providing steric bulk, thus sustaining the active conformation (Fig. 6). Dominant-positive activation by TRAF6 overexpression may result from the increased probability of intermolecular associations such as between the RZ and MATH domains (Fig. 4A), leading to disruption of the inhibitory intramolecular interaction (Fig. 6). Since several TRAF family molecules possess similar structures and have been reported to form homotypic heteromers, the activation model presented here may be generally applicable to other TRAFs.

ACKNOWLEDGMENTS

This work was supported by funds to P.E.A. from the Bayer School of Natural and Environmental Sciences at Duquesne University and to D.L.G. from NIH grant AR057310.

REFERENCES

Aggarwal BB. 2003. Signalling pathways of the TNF superfamily: A double-edged sword. Nat Rev Immunol 3:745–756.

Baud V, Liu Z-G, Bennett B, Suzuki N, Xia Y, Karin M. 1999. 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 13:1297–1308.

Chung JY, Park YC, Ye H, Wu H. 2002. All TRAFs are not created equal: Common and distinct molecular mechanisms of TRAF-mediated signal transduction. J Cell Sci 115:679–688.

Dadgostar H, Cheng G. 2000. Membrane localization of TRAF 3 enables JNK activation. J Biol Chem 275:2539–2544.

Darnay B, Ni J, Moore PA, Aggarwal BB. 1999. Activation of NF-kB by RANK requires tumor necrosis factor receptor-associated factor (TRAF) 6 and NF-kB-inducing kinase. J Biol Chem 274:7724–7731.

Deng L, Wang C, Spencer E, Yang L, Braun A, You J, Slaughter C, Pickart C, Chen ZJ. 2000. Activation of the IkappaB kinase complex by TRAF6 requires

a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell 103:351–361.

Frossard N, Freund V, Advenier C. 2004. Nerve growth factor and its receptors in asthma and inflammation. Eur J Pharmacol 500:453–465.

Huse M, Kuriyan J. 2002. The conformational plasticity of protein kinases. Cell 109:275–282.

Jensen LE, Whitehead AS. 2003. Ubiquitin activated tumor necrosis factor receptor associated factor-6 (TRAF6) is recycled via deubiquitination. FEBS Lett 553:190–194.

Lamothe B, Besse A, Campos AD, Webster WK, Wu H, Darnay BG. 2007. Site-specific Lys-63-linked tumor necrosis factor receptor-associated factor 6 auto-ubiquitination is a critical determinant of I kappa B kinase activation. J Biol Chem 282:4102–4112.

Lomaga M, Yeh W-C, Sarosi I, Duncan G, Furlonger C, Ho A, Morony S, Capparelli C, Van G, Kaufman S, van der Heiden A, Itie A, Wakeham A, Khoo W, Sasaki T, Cao Z, Penninger J, Paige C, Lacey DL, Dunstan C, Boyle W, Goddel D, Mak T. 1999. TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. Genes Dev 13:1015–1024.

Markin CJ, Saltibus LF, Spyracopoulos L. 2008. Dynamics of the RING domain from human TRAF6 by 15N NMR spectroscopy: Implications for biological function. Biochemistry 47:10010–10017.

Ni CZ, Welsh K, Leo E, Chiou CK, Wu H, Reed JC, Ely KR. 2000. Molecular basis for CD40 signaling mediated by TRAF3. Proc Natl Acad Sci USA 97:10395–10399.

Ni CZ, Oganesyan G, Welsh K, Zhu X, Reed JC, Satterthwait AC, Cheng G, Ely KR. 2004. Key molecular contacts promote recognition of the BAFF receptor by TNF receptor-associated factor 3: Implications for intracellular signaling regulation. J Immunol 173:7394–7400.

Park YC, Ye H, Hsia C, Segal D, Rich RL, Liou HC, Myszka DG, Wu H. 2000. A novel mechanism of TRAF signaling revealed by structural and functional analyses of the TRADD-TRAF2 interaction. Cell 101:777–787.

Pham LV, Zhou HJ, Lin-Lee YC, Tamayo AT, Yoshimura LC, Fu L, Darnay BG, Ford RJ. 2008. Nuclear tumor necrosis factor receptor-associated factor 6 in lymphoid cells negatively regulates c-Myb-mediated transactivation through small ubiquitin-related modifier-1 modification. J Biol Chem 283:5081–5089.

Sanz L, Diaz-Meco MT, Nakano H, Moscat J. 2000. The atypical PKC-interacting protein p62 channels NF-kappaB activation by the IL-1-TRAF6 pathway. EMBO J 19:1576–1586.

Seibenhener ML, Babu JR, Geetha T, Wong HC, Krishna NR, Wooten MW. 2004. Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation. Mol Cell Biol 24:8055–8068.

Trompouki E, Hatzivassiliou E, Tsichritzis T, Farmer H, Ashworth A, Mosialos G. 2003. CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members. Nature 424:793–796.

Walsh MC, Kim GK, Maurizio PL, Molnar EE, Choi Y. 2008. TRAF6 auto-ubiquitination-independent activation of the NFkappaB and MAPK pathways in response to IL-1 and RANKL. PLoS One 3:e4064.

Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, Chen ZJ. 2001. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature 412:346–351.

Wang KZ, Wara-Aswapati N, Boch JA, Yoshida Y, Hu CD, Galson DL, Auron PE. 2006. TRAF6 activation of PI 3-kinase-dependent cytoskeletal changes is cooperative with Ras and is mediated by an interaction with cytoplasmic Src. J Cell Sci 119:1579–1591.

Wooten MW, Geetha T, Seibenhener ML, Babu JR, Diaz-Meco MT, Moscat J. 2005. The p62 scaffold regulates nerve growth factor-induced NF-kappaB activation by influencing TRAF6 polyubiquitination. J Biol Chem 280: 35625–35629.

Wu H, Arron JR. 2003. TRAF6, a molecular bridge spanning adaptive immunity, innate immunity and osteoimmunology. Bioessays 25:1096–1105.

Xia ZP, Chen ZJ. 2005. TRAF2: A double-edged sword? Sci STKE 2005:pe7.

Xia ZP, Sun L, Chen X, Pineda G, Jiang X, Adhikari A, Zeng W, Chen ZJ. 2009. Direct activation of protein kinases by unanchored polyubiquitin chains. Nature. 461:114–119.

Yang K, Zhu J, Sun S, Tang Y, Zhang B, Diao L, Wang C. 2004. The coiled-coil domain of TRAF6 is essential for its auto-ubiquitination. Biochem Biophys Res Commun 324:432–439.

Ye H, Park Y, Kreisman M, Kieff E, Wu H. 1999. The structural basis for the recognition of diverse receptor sequences by TRAF2. Mol Cell 4:321–330.

Ye H, Arron JR, Lamothe B, Cirilli M, Kobayashi T, Shevde NK, Segal D, Dzivenu OK, Vologodskaia M, Yim M, Du K, Singh S, Pike JW, Darnay BG, Choi Y, Wu H. 2002. Distinct molecular mechanism for initiating TRAF6 signalling. Nature 418:443–447.

Yin Q, Lin SC, Lamothe B, Lu M, Lo YC, Hura G, Zheng L, Rich RL, Campos AD, Myszka DG, Lenardo MJ, Darnay BG, Wu H. 2009. E2 interaction and dimerization in the crystal structure of TRAF6. Nat Struct Mol Biol. 16:658–666.

Yoshida Y, Kumar A, Koyama Y, Peng H, Arman A, Boch JA, Auron PE., 2004 Interleukin 1 activates STAT3/Nuclear Factor-kappaB cross-talk via a unique TRAF6- and p65-dependent mechanism. J Biol Chem 279:1768–1776.