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TRAF6 is Autoinhibited by an Intramolecular Interaction Which is Counteracted by *Trans*-ubiquitination

Kent Z. Q. Wang¹, Deborah L. Galson², and Philip E. Auron^{1,*}

¹Department of Biological Sciences, Duquesne University, Pittsburgh, PA 15282

²Department of Medicine, University of Pittsburgh, Pittsburgh, PA 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 autoubiquitination is now demonstrated to act in *trans* and requires both the RING-Zinc fingers region and coiled-coil domain. We also observed that both the RING-Zinc 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 mutants is insufficient for NF- κ B activation.

Keywords

TRAF6; E3 ubiquitin ligase; Ubiquitination; NF- κ B

TRAF family proteins are ubiquitin E3 ligases that promote TNFR and TIR signaling events, resulting in the induction of numerous genes [1; 2; 3]. One of the most widely studied TRAF-regulated transcription factors is NF- κ B, which is activated by I κ B kinase (IKK) modification and subsequent proteasomal degradation of the inhibitory I κ B protein [4]. 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 [3; 5; 6; 7]. 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- κ B activation [8]. It has also been suggested that K63-linked polyubiquitin chains anchored to TRAF6 are specific signals for downstream interaction with the IKK system [9]. However, this concept has recently been disputed by studies demonstrating that auto-ubiquitination is dispensable

*To whom correspondence should be addressed: Philip E. Auron, PhD 600 Forbes Ave., Department of Biological Sciences, Duquesne University Pittsburgh, PA 15282 auronp@duq.edu Fax: +1 412 396 5907 .

for NF- κ B activation [10]. Furthermore, unlike some signaling molecules, wtTRAF6 overexpression acts as a dominant-positive *via* an unknown mechanism [11].

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 [3] and downstream signaling [12]. TRAF6 poly-ubiquitination 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.

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- κ B luciferase reporter has been described previously (Yoshida, 2003). The HcRed1- κ B reporter was constructed by replacing the luciferase coding sequence in the NF- κ B/pGL2 used for the luciferase assays with HcRed1 cDNA derived from HcRed1-Nuc (BD Biosciences) inserted into the Hind III and MfIMI restriction sites in the NF- κ B/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 EcoRI and EcoRV 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 Δ R; TRAF6(156-522) as Δ RZ1; TRAF6(274-522) as Δ RZ; and TRAF6(274-345) as Δ cc. 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- κ BpGL2) was co-transfected with indicated expression plasmids into 293 cells. After 24 hours, the transfected cells were lysed and analyzed for luciferase

activity analysis using a Veritas luminometer (Turner BioSystems, Sunnyvale, CA) according to the manufacturer's procedures.

Analysis of NF- κ B activation with live cell imaging

The HcRed1- κ B 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 -50C ultra-cooled Hamamatsu C9100-02 Electron Multiplier CCD camera and a Melles Griot Kr/Ar Laser. Images were collected using the Hamamatsu-Compix Simple PCI software suite.

Immunoprecipitation and Western blot

After indicated treatments, cell lysate supernatant (200 μ L per 10^6 cells) was incubated with a specific antibody and protein A/G conjugated agarose slurry (Santa Cruz Biotechnology). Adsorbed proteins were eluted with 1XSDS-PAGE sample buffer, loaded into a precast 4-12% gradient SDS-polyacrylamide 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- κ B activity of TRAF6 and derived mutants

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 [13]. This is similar to what has been reported by others for endogenous TRAF6 in IL-1 treated HepG2 cells [14]. 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 [15]. In order to determine whether any of the known TRAF6 structural domains (Fig. 1A, B) dictate specific sub-cellular localization that correlate with activity, we have expressed various dual YFP and FLAG-tagged mutants either alone or along with a constitutively expressed nuclear-localizing HcRed1 marker and examined TRAF6 sub-cellular localization along with ubiquitination (Fig. 1C-E) and NF- κ B (Fig. 2A) activities. Fig. 1E also presents confocal sections of 293 cells ectopically expressing wtTRAF6 and mutants along with a summary of their auto-ubiquitination status and NF- κ B activities. In addition to the quantitative luciferase-based NF- κ B activity (Fig. 2A), we have also designed a fluorescent assay for determination of NF- κ B activity in single living cells. This assay utilizes the co-transfection of an NF- κ B-driven reporter expressing a nuclear-localizing HcRed1 marker instead of luciferase. This approach permits analysis of individual cell NF- κ B activity to be correlated with sub-cellular localization of the tagged TRAF6 activators. As shown in Fig. 1F, there is strong correlation between the HcRed1- κ B nuclear signal and NF- κ B luciferase activity (Fig. 1F, 2A), when this reporter is co-expressed along with either untagged or YFP-FLAG-tagged TRAF6 and mutants.

Consistent with our previous report [13], 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, approximately 5% of the cells also revealed nuclear localization (not shown), supporting a recent report of TRAF6 nuclear function [16]. Ectopic expression of wtTRAF6 also revealed a high level of both auto-ubiquitination and NF- κ B activities (Fig. 1C-F, 2A). Lysine 124 of TRAF6 has been reported to be critical for ubiquitination and activity [17], yet K124R-YFP unexpectedly exhibited a sub-cellular

localization pattern, ubiquitination and NF- κ B activities similar to that of the wild-type (Fig. 1C-F) [10]. Furthermore, in contrast to a report that residues 98-100 are important for auto-ubiquitination [18], mR-YFP encoding two substitutions, I98L and D100G, unexpectedly generated wild-type cellular localization along with moderate ubiquitination and NF- κ B 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 [19]. The observation that RZcc is capable of ubiquitination and NF- κ B activation (Fig. 1C-F) supports previous reports that the RING-Zinc fingers plus the coiled-coil domain, in the absence of the MATH domain, are collectively sufficient for auto-ubiquitination and downstream signaling [20; 21]. A summary of statistical results related to the correlation of sequestosome formation with the ubiquitination of various TRAF6 proteins and their NF- κ B activity is available in the additional supporting information (Fig. S2. 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 (Δ R-, μ RZ1-, μ cc-, 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 Δ R-, μ RZ1- and μ cc-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 polyubiquitinated 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 Δ cc and RZ were individually co-expressed with one of each of the RZ-, μ R-, 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 sub-cellular localization for YFP-tagged muteins induced by *trans*-ubiquitination. For example, when ccMATH-YFP was co-expressed with either un-tagged RZ or μ cc (Fig. 3C), as compared to that of ccMATH-YFP expressed alone (Fig. 1E), and when μ R-YFP was co-expressed with untagged RZ (Fig. 3C), as compared to that of μ R-YFP expressed alone (Fig. 1E). This supports a mechanism in which RZ collaborates with the coiled-coil domain in the recruitment of the TRAF6-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- κ B 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- κ B luciferase reporter. As expected, combination of two incapable of *trans*-ubiquitination muteins (RZ + MATH, Δ R + ccMATH, and Δ cc + MATH) did not yield significant NF- κ B activity. However, none of the *trans*-ubiquitination-capable pairings (RZ + ccMATH, Δ cc + ccMATH and Δ cc + Δ R) rescued NF- κ B activity (Fig. 3B-C, 2B). Therefore, ubiquitination appears to be necessary, but insufficient, for NF- κ B activation, suggesting a disconnection between ubiquitination and downstream signaling.

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- κ B was significantly inhibited by MATH- and ccMATH-YFP, but not significantly by Δ R- Δ cc-, 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 [22]. 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 [8]. One such functional target may be the RZ region, which functions in activating downstream pathways [12]. Such reasoning is also supported by NF- κ B luciferase reporter assay, in which Δ cc showed minimal inhibition on TRAF6 activation of NF- κ B, 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&4). In addition, untagged ectopically expressed RZ mutant 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.

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 mutant. Fig. 4B (lanes 1&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), Δ R-, Δ RZ1-, and Δ cc-YFP-FLAG are 90, 75, 71 and 81 KDa, respectively) (Fig. 3B, lanes I-III of 1st & 2nd panels), suggesting disruption of TRAF6 multimers upon polyubiquitination. Recalling the high correlation between ubiquitination and NF- κ B 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- κ B 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- κ B or luciferase reporters. The micrographs reveal an initial increase in sequestosomes correlating with increased expression of TRAF6 and NF- κ B 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- κ B activity in the presence of super-sized sequestosomes does not appear to be associated either with a generalized mechanism of protein over-expression 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- κ B activity. This is consistent with reports of TRAF6 recycling by deubiquitination [23; 24], 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- κ B activity [13], we hypothesize that overexpression of TRAF6 results in an increase in NF- κ B 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.

Discussion

Previous independent reports demonstrated that Ubc13 E2 ubiquitin conjugase can interact with both the RING and first Zinc finger [19] as well as the coiled-coil domain [21] 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- κ B 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- κ B activation. This argument is supported by our data showing that *trans*-ubiquitination mediated by two TRAF6 muteins is insufficient for NF- κ B activity (Fig. 2B & 3C). This is also consistent with a report that lysine-deficient TRAF6 demonstrates little or no significant loss of NF- κ B activity [10]. 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- κ B pathway [25]. 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 [26]. 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 no report showing any full-length TRAF crystal structure, we modeled a full-length TRAF6 structure based on the known X-ray structure data [8; 27; 28; 29; 30]. This suggests an extended trimer, primarily self-associated *via* the coiled-coil domain. In this model, the RING-Zinc 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 [8] may result in the disruption of this interaction and conversion to an “open” state. This would expose the RING-Zinc 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 over-expression 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Sub-cellular 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 6well tissue culture plates. 24 hrs after transfection, transfected cells were lysed and immuno-precipitated with anti-FLAG antibody. Immuno-precipitated proteins containing either wtTRAF6 or derived muteins were analysed by Western blot for polyubiquitination. (D) Immuno-precipitated 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) Sub-cellular localization presented as confocal microscopic sections of 293 cells ectopically expressing YFP-FLAG-tagged wtTRAF6 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 2-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 hrs post-transfection. **(A)** NF-κB luciferase activity from ectopically expressed wtTRAF6 and derived mutants. **(B)** NF-κB luciferase activity from wtTRAF6 co-expressed with inactive mutants as well as from pairs of inactive mutants co-expressed in the absence of wtTRAF6. Results shown in **(A)** and **(B)** are averages of three independent experiments.

Fig. 3. Trans-ubiquitination of inactive TRAF6 mutants

Each indicated expression plasmid (1 μg/well) was transiently transfected into 293T cells that were pre-seeded in 6-well tissue culture plates. All analyses were performed 24 hrs 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 mutants, as indicated. **(B)** Western blots showing *Trans*-ubiquitination of wtTRAF6 derived mutants. **(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 mutants, 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 mutants.

Fig. 4. Interaction between N- and C-termini of TRAF6

Each indicated expression plasmid (1 μ g/well) was transiently transfected into 293T cells that were pre-seeded in 6-well tissue culture plates. All analyses were performed 24 hrs 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.

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- κ B live-cell (**A**) or luciferase NF- κ B (**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.

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.