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Molecular Mechanisms of TRAF6 Ubiquitination and Activation

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MOLECULAR MECHANISMS OF TRAF6 UBIQUITINATION AND ACTIVATION

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Zhi Qiang Kent Wang

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Approved December 18, 2009

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ABSTRACT

MOLECULAR MECHANISMS OF TRAF6

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The Tumor Necrosis Factor (TNF) Receptor Associated Factor 6 (TRAF6) is an intracellular signal transducers, being responsible for mediating many of the activation events initiated by TNF receptor (TNFR) and Toll-like/Interleukin-1 and 18 receptor (TIR) families, in which TRAF6 plays central roles in numerous biological processes including innate and adaptive immunity, osteoclastogenesis and bone development, CD40 signaling, neuronal cell development, and cancer cell progression.

Acting as an E3 ubiquitin ligase, TRAF6 catalyzes lysine 63 linked poly-ubiquitination of itself and many other signal transducers upon association with upstream effectors possessing a short TRAF Interaction Motif (TIM) peptide sequence in the NF-κB signal transduction pathway. Ectopic over-expression of TRAF6 acts as a dominant-positive. However, the mechanism of TRAF6 activation by upstream activators or over-

expression is unclear. This motivated our enthusiasm to study the role played by ubiquitination for TRAF6 in NF-κB signaling.

We now demonstrate that two critical regions of TRAF6, the MATH domain required for TIM activator binding and the RING-Zinc region for downstream signaling, mutually interact and render the molecule structurally closed and inactive. Our results implicate that auto-ubiquitination disrupts such interaction, thus providing a means of sustaining the open conformation necessary for downstream signaling. However, excessive ubiquitination induced by TRAF6 over-expression results in formation of large cytoplasmic sequestosomes and its inactivation. Furthermore, the inferred *cis* nature of TRAF auto-ubiquitination is now demonstrated to act in *trans* and is regulated *via* its RING-Zinc and coiled-coil domains. We also demonstrate that both the RING-Zinc region and MATH domain of TRAF6 can be targeted for ubiquitination, but *trans*-ubiquitination of TRAF6 muteins is incapable of activating the NF-κB pathway, suggesting that ubiquitination, alone, is insufficient for activity.

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I have been in the Auron lab since February 2003 as a Research Associate at the University of Pittsburgh. For the past seven years, he has guided me in many respects. I have not only learned knowledge, research skills, and scientific thinking from his supervision, but also greatly improved my English and gained some essential social skills from his teaching and humor, all of which will benefit the rest of my career.

I should address my special appreciation to Dr. Deborah L. Galson, who was actually my first advisor in my research work when I joined the Auron lab. She has also taught me many useful techniques and given me tremendous support.

Recalling the scientific path I have taken in the past two decades of my career, I have experienced many areas including basic research, translational research, R & D of new medications, teaching and management in industry and academic institutions, and

co-authored about thirty research articles and abstracts. There is a long list of acknowledgements among my advisors, colleagues, and friends who have provided me support. They have laid solid foundations for my achievements. I regret that I won't be able to acknowledge them here individually.

The last but certainly not the least, I would like to dedicate my dissertation to my family members: my wife, Nancy Huang, and my son, David Wang. It would have been impossible for me to finish this work without their love, understanding, and support. Nancy has been troubleshooting numerous hurdles in my life and experiments. David, a college sophomore, always makes me proud of him for his talent and diligence. He has also provided me great hands in preparing my dissertation.

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LIST OF ABBREVIATIONS

AP-1	Activator protein-1
APCs	Antigen-presenting cells
BCL10	B cell lymphoma 10
C/EBPβ	CCAAT/enhancer-binding protein β
CYLD	Cylindromatosis
DAG	Diacyl-grycerol
HDAC	Histone deacetylase
IKKγ	IκB kinase γ
IL-1	Interleukine-1
IL1RI	IL-1 type I receptor
IL1RAcp	IL1R interacting accessory protein
IL-6	Interleukine-6
IP3	Inositol triphosphate
IP buffer	Cell lysis buffer for immuno-precipitation
IRAK	IL-1 receptor associated kinase
IRF5	Interferon regulatory factor 5
LPS	Lipopolysacharide
LBP	LPS binding protein
LILRE	LPS and IL-1 responsive element
MAPK	Mitogen-activated protein kinase
MHC I	Class I major histocompatibility complex

MATH	Meprin and TRAF-C homology domain
NF-κB	Nuclear factor-κB
NIK	NF-κB inducing kinase
NGF	Nerve growth factor
NK cells	Natural killer cells
NMR	Nuclear magnetic resonance
PAMPs	Pathogen associated molecular patterns
PH-Akt-GFP	GFP conjugated pleckstrin homology domain of Akt
PI3K	Phosphoinositol 3-kinase
PI(4,5)P2P	Phosphatidylinositol 4, 5-bisphosphate
PI(3,4,5)P3	Phosphatidylinositol 3, 4, 5-trisphosphate
PKB/Akt	Protein kinase B
PKCΘ	Protein kinase C Theta
PLC γ1	Phopholipase C γ1
PRRs	Pattern recognition receptors
RANK	Receptor activator for NF-κB
RANKL	Receptor activator for NF-κB ligand
RING	Really interesting gene domain
SUMO-1	Small ubiquitin-related modifier-1
TAK1	Transforming growth factor beta (TGFβ) activated kinase 1
TAB1	TAK1 binding protein 1
TAB2	TAK1 binding protein 2
TGβRI	Transforming growth factor beta (TGFβ) receptor type I

TGFβ	Transforming growth factor beta
TLR	Toll like receptor
TLR4	Toll like receptor 4
TNFα	Tumor necrosis factor alpha
TRAF6	Tumor necrosis factor receptor associated factor 6
TRAP	Tartrate resistant acidic phosphatase
T_{reg} cells	$CD4^{+}CD25^{+}$ regulatory T cells

INTRODUCTION

Tumor necrosis factor receptor associated factor 6 (TRAF6) has emerged as a critical signaling adaptor molecule involved in a wide spectrum of biological processes including innate and adaptive immunity, osteoclastogenesis and bone development, CD40 signaling, neuronal cell development, cancer cell progression, and gene transcription regulation (Gravallese et al., 2001; Hostager, 2007; Inoue et al., 2007; Kashiwamura et al., 2002; Roux and Barker, 2002; Vallabhapurapu and Karin, 2009; Wu and Arron, 2003). Acting as an E3 ubiquitin ligase and a transcription factor, TRAF6 interacts with different signaling molecules located in the plasma membrane, cytoplasm, proteasome and nucleus. Therefore, it represents an important target in the regulation of many pathological conditions such as inflammation, Parkinson's disease, cancer, and bone diseases.

TRAF6 is a unique member in the TRAF family

There are seven TRAF proteins found in humans. Accumulated evidence shows that they have emerged as important signal transducers and regulators for the TNF receptor super family and the IL1R/TLR super family (Arron et al., 2002; Takeuchi et al., 1996; Xu et al., 2004). The major functions of different TRAF members in regulation of various signaling pathways are summarized in Table 1(Chung et al., 2002).

Table 1. Summary of TRAF Functions

TRAFs	Associated Receptor Family	Functions
TRAF1	TNFR	Apoptotic protection; Feedback regulation of receptor signaling
TRAF2	TNFR	CD40 signaling, RANK signaling; NF-κB activation; JNK activation; Perinatal survival
TRAF3	TNFR/TLR	T-cell-dependent antigen response; CD40 signaling; Perinatal survival
TRAF4	Unclear	Tracheal formation; cancer cell proliferation and metastasis
TRAF5	TNFR	CD27 and CD40 signaling; NF-κB activation
TRAF6	TNFR/TIR	IL-1/LPS/CD40L signaling; Bone metabolism; TCR signaling; NF-κB activation; MAPK pathways; Transcription regulation; Perinatal survival
TRAF7	TNFR-like	NF-κB and AP-1 activation; Inhibiting c-Myb signaling; Apoptosis

Notes: TRAF1 does not contain a RING domain; TRAF7 contains a WD40 instead of a MATH domain.

Modified from Chung, *etal* (2002), *J. Cell Sci.*, 115:679-87

The TRAF proteins share great similarity in their primary structures and are characterized by the presence of three functional domains. Most TRAF molecules (except TRAF1) contain a RING (Really Interesting New Gene) domain and several zinc finger motifs in their N-terminus, which is believed to be important for downstream signaling events. The RING domain is also suggested for the binding of ubiquitin conjugating enzyme E2 (Uev1a–Ubc13) (Pickart, 2001). The C-termini of TRAF molecules (except TRAF7) contain a meprin and TRAF-C homology (MATH) domain and is thought to be engaged in upstream interactions with receptors and other signaling proteins (Chung et al., 2002). There is an alpha-helix coiled-coil region located between the zinc finger motifs and MATH domain (except TRAF7), and it has been suggested to be involved in multimerization and important for TRAFs to function (Baud et al., 1999b; Park et al., 1999; Yang et al., 2004).

The humanTRAF6 gene is located on chromosome 11 (11P12). TRAF6 protein was first identified as a unique signal transducer in the IL1R signal transduction pathway (Cao et al., 1996) and it has been the most characterized protein that is involved in multiple signaling and cellular regulatory processes in the TRAF family. A scheme of the primary structure of TRAF6 is shown in Figure 1.

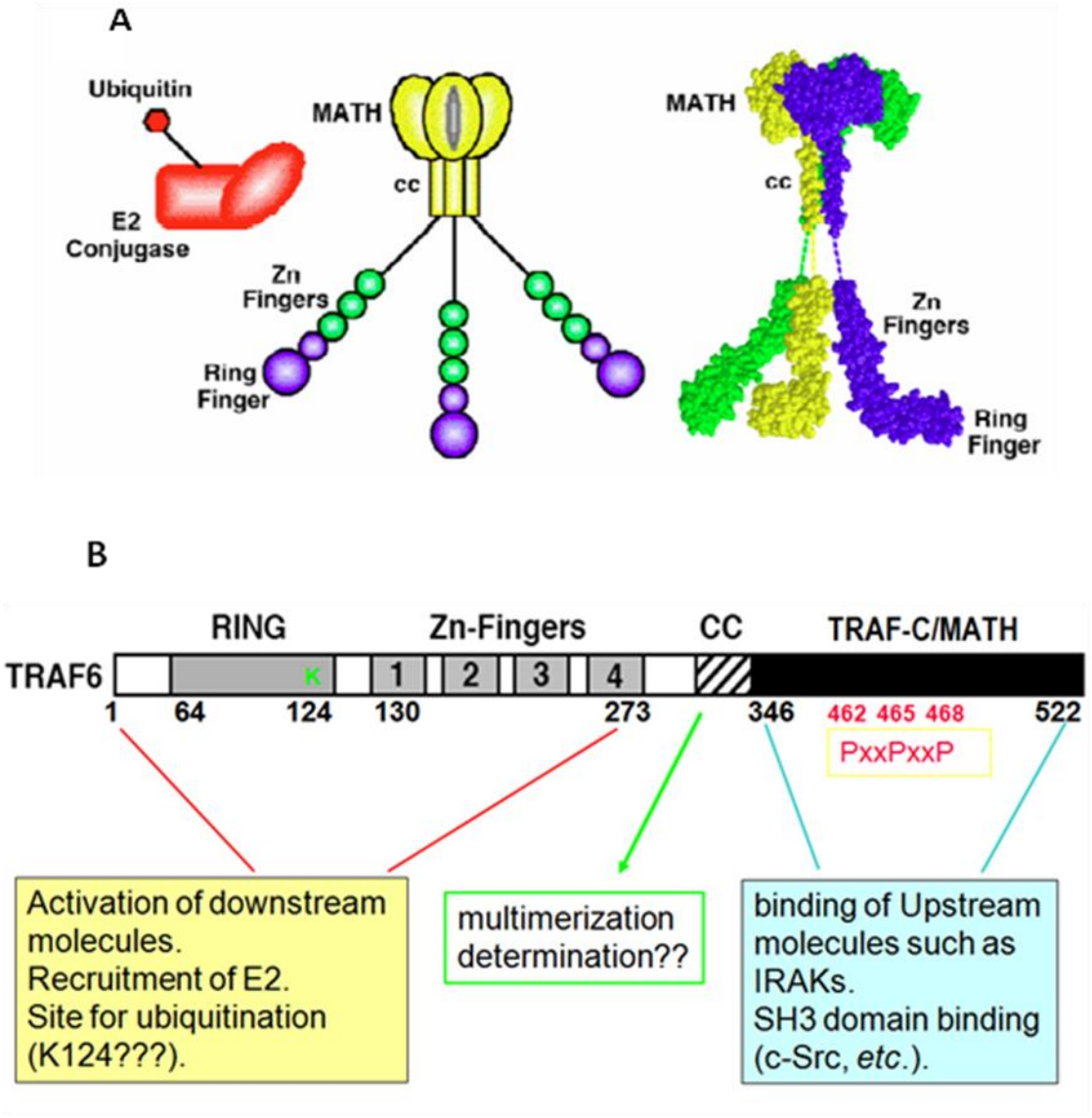


Figure 1. Demonstration of TRAF6 functional domains.

A. Cartoon representation of the proposed "Open" structure of TRAF6 with labeled structural domains, derived from various X-ray structural studies referenced in text (Middle panel) and a composite molecular structure derived from RCSB PDB coordinate datasets 1AC9 and 3HCS (right panel). Left panel is a representation of the E2 ubiquitin conjugating enzyme with covalently attached ubiquitin (Red). B. Brief indications of each functional domain/motif.

Crystallography of the TRAF6 MATH domain containing residues 346-504 shows a monomer structure (Ye et al., 2002). Its outline looks very similar to the MATH domain of TRAF2. However, the TRAF2 crystal structure is trimeric (McWhirter et al., 1999; Park et al., 1999), possibly because the crystallized fragment of TRAF2 contains both coiled-coil and MATH domains. A similar TRAF3 protein is also trimeric. This might implicate the coiled-coil domain in regulation of the multimerization of TRAF molecules. Although great similarities exist between TRAF6 and TRAF2, the divergent peptide binding groove in the MATH domain of TRAF6, as compared to other TRAFs, renders unique biological functions to this protein; it does not interact with peptide motifs that are recognized by TRAF1, -2, -3 or -5. A comparison of crystal structures between TRAF6 and TRAF2 in peptide binding revealed that the chain direction of bound TRAF6-binding peptides shows a 40^{o} rotation from that of the TRAF2-binding peptides. As a result, side chains of TRAF6-binding peptides interact with surface pockets on TRAF6 that are completely different from those on TRAF2 (Ye et al., 2002) (Fig. 2).

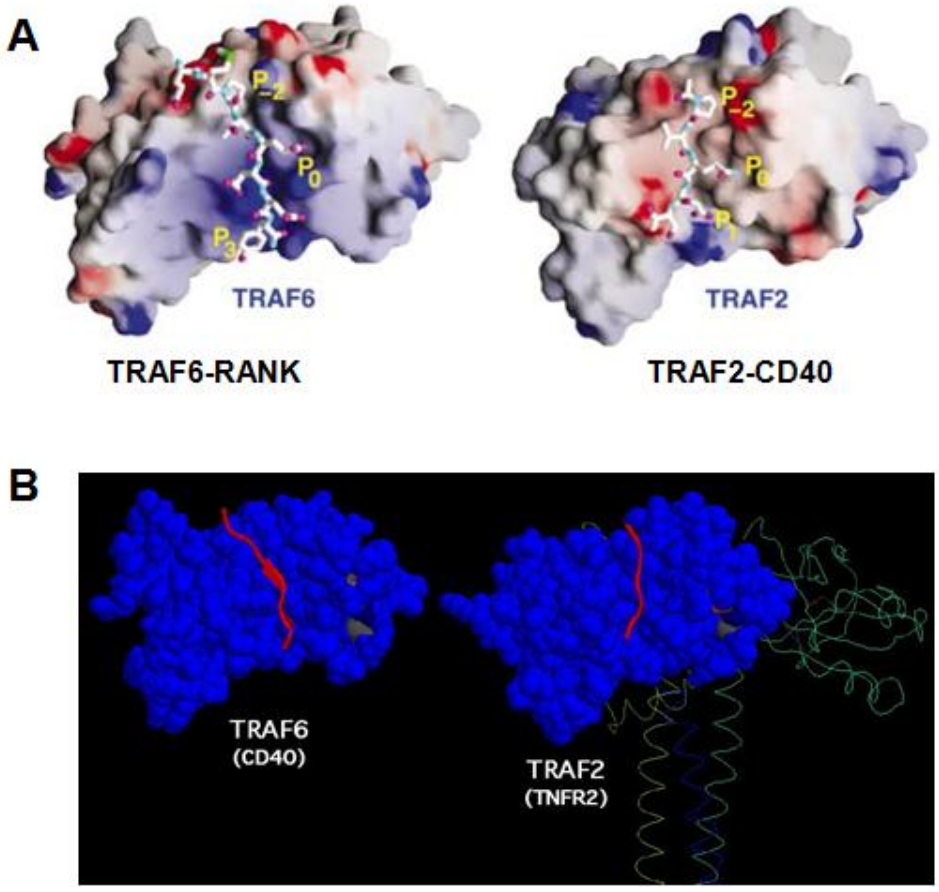


Figure 2. Peptide binding grooves located within the MATH domains of TRAF6 and TRAF2.

(A). Surface representation of TRAF6 and TRAF2 shown with the bound RANK peptide and CD40 peptide (Adopted from Ye et al., 2002, *Nature*. 418, 443-7).

(B). The X-ray crystal structure coordinates for the TRAF6 MATH monomer bound to a CD40 TRAF-binding peptide (Ye et al., 2002) is orientationally aligned with the coordinates for the TRAF2 MATH domain plus coiled-coil region trimer bound to the TNFR2-binding peptide (Park et al., 1999). The alignment was accomplished by superimposition of the MATH domain peptide backbones (Wang et al., 2006, *J. Cell Sci.,* 119, 1579-91).

Ubiquitin modification of TRAF6

Acting as an E3 ubiquitin ligase, TRAF6 forms a complex with ubiquitin conjugating enzyme Ubc13 and Ubc-like protein Uev1A, which results in TRAF6 auto-ubiquitination or perhaps *trans*-ubiquitination and activation (Wang et al., 2001). Upon activation, TRAF6 activates many signal transducers and cell surface receptors such as sequestosome 1/P62, TAK1, IKB kinase (IKK) and interferon regulatory factor 5 (IRF5) through a non-degradative K63-linked polyubiquitination (Balkhi et al., 2008; Rong et al., 2007; Seibenhener et al., 2004; Wang et al., 2001). However, TRAF6 can also induce proteasomal degradation of proteins such as RIP1 and IRAK1 mediated by K48-linked polyubiquitination (Newton et al., 2008). Interestingly, TRAF6 binds to K48-linked polyubiquitinated proteins like IRAK1 and translocates together into the proteasome where IRAK1 undergoes proteasomal degradation while TRAF6 is recycled through deubiquitination, rather than by degradation. Cylindromatosis (CYLD) has been shown to mediate deubiquitination of K63-linked-TRAF6 as well as -TRAF2 and -Nemo (Jensen and Whitehead, 2003; Kovalenko et al., 2003; Trompouki et al., 2003; Wooten et al., 2008; Yoshida et al., 2005). A20 has also emerged as an ubiquitin editing enzyme which specifically replaces K-63 linked poly-ubiquitin with K48 linked poly-ubiquitin on its target proteins (Coornaert et al., 2009; Sun, 2008).

Wooff *etal* employed a yeast two hybrid system and demonstrated a physical interaction between the RING domain of TRAF6 and the sub-unit of E2 ubiquitin conjugating enzyme, Ubc13. They also disclosed the necessity of the integrity of the Zn^{2+} finger motif inside the RING domain. A mutation that disrupts the conformation of a Zn^{2+} finger motif (TRAF6C70S) abolishes the interaction of TRAF6 and Ubc13 (Wooff

et al., 2004). A similar mutant construct (TRAF6C70A) generated by another group has been confirmed to be disabled for the auto-ubiquitination of TRAF6 (Lamothe, 2007). Recent studies by means of nuclear magnetic resonance (NMR) spectroscopy have implicated that residues 98-100 within the RING domain of TRAF6 physically provide a docking site for the recruitment of Ubc13 (Markin et al., 2008; Mercier et al., 2007). The exploration of K63-linked ubiquitination site(s) of TRAF6 has revealed that residue K124 largely mediates the auto-ubiquitination of TRAF6, and a mutation of TRAF6(K124R) remarkably reduces the auto-ubiquitination of TRAF6 and its capability for NF-κB activation as well as osteoclast differentiation from either RAW264.7 cells or bone marrow derived monocytes (Lamothe et al., 2007). Our preliminary studies, however, with the same mutant construct do not seem to support their results (more detail will be addressed in results and discussion sections).

On the other hand, TRAF6 can be sumoylated by small ubiquitin-related modifier-1 (SUMO-1), a process similar to ubiquitination, and appears as a repressor of gene transcription like many other nucleocytoplasmic proteins (Gill, 2003, 2005). Pharm *etal* reported that besides cytoplasmic localization, TRAF6 is also found in the nuclei of both normal and malignant B lymphocytes after CD40 signaling. Further studies show that nuclear TRAF6 is modified by SUMO-1 at lysines 124, 142 and 453, which binds c-Myb and enters the nucleus through the nuclear pore complex involving RanGap-1, a Ran GTPase-activating enzyme. In nuclei, TRAF6 interacts with histone deacetylase (HDAC) 1, and localizes on putative c-Myb binding sites of c-Myb gene promoter, resulting in the repression of c-Myb-mediated c-Myb gene transcription (Pham et al., 2008).

TRAF6 regulates inflammatory response initiated from toll like receptor 4 (TLR4)

Inflammation is a complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. Innate immunity acts as the first barrier to defend against acute inflammation induced by foreign pathogens, through the activation of immune cells such as macrophages, neutrophils, and natural killer (NK) cells in the body (Gallo and Nizet, 2008). Invading foreign pathogens are recognized by receptors expressed in immune cells. Different pathogens have different pathogen associated molecular patterns (PAMPs) on their surfaces, which can transmit inflammatory signals into target cells that recognize them with corresponding pattern recognition receptors (PRRs) (Kanneganti et al., 2007; Kawai and Akira, 2009; Medzhitov, 2009). Toll like receptors (TLRs) are a family of the most documented PRRs, which consist of 11 subfamily members in humans (Kaisho and Akira, 2006; Kawai and Akira, 2007; Ozato et al., 2002; Roach et al., 2005). Recognition patterns between the pathogen ligands and TLRs are outlined in Figure 3.

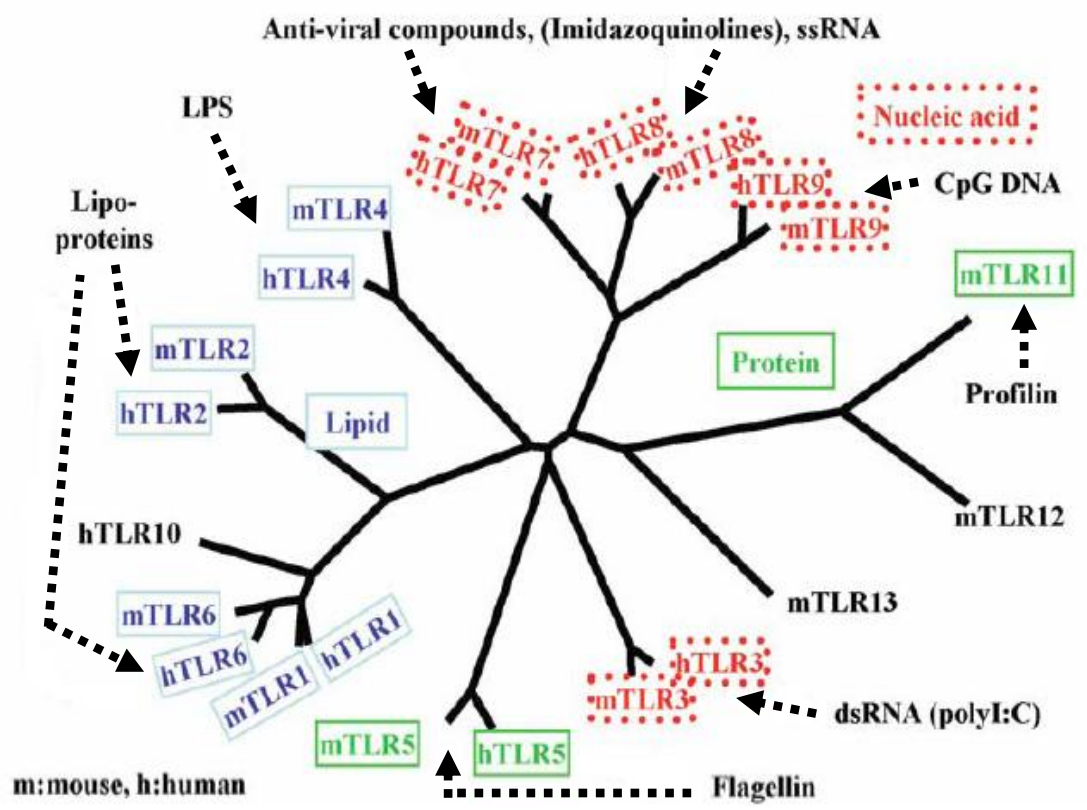


Figure 3. Phylogenetic tree of human and murine TLRs.

Human and murine TLRs are connected with solid lines on the basis of the phylogenetic analysis of their amino acid structures. Branch length is proportional to evolutionary distances. Dashed arrows indicate representative ligands. Human TLR8 can function as a sensor, whereas there are no reports on the function of murine TLR8. h, Human; m, murine. (Adapted from: Kaisho and Akira (2006)) *J Allergy Clin Immunol*, 117, 979-87.)

TLR4 is a plasma membrane receptor expressed in a variety of cell types such as monocytes, macrophages, endothelia, fibroblasts, and epithelia (Medzhitov, 2001). The Gram negative bacterial cell wall component, LPS, specifically binds to TLR4 together with LPS binding protein (LBP), CD14 and MD2, which induces dimerization of TLR4. The stimulatory signal is transmitted inside cells through the cytoplasmic TIR domain of TLR4, which can recruit adaptor molecules of either TIRAP-MyD88 (referred to as the MyD88 dependent pathway) (Horng et al., 2001; Medzhitov et al., 1998; O'Neill et al., 2003) or TRAM-TRIF (referred to as the MyD88 independent pathway) (Fitzgerald et al., 2003). In the MyD88 dependent pathway, regulatory molecules that are involved in the signaling network include the IL-1 receptor associated kinase (IRAK) family and TRAF6. It ultimately activates transcription factors, nuclear factor-κB (NF-κB), resulting in transcription activation of a profile of inflammatory cytokines (Fig. 4) such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα). Vigorous responses induced by severe bacterial infection may cause apoptosis and necrosis of the responding cells.

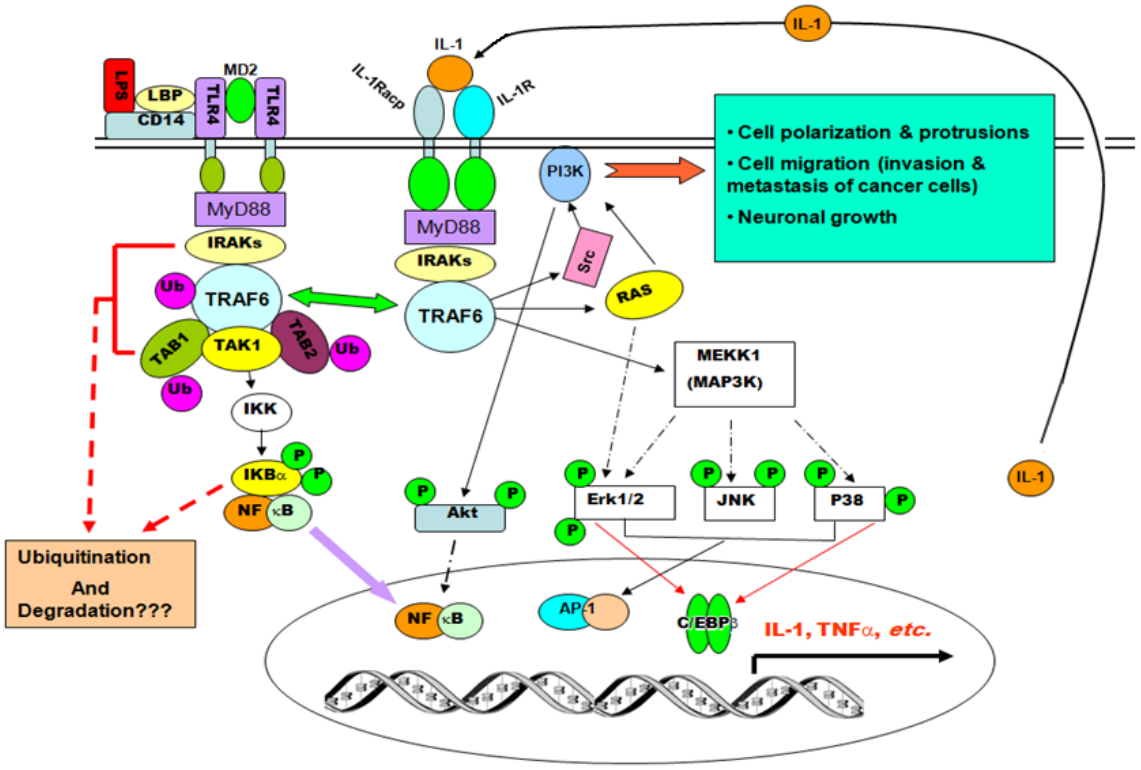


Figure 4. Examples of TLR4/IL1R signal transduction pathways.

The scheme shows the Gram negative bacterial cell wall component, LPS initiates an inflammatory stimulation to TLR4 in the plasma membrane and signal transduction via the MyD88 dependent pathway. IL-1 is produced upon primary inflammatory response and ignites second wave of inflammatory response to adjacent tissues.

TRAF6 is pivotal in mediating interleukin 1 (IL-1) inflammatory signaling

IL-1β is one of the cytokines rapidly expressed in response to LPS stimulation (Fenton et al., 1987; Fenton et al., 1988; O'Neill, 2002). As this cytoplasmic protein is channeled out of living cells (Mariathasan et al., 2004; Perregaux and Gabel, 1998) or leaked out from necrotic cells (Kornbluth and Edgington, 1986), IL-1β ignites further rounds of inflammatory stimulation to adjacent tissues (Boch et al., 2001; Dinarello, 2009).

IL-1 signaling is transmitted into the responding cell through IL-1 type I receptor (IL1RI) (Bird et al., 1988; Solari, 1990). TRAF6 is a unique intra-cellular signal transducer and plays pivotal roles for IL1RI inflammatory signaling. Upon IL-1 engagement, IL-1RI forms a heterodimer with IL1R accessory protein (IL1RAcp) and recruits MyD88 onto the cytoplasmic TIR domains of the receptor complex (Dunne et al., 2003). As IRAK is recruited to the receptor complex in the cell membrane through the death domain interaction between MyD88 and IRAK, TRAF6 is thought to transiently move to the membrane via its physical interaction with IRAK, which simultaneously induces engagement of Transforming growth factor beta (TGFβ) activated kinase 1 (TAK1) binding protein 1 and 2 (TAB1, TAB2) as well as TAK1 to TRAF6. The IRAK-TRAF6-TAB1-TAB2-TAK1 complex then immediately translocates into the cytosol (Li and Qin, 2005) where TRAF6 induces ubiquitination of multiple downstream molecules such as TAB2 (Kishida et al., 2005) and IκB kinase γ (IKKγ) (Deng et al., 2000; Sebban-Benin et al., 2007). It further results in the activation of a ubiquitous transcription factor, NF-κB (p50/p65), by inducing phosphorylation and proteasomal degradation of IκB subunits (Fig. 4) (Ghosh et al., 1998; Li and Verma, 2002; Yaron et al., 1998). TAK1 can

also activate the mitogen-activated protein kinase (MAPK) signaling cascade through phosphorylation and activation of a MAPK upstream molecule, MEKK1 (Baud, 1999), which leads to the activation of many other transcription factors like activator protein-1 (AP-1) and CCAAT/enhancer-binding protein β (C/EBPβ) (Adhikari, 2007).

TRAF6 and T-cell receptor (TCR) signaling

T-cells recognize foreign antigens on antigen-presenting cells (APCs) in the context of a processed peptide bound to class I major histocompatibility complex (MHC I). The recognition of an antigen takes place at the T-cell:APC contact site where an "immune synapse" is formed by recruitment of multiple signaling modulators to the cytoplasmic domain (CD3) of TCR. It has been shown that NF-κB plays critical roles in mediation of T cell activation. During TCR signaling, tyrosine kinase Zap70 moves to and phosphorylates CD3 (Straus and Weiss, 1993), which in turn recruits engagement of SLP76, LAT and phopholipase C γ1 (PLC γ1) (Braiman et al., 2006; Yablonski et al., 2001). PLC γ1 cleaves phosphatidylinositol 4, 5-bisphosphate (PI(4,5)P2) into inositol triphosphate (IP3) and diacyl-glycerol (DAG). Protein kinase C Theta (PKCΘ) then binds to DAG and induces clustering of CARMA1, CARD11, B cell lymphoma 10 (BCL10), MALT1, caspase 8, and TRAF6 in membrane rafts (Bidere et al., 2006; McCully and Pomerantz, 2008; Misra et al., 2007), which mediate IKK activation by facilitating the K63-linked non-degradative polyubiquitination of NEMO in TCR signaling (Zhou et al., 2004). TRAF6 involvement of NF-κB activation as an E3 ubiquitin ligase in the TCR signaling was first demonstrated by Chen group (Sun et al., 2004). They set up in vitro studies with Jurkat T cell extract and revealed that TRAF6 physically interacts with

MALT1, which induces ubiquitination of TRAF6 and activation of its downstream pathways. Similar to ubiquitination and activation of IKKγ in the TLR4/IL-1R pathways, TRAF6 utilizes the same components such as TAB1, TAB2 and TAK1 for the activation of IKKγ in the CTR signaling, resulting in degradation of IκBα and activation of NF-κB (Fig. 5).

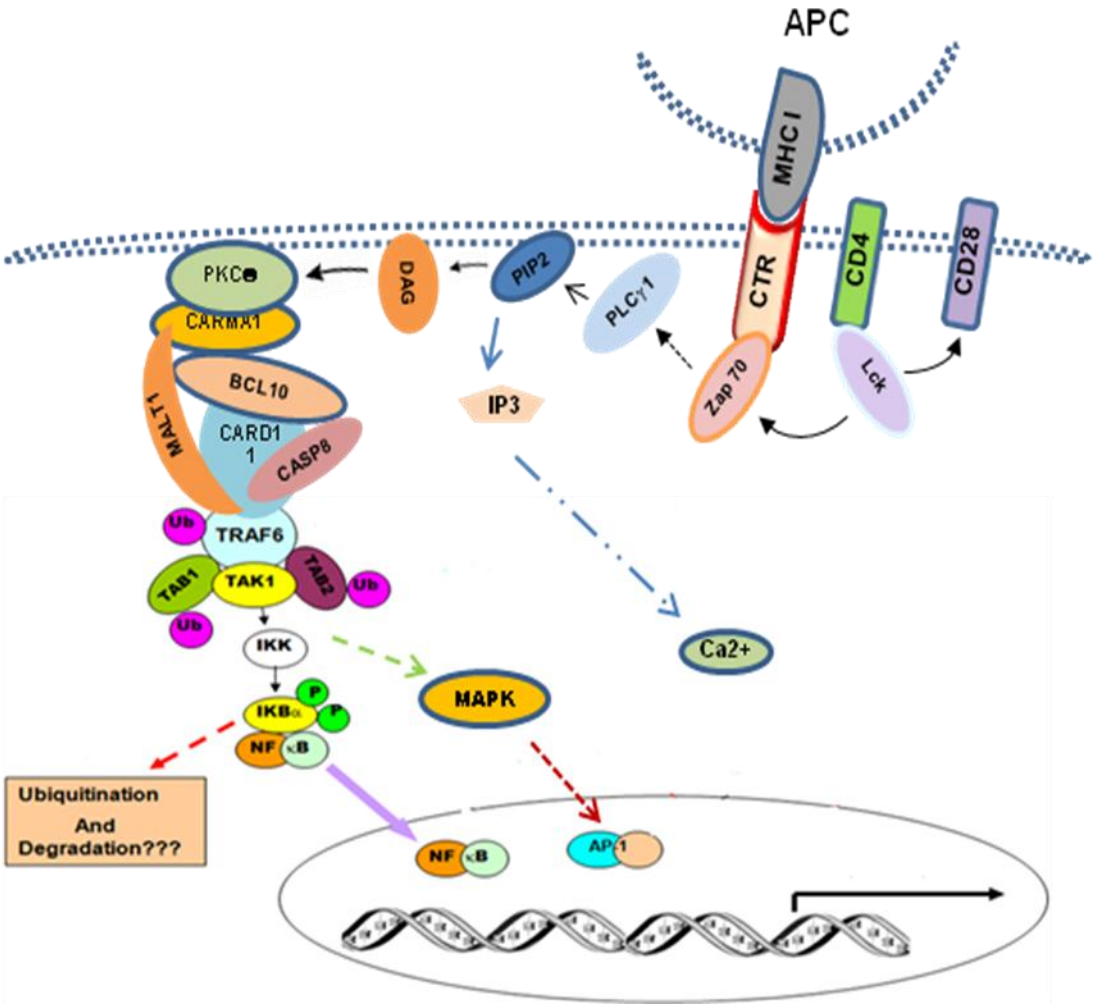


Figure 5. Activation of NF-κB through T-cell receptor signaling.

Scheme shows several signaling events initiated from engagement of TCR in the plasma membrane. NF-κB plays central roles in many different types of T cell proliferation and activation.

A TRAF6 knockout mouse model first developed by the Mak group showed that the $TRAF6^{-/-}$ cells had minimal response to IL-1, LPS, and CD40 induction of the NF-κB pathway (Lomaga et al., 1999). To more precisely investigate the roles of TRAF6 in T cell regulation, the Choi group generated a mouse model with TRAF6 deletion specific to T cells (TRAT6-ΔT) (King et al., 2006). TRAT6-ΔT mice were born at normal mendelian ratios and appeared healthy and viable at birth. By 10–12 weeks, however, these mice showed signs of systemic inflammatory disease, including mononuclear cell infiltrates in the intestine, liver, lung and kidney as well as the presence of autoreactive T cells. TRAT6-ΔT T cells also exhibit CD28-independent proliferation through hyperactivation of the PI3K dependent pathway and are resistant to suppression by $CD4^{+}CD25^{+}$ regulatory T cells (T_{reg}). They further confirmed that TRAT6-ΔT T cells were resistant to anergizing signals both in vitro and in vivo. Resistance to anergy was correlated with decreased expression of another mediator of T cell anergy, an E3 ubiquitin ligase, Cbl-b. These implicate that TRAF6 plays roles as a negative modulator in the maintenance of peripheral tolerance (King et al., 2008; King et al., 2006; Lin and Mak, 2007). More recently, TRAF6 has been shown to regulate $CD8^{+}$ memory T cell development after infection by modulating fatty acid metabolism. Investigators reported that mice with a T-cell-specific deletion of TRAF6 mount robust antigen-specific effector T cell responses but have a profound defect in their ability to generate memory T cells characterized by the disappearance of antigen-specific cells in the weeks after primary immunization. Microarray analyses revealed that TRAF6-deficient CD8 T cells exhibit altered expression of genes that regulate fatty acid metabolism (Pearce et al., 2009).

TRAF6 is involved in regulation of osteoclastogenesis

Bone remodeling is a normal process that involves the resorption of bone by osteoclasts and the synthesis of bone matrix by osteoblasts in order to maintain tissue integrity. Many bone diseases caused by disregulation of bone mass are due to an imbalance in the ratio of osteoblasts to osteoclasts. Osteoclasts originate from bone marrow hematopoietic cells (Suda et al., 1992). A cell surface protein called receptor activator for NF-κB (RANK) that transmits stimulatory signal from the RANK ligand (RANKL) is indispensable for differentiation and activation of osteoclasts (Burgess et al., 1999; Lacey et al., 1998). TRAF6 regulating RANK signaling was first demonstrated by in vitro protein-protein interaction studies (Darnay et al., 1998; Wong et al., 1998). TRAF6 and RANK both belong to the TNFR superfamily. Alignment of amino acid sequences revealed that RANK contains three putative TRAF binding motifs in its cytoplasmic domain but only the motif containing amino acid residues 340-470 (RQXPXEXE) has been confirmed to be unique and necessary for the binding of TRAF6 as well as activation of NF-κB *via* NF-κB inducing kinase (NIK) (Darnay et al., 1999). Mizukami *etal* further disclosed that TRAF6 could play roles similar to its function in the TLR4/IL-1R signaling pathways, in which it acts as an E3 ubiquitin ligase to recruit TAB2 to the plasma membrane upon engagement of RANK, where TAB2 is ubiquitinated and activated by TRAF6 resulting in the activation of NF-κB through IKK and activation of MAPK pathways through the phosphorylation of MEKK1 by the TRAF6-TAB2-TAK1 complex (Mizukami et al., 2002). On the other hand, TRAF6 can also recruit and activate c-Src to the receptor complex during RANK signaling, which in turn induces activation of phosphoinositol 3-kinase (PI3K) and protein kinase B

(PKB/Akt). Such event provides another survival signal in osteoclasts (Wong et al., 1999). Ha *etal* reported that RANK-TRAF6-Src complex exists in membrane raft microdomains upon RANKL stimulation in osteoclasts. Interruption of membrane rafts with specific chemical inhibitors abolishes formation of the RANK-TRAF6-Src complex and activation of Akt, leading to survival reduction of osteoclasts. They also showed that the integrity of membrane rafts is necessary for actin ring formation and bone absorption (Ha et al., 2003b). The direct evidence for TRAF6 regulating osteoclastogenesis was obtained in a TRAF6 knockout mouse model. Lomaga *etal* reported that mice deficient in TRAF6 appeared normal at birth. However, mice that survived more than two weeks displayed osteopetrosis with defects in bone remodeling and tooth eruption due to impaired osteoclast function. Although $TRAF6^{-/-}$ mice were capable of producing tartrate resistant acidic phosphatase (TRAP) positive osteoclasts, they displayed abnormalities in adhesion zones and ruffled border (Lomaga et al., 1999). Therefore, TRAF6 pays a pivotal role in the differentiation and activation of osteoclasts (Asagiri and Takayanagi, 2007; Rong et al., 2007; Takayanagi, 2005; Tanaka et al., 2005).

TRAF6 induces PI3K-dependent cellular polarization in IL-1β signaling

Similar to the observations of TRAF6 activation of PI3K in RANK signaling, my previous studies in Prof. Auron's laboratory at the University of Pittsburgh also demonstrated that TRAF6 could induce PI3 kinase-dependent cytoskeletal changes mediated by c-Src in IL-1β signaling (Wang et al., 2006). Employing the GFP conjugated pleckstrin homology domain of Akt (PH-Akt-GFP) as a reporter of phosphatidylinositol 3, 4, 5-trisphosphate (PI(3,4,5)P3), we observed during live cell imaging that IL-1β

induced cytoskeletal changes, and the green fluorescent signal (PH-Akt-GFP) localized to the cell membrane and filopodia like cellular protrusions (Fig. 6A).

In order to test if this was regulated by PI3K, stimulated cells were further treated with a specific inhibitor of PI3K, Ly294002. We confirmed that fluorescence was withdrawn from the protrusions into the cytoplasm (Fig. 6B).

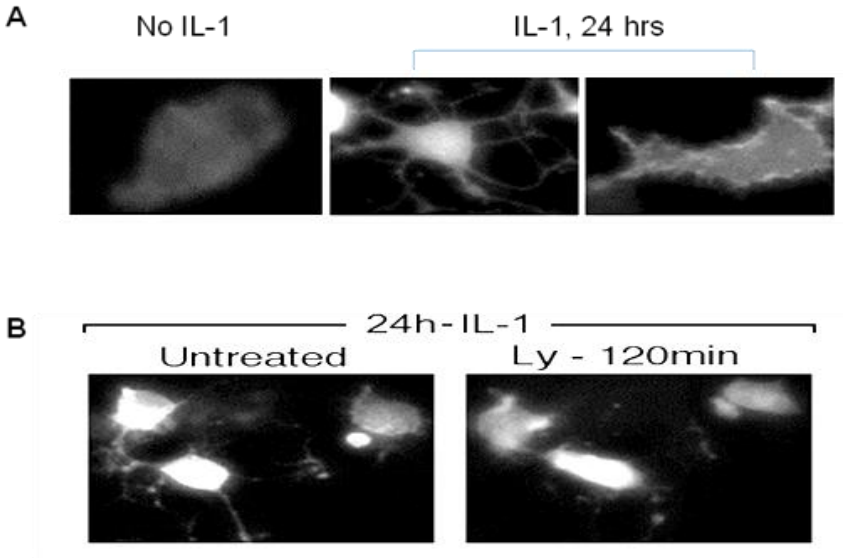


Figure 6. IL-1 induces PI3K-dependent cytoskeletal changes.

A. HEK293R cells were transfected with a PH-Akt-GFP expression vector reporter and then stimulated with IL-1β to a final concentration of 10 ng/mL for 24 hrs. B. IL-1 stimulated HEK293R cells were treated with Ly294002 to a final concentration of 25 μM for 120 min.

A similar phenomenon was observed when HEK293R cells were co-transfected with expression plasmids coding either for PH-Akt-GFP and P110 (a catalytic subunit of PI3K) or PH-Akt-GFP and TRAF6, which is the only TRAF family member involved in IL-1R signaling, and can act as a constitutively active surrogate for IL-1β (Fig. 7).

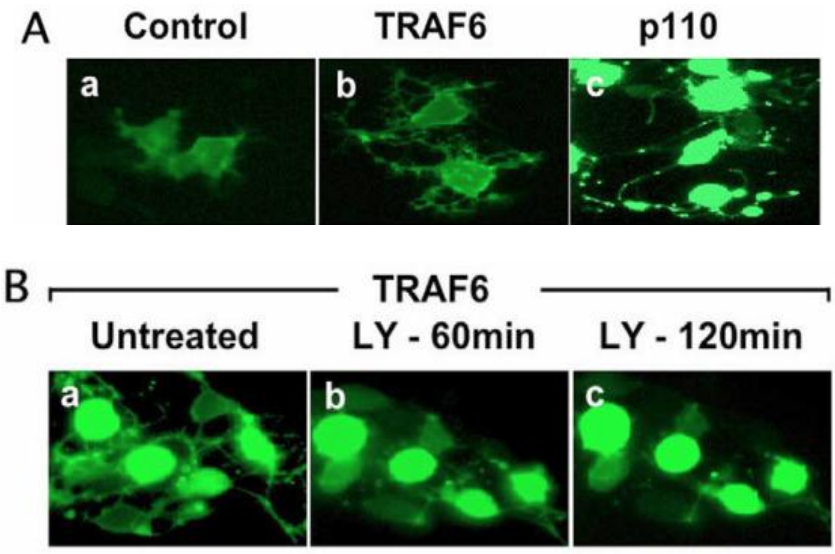


Figure 7. TRAF6 induces translocation of PH-Akt-GFP to cell membranes and protrusions in a PI3K dependent pathway.

A. A PH-Akt-GFP expression vector reporter was co- transfected with expression vectors encoding a second activator protein, p110 subunit of PI3K or TRAF6 into the HEK293 cells.

B. PH-Akt-GFP expression vector reporter co-transfected with a TRAF6 expression vector into HEK293 cells for 24 hrs (a), then treated with Ly294002 (LY) for indicated times (b & c).

It has been well documented that cellular protrusions such as filopodia and lamellipodia are formed by the polymerization of cytoplasmic G actin, and PI 3-kinase is a key positive regulator for these processes (Janmey and Lindberg, 2004; Revenu et al., 2004). We therefore stained the cells co-transfected with TRAF6 and PH-Akt-GFP with

phalloidin-TRITC in order to visualize filamentous actin (F-actin). As demonstrated in Figure 8A, the PH-Akt-GFP signal (green) coincided with the F-actin (red) staining. Furthermore, treatment at 24 hours post transfection of PH-Akt-GFP and TRAF6 constructs with a specific F-actin depolymerization reagent, cytochalasin D, resulted in a significant decrease in PH-Akt-GFP containing protrusions (Fig. 8B). This further demonstrates that PH-Akt-GFP localizes into F-actin containing structures.

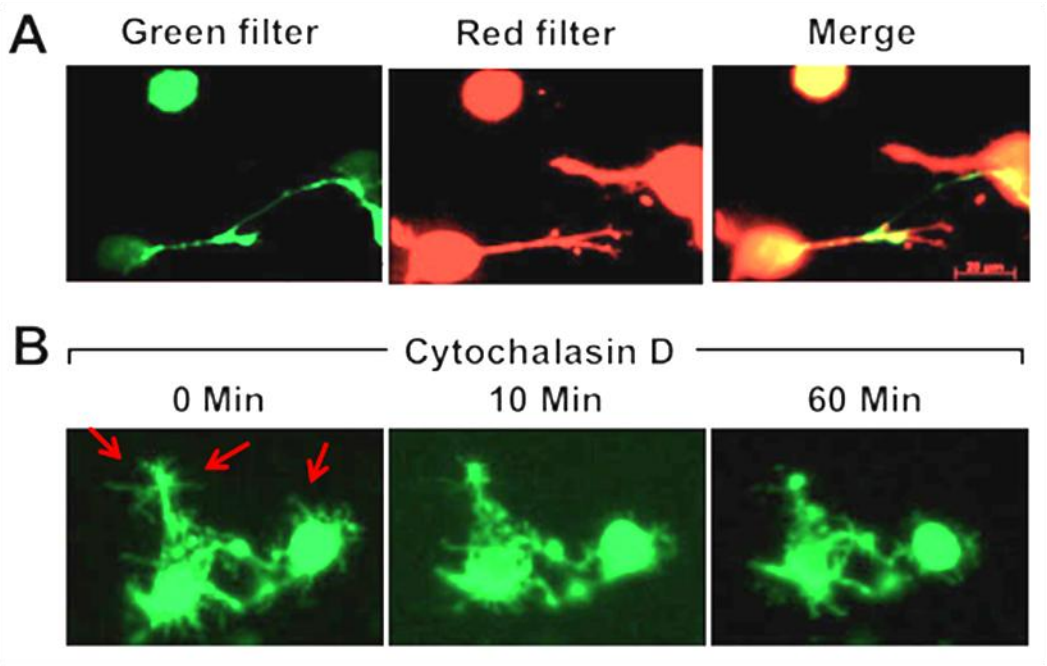


Figure 8. TRAF6 induces the localization of PH-Akt-GFP to actin branch points.

A. HEK293 cells were co-transfected with a PH-Akt-GFP expression vector reporter and a TRAF6 expression vector for 24 hours, followed by fixation and phalloidin-TRITC staining. GFP (green), FITC (red) and merged images were used to determine signal co-localization (yellow). B. HEK293 cells were cotransfected with PH-Akt-GFP and TRAF6 expression vectors for 24 hours, then treated for the indicated times with 5 μM cytochalasin D.

Taking advantage of the technique of bimolecular fluorescence complementation (BiFC) or split-GFP, which only generates a fluorescent signal upon protein-protein interaction (Hu et al., 2002), we were also able to detect the direct interaction of TRAF6 and c-Src via the poly-proline motif in the TRAF-C/MATH domain of TRAF6 and the SH3 domain of c-Src in live cells (Fig. 9).

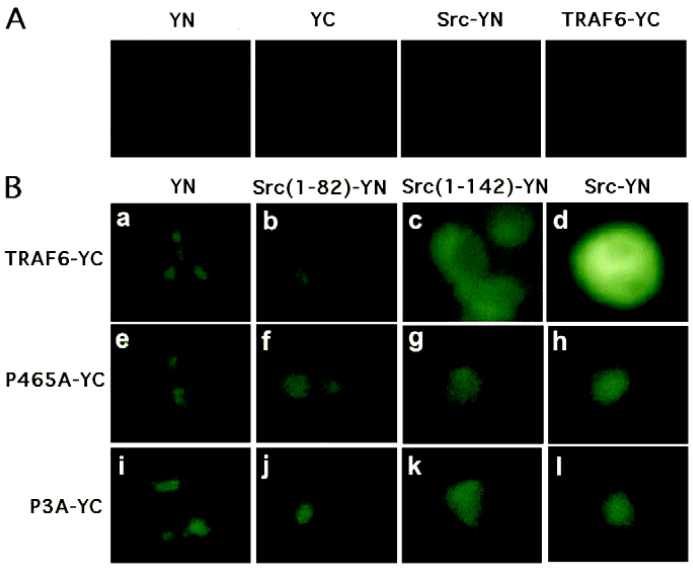


Figure 9. Examination of subcellular localization and protein-protein interaction in living cells for de novo-expressed Src and TRAF6.

Bimolecular fluorescence complementation (BiFC) involving split YFP tags was used to detect intracellular protein-protein interaction betweenTRAF6 and Src that could be compared with the expression pattern for these proteins tagged with full-length YFP in HEK293T cells transiently transfected for 24 hours. A. Transfection of 200 ng of individual, uncomplemented BiFC vectors; –YN, N-terminal YFP fragment (aa 1-154), –YC, C-terminal YFP fragment (aa 155-238). B. Transfection of 200 ng of each BiFC vector complementary pair (–YN + –YC). Fluorescence signals in c and d implicated protein-protein interaction, whereas other combinations only showed background noise.

HYPOTHESIS AND SPECIFIC AIMS

The unique structural characteristics of TRAF6 are critical for the many important functions of this protein in mediating signaling events. Accumulated evidence has suggested that auto-ubiquitination of TRAF6 plays central roles for its activation, in which the K63-linked poly-ubiquitin chains attached to TRAF6 interact with the ubiquitin binding domain(s) of its downstream signaling regulators such as p62, TAB1, and TAB2 (Kanayama et al., 2004; Wooten et al., 2001). However, the molecular regulatory mechanism of TRAF6 auto-ubiquitination has not yet been elucidated. Meanwhile, it will be intriguing to test whether ubiquitination of TRAF6 affects its cellular distribution and consequently induces certain biological functions. Intriguingly, unlike some signaling molecules which require upstream activator(s) for function, ectopic over-expression of TRAF6 acts as dominant positive for downstream pathways *via* an unknown mechanism (Dadgostar and Cheng, 2000).

We hypothesize that different functional domains within TRAF6 may cooperate with each other to determine its sub-cellular localization and dynamically transmit different signaling events as well. We also speculate that TRAF6 activation by upstream activators or dominant positive exhibited by TRAF6 over-expression may involve conformational change in some of these domains, resulting in its auto-ubiquitination. Many TRAF members have been crystallized and display a trimeric conformation. We therefore hypothesize that TRAF6 auto-ubiquitination upon activation may be regulated inter-molecularly.

In this study, we set out to systematically explore the involvement of different functional domains of TRAF6 in regulating its "auto" ubiquitination and activity as well as to further sort out any correlation between ubiquitination of TRAF6, its cellular localization, and activation of the NF-κB pathway. To test these, we engineer different types of expression plasmids coding for TRAF6 mutants and analyze the roles of different functional domains. Each TRAF6 mutant coding sequence is inserted into non-tagged pcDNA3.1(+) and YFP-FLAG dual-tagged pFLAG CMV 5a-YFP expression vectors. These YFP-tagged mutant plasmids are ectopically expressed in 293 cell lines for examination of cellular localization by confocal microscopic techniques. The FLAG and YFP tags in each mutant protein (mutein) can also be recognized by either anti-FLAG or anti-GFP antibodies for immuno-precipitation, and western blot analysis, for the determination of expression level and ubiquitination. We also use corresponding un-tagged muteins, although some of them may not be recognized by the anti-TRAF6 antibody for co-immunoprecipitation and western blot analysis, to ensure the consistency of NF-κB activity with their YFP-tagged counterparts.

- ***Aim 1.*** Engineer TRAF6 mutant constructs containing different functional domains or point mutation in pcDNA3.1(+) and pFLAG CMV 5a-YFP expression vectors.
- ***Aim 2***. Evaluate contribution of different functional domains of TRAF6 to its cellular distribution and ubiquitination.
- ***Aim 3***. Establish assays detecting activation of NF-κB to evaluate correlation among cellular localization, ubiquitination, and activity of TRAF6.

Aim 4. Explore possible molecular regulatory mechanisms of TRAF6 poly-ubiquitination and the effect on NF-κB activation.

MATERIALS AND METHODS

Reagents and antibodies

ANTI-FLAG® M2 Affinity Gel #A2220), and ANTI-FLAG® M2 (Clone M2, #F1804) were purchased from Sigma Aldrich. Protein A/G plus-agarose (#SC-2003), ubiquitin antibody (P4D1) (#SC-8017), and TRAF6 (H-274) (#SC-7221) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). FuGENE HD transfection reagent (#04709705001), green fluorescent protein (GFP) antibody (#11814460001), expand high fidelity PCR system (#11732641001), and rapid DNA ligation kit (#11635379001) were purchased from Roche Applied Science. QuickChange XL site-directed mutagenesis kit (#200517-5) was purchased from Stratagene. All DNA restriction enzymes used for cloning were purchased from New England Biolab. Luciferase assay system (#E1531), horseradish peroxidase conjugated anti-mouse antibody (#W402B) and horse radish peroxidase conjugated anti-rabbit antibody (#W401B) were purchased from Promega. QIAprep Spin Miniprep Kit (#27104), QIAquick PCR Purification Kit (#28104), and EndoFree Plasmid Maxi Kit (#12362) were purchased from Qiagen. SuperSignal West Pico Chemiluminescent Substrate (#34077) was purchased from Thermo Fisher Scientific. 4-12% (BMA59524) and 8-16% Gradient polyacryamide precast gels (BMA59521) were purchased from Lonza. MG132 (#474790) was purchased from Calbiochem.

Cell Lines

HEK293 (CRL-1573) and HEK293T (CRL-11268) cells were from American Type Cell Collection (Manassas, VA) and were cultured in EMEM medium supplemented with 10% heat inactivated fetal bovine serum and 2 mg/ml L-Glutamine.

Reporters and expression plasmids

The NF-κB luciferase reporter, NF-κB/pGL2, contains a c-*fos* core promoter and four tandem repeats of NF-κB p65/p50 heterodimer binding sequences (Yoshida et al., 2004). The NF-κB/HcRed1-Nuc reporter (HcRed1-κB) was constructed by replacing the luciferase coding sequence in the NF-κB/pGL2 with the HcRed1 cDNA ligated to a SV40 nuclear localization signal (SV40NLS) by PCR and cloning procedures using the pHcRed1/Nuc vector (Clontech, Mountain View, CA) as a template. Sequences of the primer pair were: forward primer, 5'-acta aag ctt ATG GTG AGC GGC CTG CTG AAG GAG-3'; reverse primer, 5'-atca cca acc TAT GGA ACC ACA ACT AGA ATG CAT TG-3'. The PCR product was inserted into the Hind III and MflMI restriction sites in the NF-κB/pGL2 vector (Fig. 10).

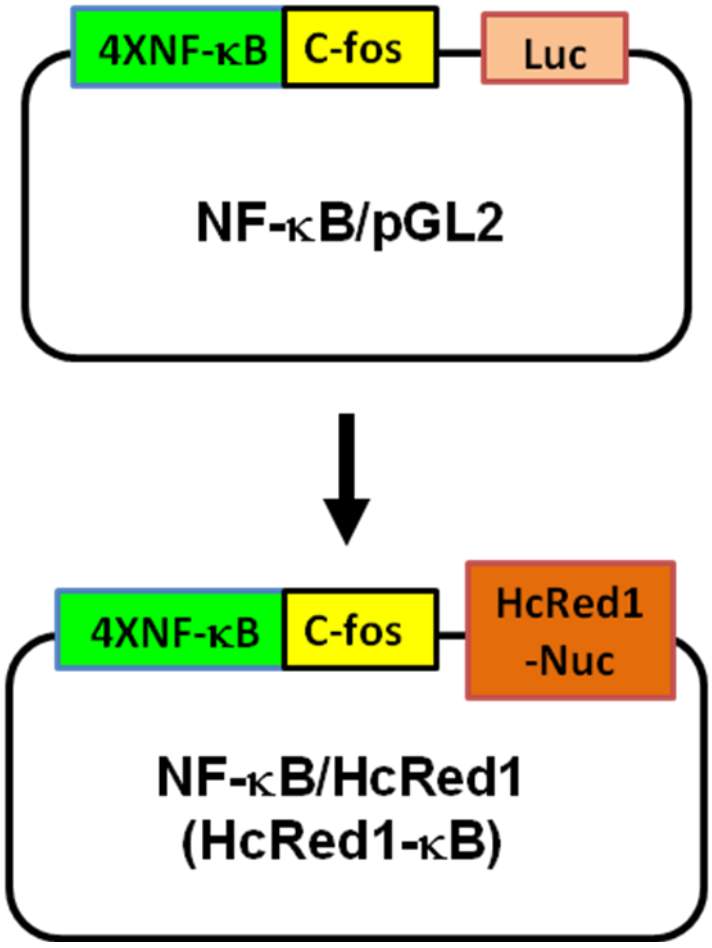


Figure 10. Maps of NF-kB/pGL2 and NF-kB/HcRed1 reporter vectors.

The NF-κB/pGL2 reporter was constructed with the promoter-less pGL2 basic plasmid by inserting 4 tandems repeats of a classic NF-κB consensus sequence as described previously (Yoshida et al., 2004). The NF-κB/HcRed1 reporter was engineered by replacing the luciferase coding sequence with an insert coding for a nuclear localizing HcRed1protein.

The human wild type TRAF6 and TRAF6(1-273) coding sequences, designated as RZ, were inserted into the Eco RI and Eco RV sites of the pcDNA3.1(+) vector as described previously (Yoshida, 2004). The sequences coding for: TRAF6(1-358) is designated 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 were generated by PCR using the wild type TRAF6pcNDA3.1 as template and subcloned into pcDNA3.1(+) vector between the Eco RI and Eco RV restriction sites. The primer pairs used for PCR reactions are shown in Table 2.

Table 2. Primer sequences of TRAF6 mutant constructs in pcDNA3.1(+)

Construct	Primer	Sequence
RZC	Forward	5'-CAGAATTCGTCGACAATGAGTCTGCTAAACTGT-3'
	Reverse	5'-CCAGATATCCTAGCCAATCTTCCAAATATAAAT-3'
MATH	Forward	5'-CCAGAATTCATGGCACAGCAGTGCAATGGAATTTAT-3'
	Reverse	5'-CCAGATATCCTATACCCCTGCATCAGTACTTCG-3'
ΔR	Forward	5'-CCACGAATTCATGGAGTTTGCTCTTATGGATTGTCC-3'
	Reverse	5'- CCAGATATCCTATACCCCTGCATCAGTACTTCG-3'
ΔRZ	Forward	5'-CCACGAATTCATGGCCCAGGCTGTTCATAGTTTGAGC-3'
	Reverse	5'- CCAGATATCCTATACCCCTGCATCAGTACTTCG-3'
ΔRZ1	Forward	5'-CCACGAATTCATGCTGGAGATTCTTTCTCTGATGGTG-3'
	Reverse	5'- CCAGATATCCTATACCCCTGCATCAGTACTTCG-3'
ΔZ1	Forward	5'-CAGAATTCGTCGACAATGAGTCTGCTAAACTGT-3'
	Linker	5'-GCATTGGGGACAATCCATTTTTGCAAAATTGTCTGG-3'
	Reverse	5'- CCAGATATCCTATACCCCTGCATCAGTACTTCG -3'
Δcc	Forward	5'- CAGAATTCGTCGACAATGAGTCTGCTAAACTGT-3'
	Linker	5'-TCACACATGAGAATGTTGGCACAGCAGTGCAATGGA-3'
	Reverse	5'- CCAGATATCCTATACCCCTGCATCAGTACTTCG-3'

TRAF6(K124R)pcDNA3.1 designated as K124R and TRAF6(I98L, D100G)pcDNA3.1 designated as mR were engineered by a QuickChange II XL site-directed mutagenesis kit (Stratagene) with the wild type TRAF6pcDNA3.1 as a template. The primer sequences for these constructs are shown in Table 3.

Table 3. Site-directed mutagenesis primers of the TRAF6 mutant constructs

Construct	Primer	Sequence
K124R	Forward	5'-CCAGACAATTTTGCAAGACGTGAGATTCTTTCTCTGATGG–3'
	Reverse	5'-CCATCAGAGAAAGAATCTCACGTCTTGCAAAATTGTCTGG–3'
mR	Forward	5'-GCCTGCATCATAAAATCACTAAGGGGTGCAGGTCACAAATGTCC-3'
	Reverse	5'-GGACATTTGTGACCTGCACCCCTTAGTGATTTTATGATGCAGGC-3'

The pFLAG CMV 5a-YFP control vector was generated by inserting an EYFP coding sequence into the Eco RV and Bam HI sites of the pFLAG CMV 5a plasmid (Sigma Aldrich) (Fig. 11). The EYFP DNA fragment was PCR amplified with the EYFP C1 plasmid (Clontech) as a template and the primer pair used for the PCR were 5'-CCTGATATCCA TGGTGAGCA AGGGCGAG-3' (forward) and 5'-CGGGGATCCGGCCATGATA TAG ACGTTGTG-3' (reverse).

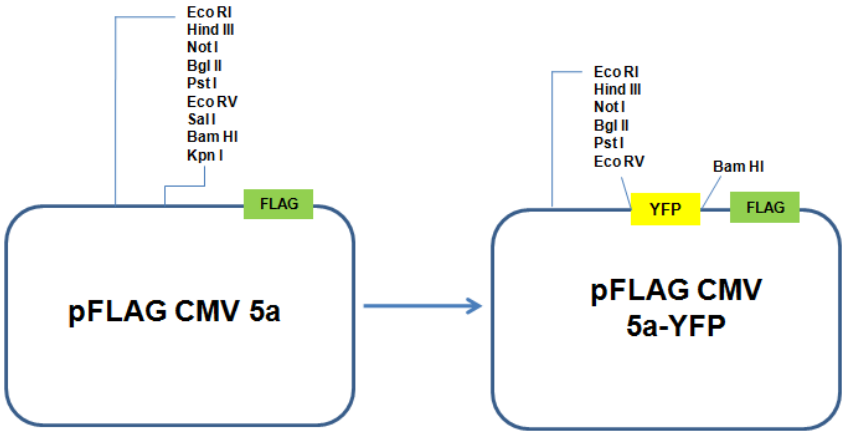


Figure 11. Construction of pFLAG CMV 5a-YFP vector.

The pFLAG CMV 5a-YFP vector was constructed by inserting an EYFP coding sequence into the Eco RV and Bam HI sites in the pFLAG-CMV 5a plasmid. The FLAG tag coding sequence is maintained and will be co-expressed along with the EYFP in mammalian systems. Therefore, a recombinant protein expressed from this parental construct will be YFP-FLAG dual tagged to its C-terminus.

The YFP conjugated TRAF6 wild type and mutant constructs were generated with PCR and subcloning strategies and inserted into the Eco RI and Eco RV restriction sites in the pFLAG CMV 5a-YFP vector. The primer sequences for these constructs are shown in Table 4.

Table 4. Primer sequences for the TRAF6-YFP and mutant constructs

Construct	Primer	Sequence
TRAF6	Forward	5'-CAGAATTCGTCGACAATGAGTCTGCTAAACTGT-3'
	Reverse	5'-CCAGATATCGGTACCCCTGCATCAGTACTTCG-3'
RZ	Forward	5'-CAGAATTCGTCGACAATGAGTCTGCTAAACTGT-3'
	Reverse	5'-CCAGATATCGGCAACATTCTCATGTGTGACTG-3'
RZcc	Forward	5'-CAGAATTCGTCGACAATGAGTCTGCTAAACTGT-3'
	Reverse	5'-CCAGATATCGGGCCAATCTTCCAAATATAAAT-3'
Δcc	Forward	5'- CAGAATTCGTCGACAATGAGTCTGCTAAACTGT-3'
	Linker	5'-TCACACATGAGAATGTTGGCACAGCAGTGCAATGGA-3'
	Reverse	5'-CCAGATATCGGTACCCCTGCATCAGTACTTCG-3'
MATH	Forward	5'-CCAGAATTCATGGCACAGCAGTGCAATGGAATTTAT-3'
	Reverse	5'-CCAGATATCGGTACCCCTGCATCAGTACTTCG-3'
ΔR	Forward	5'-CCACGAATTCATGGAGTTTGCTCTTATGGATTGTCC-3'
	Reverse	5'-CCAGATATCGGTACCCCTGCATCAGTACTTCG-3'
ΔRZ	Forward	5'-CCACGAATTCATGGCCCAGGCTGTTCATAGTTTGAGC-3'
	Reverse	5'-CCAGATATCGGGCCAATCTTCCAAATATAAAT-3'
ΔRZ1	Forward	5'-CCACGAATTCATGCTGGAGATTCTTTCTCTGATGGTG-3'
	Reverse	5'-CCAGATATCGGTACCCCTGCATCAGTACTTCG-3'
ΔZ1	Forward	5'-CAGAATTCGTCGACAATGAGTCTGCTAAACTGT-3'
	Linker	5'-GCATTGGGGACAATCCATTTTTGCAAAATTGTCTGG-3'
	Reverse	5'-CCAGATATCGGTACCCCTGCATCAGTACTTCG-3'

The TRAF6(K124R)YFP designated K124R-YFP and TRAF6(I98L, D100G)YFP designated as mR-YFP were engineered using a QuickChange II XL site-directed mutagenesis kit (Stratagene) with the wild type TRAF6pFLAG CMV 5a-YFP as a template. The primer sequences for these constructs are the same as those being used to generate the correlative mutant constructs in the pcDNA3.1 and shown in Table 2.

The human Sequestosome 1/P62 expression plasmid (P62pcDNA3.1) was kindly provided by Dr. Deborah Galson at the University of Pittsburgh. We re-engineered a HcRed1 tagged P62 expression plasmid as follows: 1). Generated pFLAG CMV 5a-HcRed1 by replacing the EYFP coding sequence with the HcRed1 coding sequence from pFLAG CMV 5a-YFP. The HcRed1 coding sequence franked with Eco RV and Bam HI restriction sites, but without a nuclear localization signal (NLS) attached to, was produced by PCR reaction from the HcRed1/Nuc vector (Clontech). 2). The P62 coding sequence was generated by PCR from P62pcDNA3.1 and inserted into the Eco RI and Bgl II sites of the pFLAG CMV 5a-HcRed1 plasmid. The primer sequences used for HcRed1 PCR were CTG ATA TCC GGA GGA GGA ATG GTG AGC GGC CTG CTG AAG GAG (sense) and CTG GGA TCC ACC ACC ACC GTT GGC CTT CTC GGG CAG GTC GCT GTA C (anti-sense), and the primer sequences used for P62 PCR were CCA GAA TTC ATG GCG TCG CTC ACC GTG AAG (sense) and CAT AGA TCT AT CAA CGG CGG GGG ATG CTT TGA (anti-sense).

Cell transfection

Reporters or expression plasmids were transfected into cells with FuGENE HD transfection reagents (Roche Applied Science, Indianapolis, IN). The experimental procedures are briefly described as follows. Cells were seeded with normal culture media and allowed to settle overnight. The density of cells before transfection was about 60-70% of confluence. All plasmids used for transfection were added into a tube with proper volume of serum-free Opti-MEM medium. FuGENE HD was first diluted in another tube with equal amount of serum-free Opti-MEM medium as that used for plasmids, and then transferred the FuGENE Opti-MEM medium into the tube containing the plasmids. The amount of plasmids and FuGENE HD in the mixture should be adjusted to a ratio of plasmids (μg) : FuGENE HD (μL) = 1:3, mixed gently and incubated the mixture at room temperature for 15-20 min. The plasmid-FuGENE mixtures were then added into indicated containers with pre-seeded cells. Kept cells at 37^{o}C with 5% CO_2 and cultured them for about 24 hrs before further treatment or analysis. The transfection mixture was not removed from the cell culture media.

Luciferase bulk-cell assay for NF-κB activity

A luciferase reporter (NF-κB/pGL2) was co-transfected with indicated expression plasmids into cells for 24 hours with the procedures described above. The transfected cells were then lysed with 100 μL of 1X cell lysis buffer (Promega, Madison, WI) in each well and shaken for 20 min. 20 μL of supernatant from each well was used for luciferase

activity analysis using a VERITAS luminometer (Turnaer BioSystems, Sunnyvale, CA) according to the manufacturer's procedure.

Analysis of NF-κB activation with HcRed1-κB reporter

The HcRed1-κB reporter was co-transfected with indicated TRAF6 mutant constructs according to 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^{o}C ultra-cooled Hamamatsu C9100-02 Electron Multiplier CCD camera and a Melles Griot Kr/Ar Laser and collected using the Hammamatsu-Compix Simple PCI software suite. The microscope was enclosed in a Zeiss/Pecon XL S1 LSM incubation chamber with logic-controlled temperature and CO_2 modules and custom stage inserts with optically transparent CO_2 covers designed to support a variety of single and multi-well culture plates.

Immuno-precipitation and Western blotting

Western blotting. After indicated treatments, 293 or 293T cells were detached by pipeting and transferred to microcentrifuge tubes. Spin down cells with a bench-top microcentrifuge at 5000 rpm for 3 min. Aspirated culture medium and washed cell pellets once with 1XPBS. Resuspended pellets in 50 μL of ice cold lysis buffer per 10^6 cells. Cell lysate was incubated on ice for 30 minutes and vortexed every 10 minutes, and then centrifuged at 14,000 rpm (10,000-15,500x g) at 4^{o}C for 10 minutes. Supernatant was harvested and mixed with an equal volume of 2XSDS-PAGE sample buffer supplemented with fresh 2-mercapto ethanol (Bio-Rad) and incubated at 98^{o}C for 10

minutes. Heat denatured samples were then loaded into a precast 4-12% gradient SDS-polyacylamide gel (Lonza) and run with 1X Laemmli buffer (25 mM Tris, 192m M Glycine, 0.1 % SDS; pH 8.3) at 100 v for about 2 hours. Resolved proteins were transferred from the SDS-polyacryamide gel onto a PVDF membrane in 1X Tobin buffer (3.03 g TRIS, 14.4 g glycine, 150 mL methonal per liter of solution, pH 8.3). Membranes were incubated in 5% non-fat milk with 1XTPBS (10 mM phosphate, 140 mM NaCl, 3 mM KCl, 0.05% Tween 20; pH 7.4) with slight shaking for 1 hour, and then incubated with indicated primary antibody for another 1-2 hours. Removed primary antibody and washed the membrane in 1XTTBS with slight shaking for 3X5 minutes. The membrane was then incubated in a 1:10,000 dilution of secondary antibody in 5% non-fat milk with 1XTTBS with slight shaking for 1 hour. The secondary antibody was removed and the membrane washed in 1XTTBS with slight shaking for 3X5 minutes. The membrane was incubated with chemiluminescent substrate (Thermo Scientific, Waltham, MA) at room temperature for 5 minutes. Signals were visualized by X-ray film exposure.

For immuno-precipitation, cell lysate supernatant was diluted with the same lysis buffer as described above into 200 μL per 10^6 cells. A specific antibody was added and the sample rocked at 4^oC for 2-5 hours. Added 30 μL of 50% protein A/G conjugated agarose slurry pre-absorbed by non-specific IgG into each sample tube and rocked at 4^oC overnight. Agarose beads were centrifuged at 10,000 rpm for 1 minute. Supernatant was aspirated and beads rinsed with 500 μL lysis buffer three times. 100 μL 1XSDS-PAGE sample buffer was added to each sample tube containing the agarose beads. Re-suspended agarose beads by vortexing and then incubated at 98^oC for 10 minutes. SDS-PAGE and immuno-blotting procedures were as described above for sample analysis.

RESULTS

Engineering of human TRAF6 mutant constructs

In order to collectively investigate the correlations between the structural determinants (functional domains) of TRAF6 and its cellular localization, ubiquitination, and activities, we have engineered various mutant constructs with each mutant coding sequence subcloned into pcDNA3.1(+) and pFLAG CMV 5a-YFP vectors individually. These allow us to study the biological functions of each mutant by over-expressing a plasmid that is either constructed in the pcDNA3.1(+) or pFLAG CMV 5a-YFP vector. By comparing activities generated from both sets of plasmids, we were able to ensure no significant allosteric effect from the conjugated YFP protein. The YFP-FLAG dual tagged constructs provide a convenient means of studying the cellular localization of each mutant with confocal microscopic techniques, and we can also use anti-GFP or anti-FLAG antibodies for immune-precipitation or immune-blotting of all TRAF6 mutants without worrying about the existence of the specific antigen determinant that can be recognized by the anti-TRAF6 antibody. A scheme of a collection of the TRAF6 mutant constructs is summarized in Figure12.

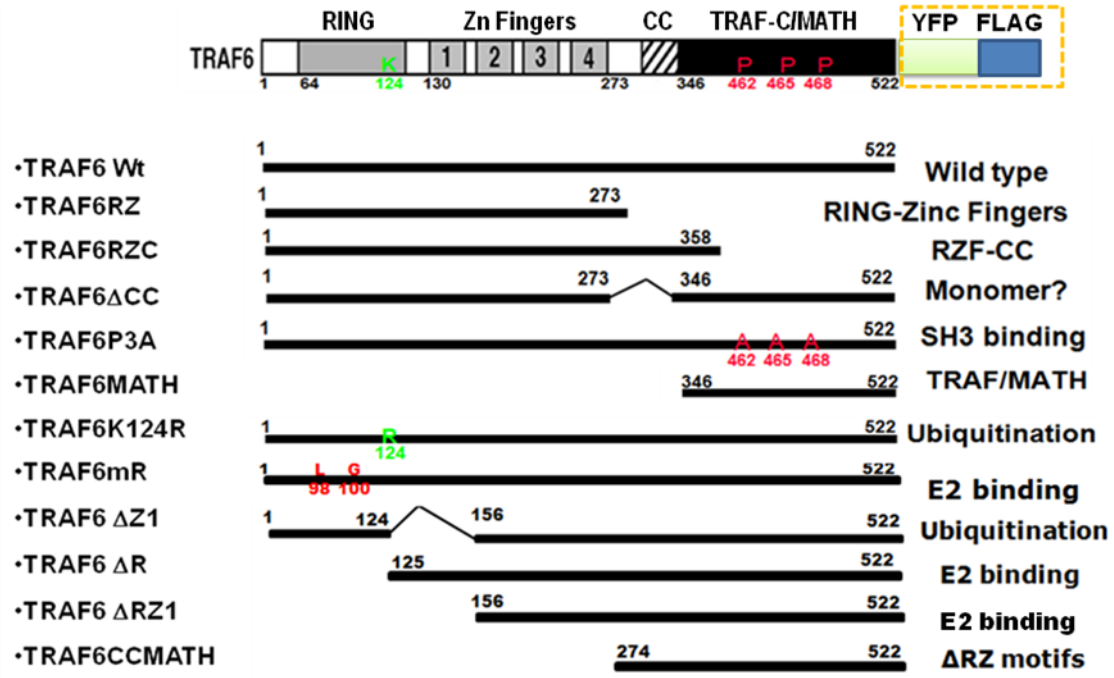


Figure12. Engineering of different TRAF6 mutant constructs.

The TRAF6 and each mutein's coding sequence were inserted into pcDNA3.1 (Non-tagged) and pFLAG CMV 5a-YFP (YFP-FLAG dual-tagged) expression vectors. The scheme shows basic structural information of each TRAF6 protein and/or possible targeted functions of indicated muteins.

Table 5. Reference molecular sizes of un-tagged and dual-tagged TRAF6 proteins

Peptides	Un-tagged Proteins (KDa)	Dual-tagged Proteins (KDa)
YFP-FLAG	N/A	29
TRAF6	61	90
RZ	32	61
RZcc	42	71
Δcc	53	81
P3A	61	90
MATH	21	50
K124R	61	90
mR	61	90
ΔZ1	57	86
ΔR	47	76
ΔRZ1	42	71
ccMATH	29	58

The formula used for the calculation: M.W. = Numbers of residues X 0.117 KD.

Details of engineering the TRAF6 mutant constructs have been described in the materials and methods section. The sequences of all constructs were confirmed by DNA sequencing analysis. The perspectives to engaging such constructs in our studies are as follows:

TRAF6 (wild type) The human *TRAF6* gene is located on chromosome 11 (11p12). The TRAF6 protein contains 522 amino acid residues. The wild type construct coding for TRAF6 is made and used as a reference for the studies of sub-cellular localization patterns and biological functions of other TRAF6 mutants.

TRAF6RZ Previous studies have suggested that the RING-Zinc fingers of TRAF6

function in the recruitment of downstream molecules, whereas the fragment only containing RING-Zinc fingers (amino acid residues 1-273) is incapable of activating downstream molecules such as NF-κB and PI3K (Wang et al., 2006; Yoshida et al., 2004). To test whether this fragment alone inside cells can be ubiquitinated or not and its cellular localization when it is over-expressed, a TRAF6RZ-YFP conjugated construct was also generated and used for our subsequent studies.

TRAF6RZC It is thought that TRAF6 may form a trimer inside cells, and multimerization of TRAF6 is required for proper function. We inserted a cDNA sequence that encodes the RING-Zinc fingers and coiled-coil domains of TRAF6 (amino acid residues: 1-358) into the pcDNA3.1 (+) and pFLAG CMV 5a-YFP plasmids, respectively. These expression constructs will be used for comparison studies to the TRAF6 wild type in cellular distribution, ubiquitination, and biological function since this C-terminus truncated mutant may maintain the capability of trimerization.

TRAF6ΔCC The mutant plasmids with the coiled-coil domain deletion are used to confirm the necessity of multimerization of TRAF6 for its regulatory activities. We will also test their impacts in cellular distribution and ubiquitination of TRAF6 in our collective studies.

TRAF6P3A There is a poly-proline motif (PXXPXXP) in the MATH domain of TRAF6. These three proline residues are located in 462, 465 and 468, respectively. Previous studies from our group and others have implicated that this putative SH3 (refer to as Src homology 3) binding motif is involved in the interaction with c-Src (Wang et al., 2006; Wong et al., 1999). The impact of this mutant in ubiquitination of TRAF6 and its activation of NF-κB will be a focus of our studies.

TRAF6MATH The MATH domain (amino acid residues 346-522) of TRAF6 is involved in receptor binding upon different signaling events. Lacking the downstream molecule recruitment elements, the MATH domain alone should play a role as a dominant negative mutant. But it is still intriguing to investigate whether the MATH domain has any contribution to the ubiquitination or a particular cellular localization of the TRAF6 protein.

TRAF6K124R It was reported that the lysine residue at 124 of mouse TRAF6 is a specific and critical site for ubiquitination, NF-κB activation and osteoclastogenesis (Lamothe et al., 2007). We intend to generate its human homologue mutant construct. It will allow us to test if it has similar effects. Moreover, we will test whether this mutant will sequester its sequestosomal localization resulting from the absence of ubiquitination.

TRAF6mR Auto-ubiquitination of TRAF6 is most likely induced by its direct binding to E2 ubiquitin conjugating enzyme, Ubc13, through the RING domain (Wooff et al., 2004). A point mutation at residue cysteine 70 within the RING domain of TRAF6 was shown to disrupt both ubiquitination and NF-κB activation (Funakoshi-Tago et al., 2008; Lamothe et al., 2007; Wooff et al., 2004). However, it is not clear whether this is due to specific interference with E2 recruitment to the RING domain. This mutation could alter the conformation of the RING domain, since cysteine 70 may be a critical site for Zn^{2+} ion binding. Markin, *et al* further showed by means of nuclear magnetic resonance (NMR) spectroscopy that residues 98-100 in the RING domain of TRAF6 were involved in a strong physical interaction with Ubc13, but the impacts on ubiquitination and biological function of TRAF6 have not been explored biochemically (Markin et al., 2008). It warrants our enthusiasm to engineer the TRAF6mR constructs that contain

double point mutations at residues 98 and 100 (I98L, D100G), hoping to investigate the correlation among ubiquitination , cellular localization, and its biological activities a mutein being potentially defective in E2 binding.

TRAF6ΔZ1 Besides the RING domain, the integrity of the first Zinc finger (amino acid residues 125-155) of TRAF6 was also suggested to be necessary for its activation of NF-κB (Lamothe et al., 2008). We generated the first Zinc finger deletion mutant constructs and attempted to test the consequences for ubiquitination and cellular distribution of TRAF6 protein.

TRAF6ΔR As addressed above, the RING domain deletion mutant (delta 1-124) should suppress auto-ubiquitination. This construct will be used as backup for the TRAF6mR constructs to confirm the relationship of the cellular distribution, ubiquitination, and regulatory activity of TRAF6 to its downstream signaling.

TRAF6ΔRZ1 With similar thoughts to engineering of the TRAF6ΔR, we constructed the RING and first Zinc finger deletion (delta 1-155) mutant plasmids and aim at the cellular localizations of this truncated peptide.

TRAF6ccMATH The constructs coding for this un-tagged or dual-tagged mutein only contain the coiled-coil and MATH domains (amino acid residues 274-522). The N-terminus RING and Zinc fingers (amino acid residues 1-273) are truncated. It has been shown that this mutein acts as a dominant-negative in the IL1R, RANK and CD40 signaling pathways by competing for the upstream activators binding to the MATH domain of the wtTRAF6 (Rothe et al., 1995; Wong et al., 1998; Yoshida et al., 2004). However, it will be interesting to test if this mutein can interfere with the dominant-positive effect of TRAF6.

Cellular distribution of TRAF6 and muteins

We have reported that TRAF6 mainly localizes in the cytoplasm and forms sequestosomal aggregation when a YFP conjugated TRAF6 construct is over-expressed in HEK293 cells (Wang et al., 2006). We have also observed that a small portion of the cells demonstrated green fluorescent signals inside the nuclei. In order to sort out which determinants / functional domains of TRAF6 dictate the localization of the protein, we have over-expressed different YFP conjugated mutant constructs in HEK293 cells.

Consistent with our previous results, TRAF6YFP is localized to cytoplasmic speckles and sequestosomal aggregations (small punctuate fluorescence spots) in the cytoplasm, with a small portion (about 5 %) of signal localizing in nuclei (Fig. 13).

The ectopically expressed TRAF6RZYFP protein (consisting of the N-terminal residues 1-273) distributes throughout the entire cell. Some YFP signal co-localizes with the nuclear localizing HcRed1 (HcRed1-Nuc) protein, which confirms that the YFP conjugated RING-Zinc fingers domain distributes in both the cytoplasm and nuclei of transfected cells. It is noted that, in contrast to that of the wild-type TRAF6 protein, there is no sequestosomal fluorescence observed (Fig. 14).

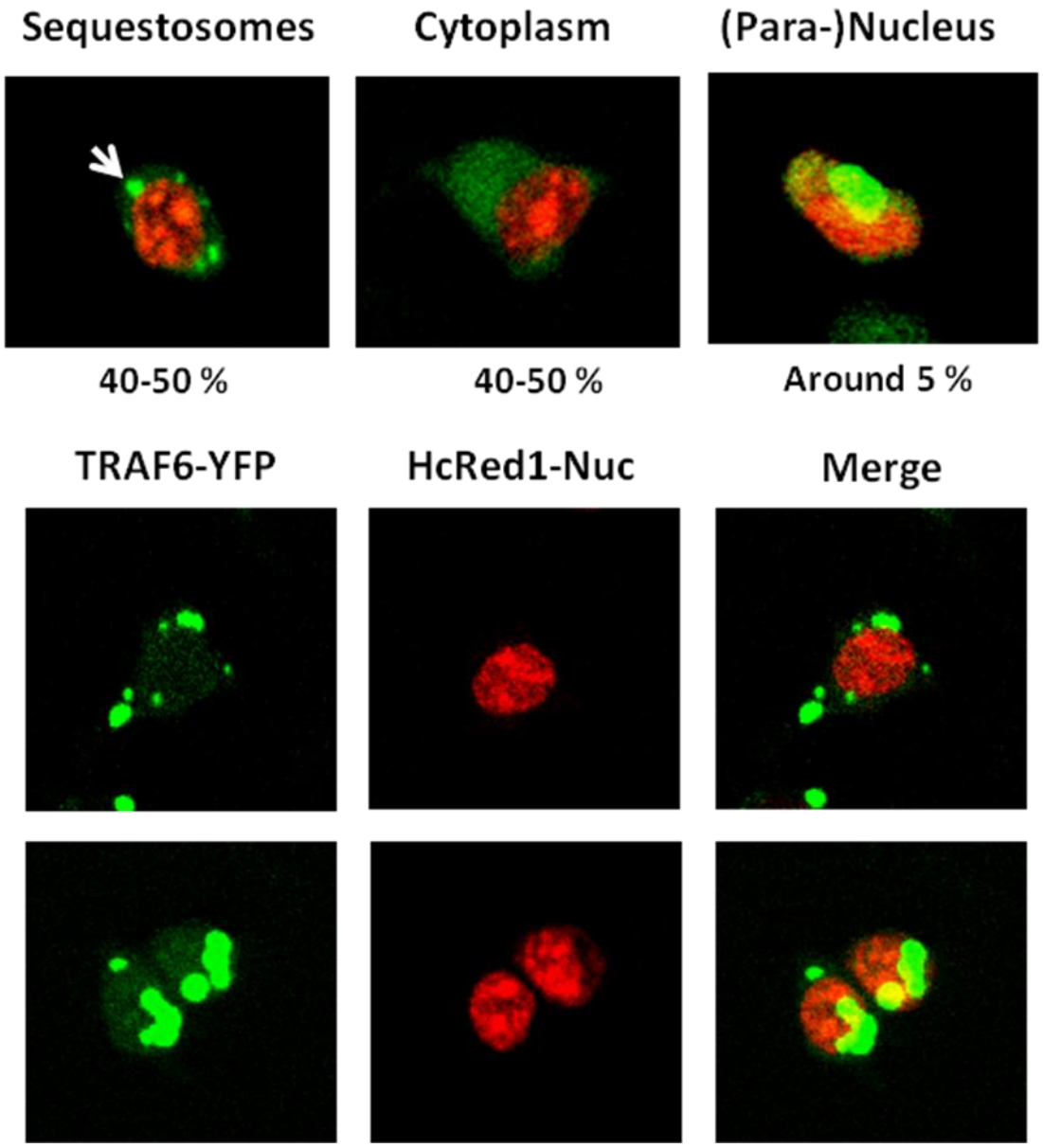


Figure 13. Sub-cellular distributions of TRAF6-YFP.

An expression plasmid (30 ng) coding for TRAF6-YFP protein was co-transfected with pHcRed1-Nuc vector (30 ng) into one well of pre-inoculated 293 cells in a 96-well tissue culture plate. Confocal images were taken after 24 hours of transfection. The YFP fluorescent signal is pseudo-colored green. The fluorescent signal of HcRed1-nuc, a nuclear localization marker, is pseudo-colored red.

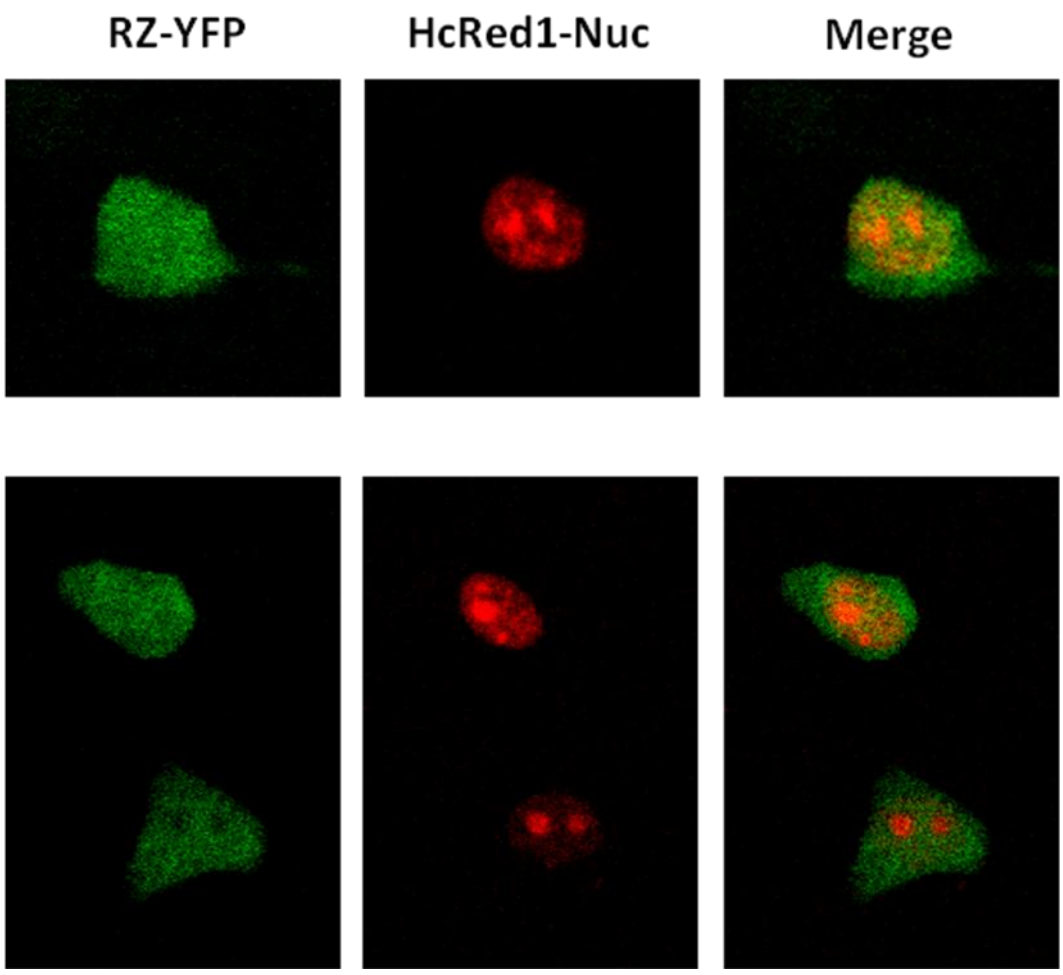


Figure 14. Sub-cellular distribution of TRAF6RZ-YFP.

An expression plasmid (30 ng) coding for TRAF6RZ-YFP mutein (pseudo-colored green) was co-transfected with pHcRed1-Nuc vector (30 ng) into one well of pre-inoculated 293 cells in a 96-well tissue culture plate. Confocal images were taken after 24 hours of transfection. The fluorescent signal of HcRed1-nuc, a nuclear localization marker, is pseudo-colored red.

Similar to TRAF6RZYFP, the TRAF6MATHYFP mutein (also designated as TRAF-C), which contains residues 346-522 in the C-terminal MATH domain of TRAF6, also displays YFP signal throughout the entire cell (Fig. 15).

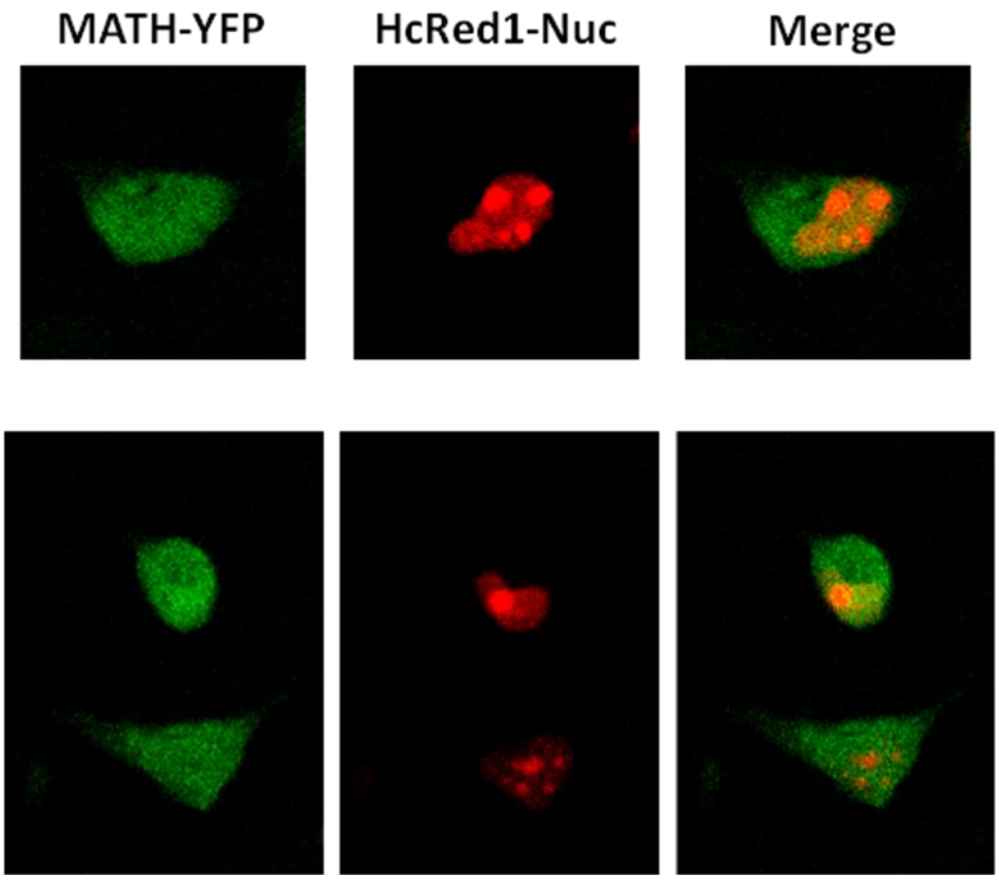


Figure 15. Sub-cellular distribution of TRAF6MATH-YFP.

An expression plasmid (30 ng) coding for TRAF6MATH-YFP (green) was co-transfected with pHcRed1-Nuc vector (30 ng) into one well of pre-inoculated 293 cells in a 96-well tissue culture plate. Confocal images were taken after 24 hours of transfection. The fluorescent signal of HcRed1-nuc, a nuclear localization marker, is pseudo-colored red.

TRAF6RZccYFP, a MATH domain truncated mutein, displays similar cellular localization patterns as those of the wild-type TRAF6YFP (Fig. 16). Since sequestosomes have been suggested to be sites where ubiquitinated TRAF6 co-localizes with proteasomes (Sanz et al., 2000), we speculate that RING-Zinc fingers along with the coiled-coil domain of TRAF6 will be sufficient for ubiquitination.

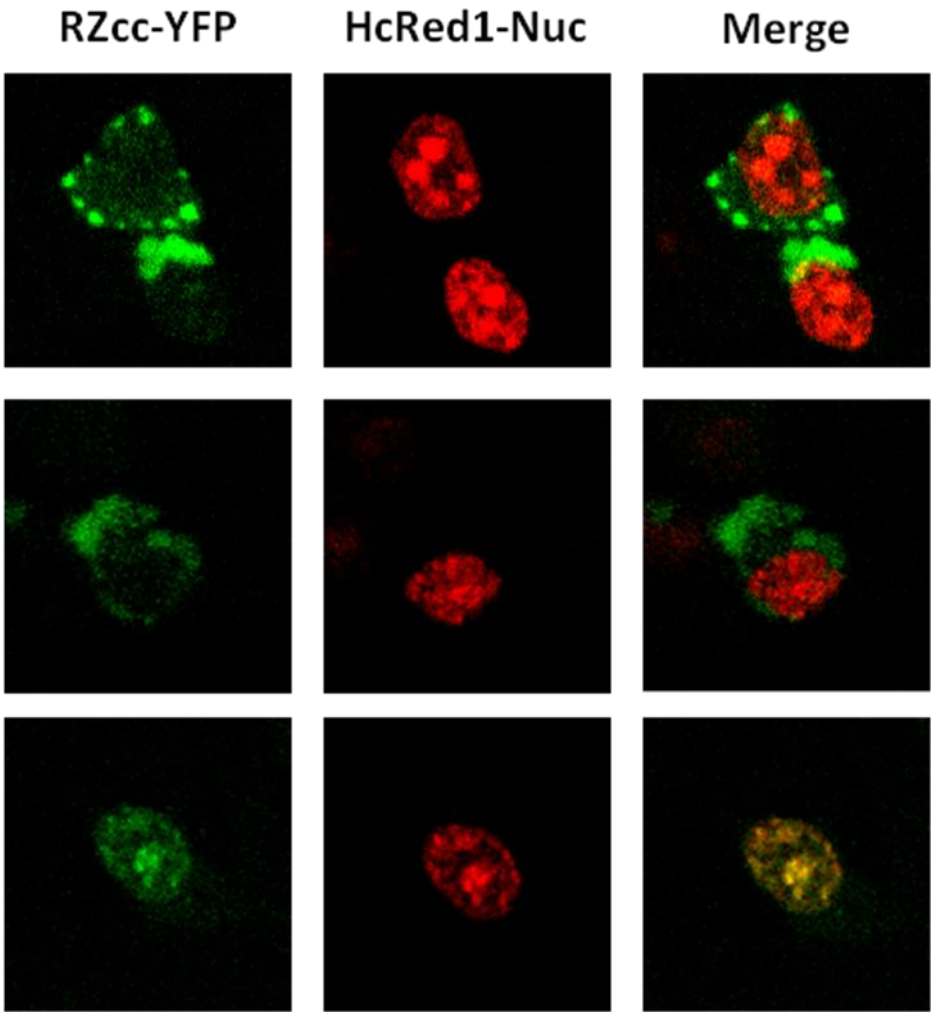


Figure 16. Sub-cellular distributions of TRAF6RZcc-YFP.

An expression plasmid (30 ng) coding for TRAF6RZcc-YFP (green) was co-transfected with pHcRed1-Nuc vector (30 ng) into one well of pre-inoculated 293 cells in a 96-well tissue culture plate. Confocal images were taken after 24 hours of transfection. The fluorescent signal of HcRed1-nuc, a nuclear localization marker, is pseudo-colored red.

The coiled-coil domain has been suggested to be involved in the multimerization of TRAF molecules (Park et al., 1999). To evaluate the cellular distribution of a TRAF6 mutein with a deletion in the coiled-coil domain, an expression plasmid coding for TRAF6ΔCCYFP protein was transfected into 293 cells. The over-expressed TRAF6ΔCCYFP protein mainly stays in the cytoplasm. Less than 2% of transfected cells showed tiny punctuate dots similar to the sequestosomal fluorescence pattern, but their sizes were smaller than those observed in the wild-type (Fig. 17), suggesting that the coiled-coiled domain may be involved in regulating ubiquitination of the TRAF6 molecule.

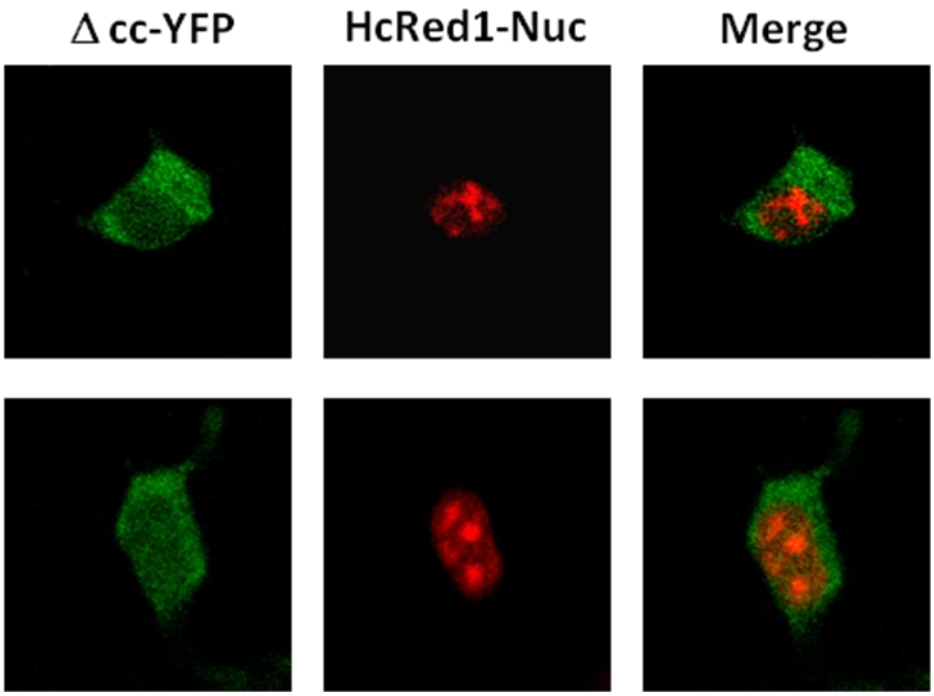


Figure 17. Sub-cellular distributions of TRAF6Δcc-YFP.

An expression plasmid (30 ng) coding for TRAF6Δcc-YFP (green) was co-transfected with pHcRed1-Nuc vector (30 ng into one well of pre-inoculated 293 cells in a 96-well tissue culture plate. Confocal images were taken after 24 hours of transfection. The fluorescent signal of HcREd1-nuc, a nuclear localization marker, is pseudo-colored red.

In agreement with our previous data (Wang et al., 2006), over-expression of the poly-proline motif mutant construct, TRAF6P3AYFP, showed minimal difference when compared to the cellular localization patterns of wild-type TRAF6YFP protein (Fig. 18). Comparison of its activity to wild-type TRAF6 protein will be discussed later (see Fig. 27-29).

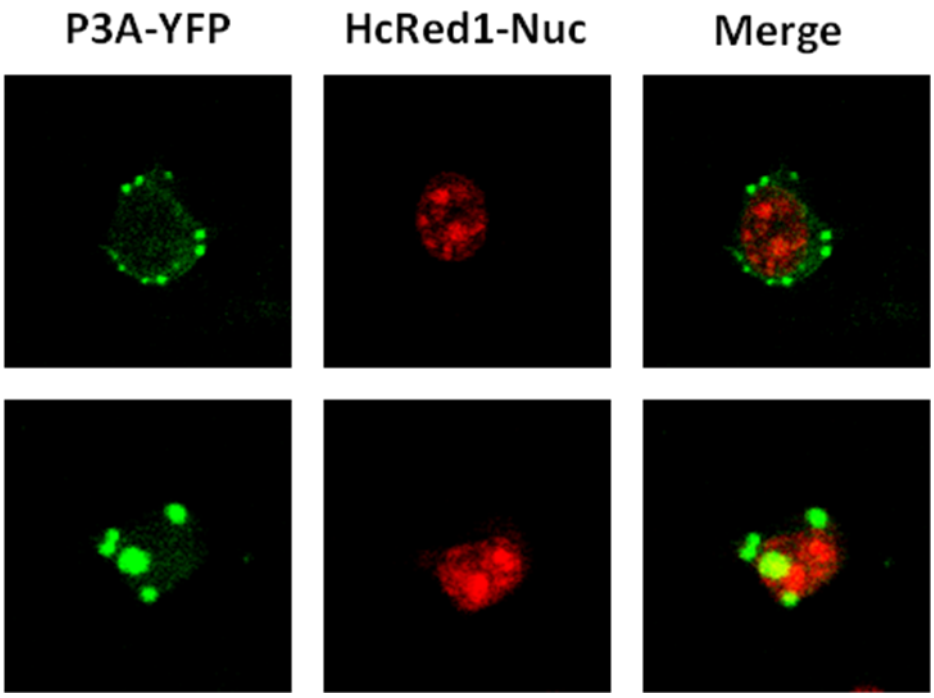


Figure 18. Sub-cellular distributions of TRAF6P3A-YFP.

An expression plasmid (30 ng) coding for TRAF6 P3A-YFP (green) was co-transfected with pHcRed1-Nuc vector (30 ng) into one well of pre-inoculated 293 cells in a 96-well tissue culture plate. Confocal images were taken after 24 hours of transfection. The YFP flruorescent signal is pseudo-colored green. The fluorescent signal of HcRed1-nuc, a nuclear localization marker, is pseudo-colored red.

The lysine 124 residue had been reported to be critical for ubiquitination and activity of TRAF6 (Lamothe, 2007).However, the human analogue mutant TRAF6K124RYFP showed very similar cellular localization patterns to the wild-type TRAF6 (Fig. 19). The inconsistency also exists in its capability of ubiquitination and activation of NF-κB, when this mutein was over expressed in HEK293 cells (more detail will be addressed in Discussion Section).

It was reported that residues 98-100 in the RING domain of TRAF6 are involved in strong physical interaction with an E2 ubiquitin conjugating enzyme, Ubc13, demonstrated by means of nuclear magnetic resonance spectroscopy (Markin et al., 2008), but investigators did not conduct any biochemical study to validate their NMR results. We engineered a mutant construct coding for the TRAF6mRYFP that consists of point mutations of I98L and D100G. However, when TRAF6mRYFP protein was ectopically expressed in HEK293 cells, we observed similar cellular localization patterns to the wild-type protein (Fig. 20). The intracellular sequestosomal aggregation of TRAF6mRYFP protein implicates that this molecule may still be ubiquitinated. Therefore, amino acid residues 98-100 of TRAF6 within the RING domain may not be a necessary binding site for Ubc13, or recruitment of Ubc13/Uev1 may also involve other domain(s).

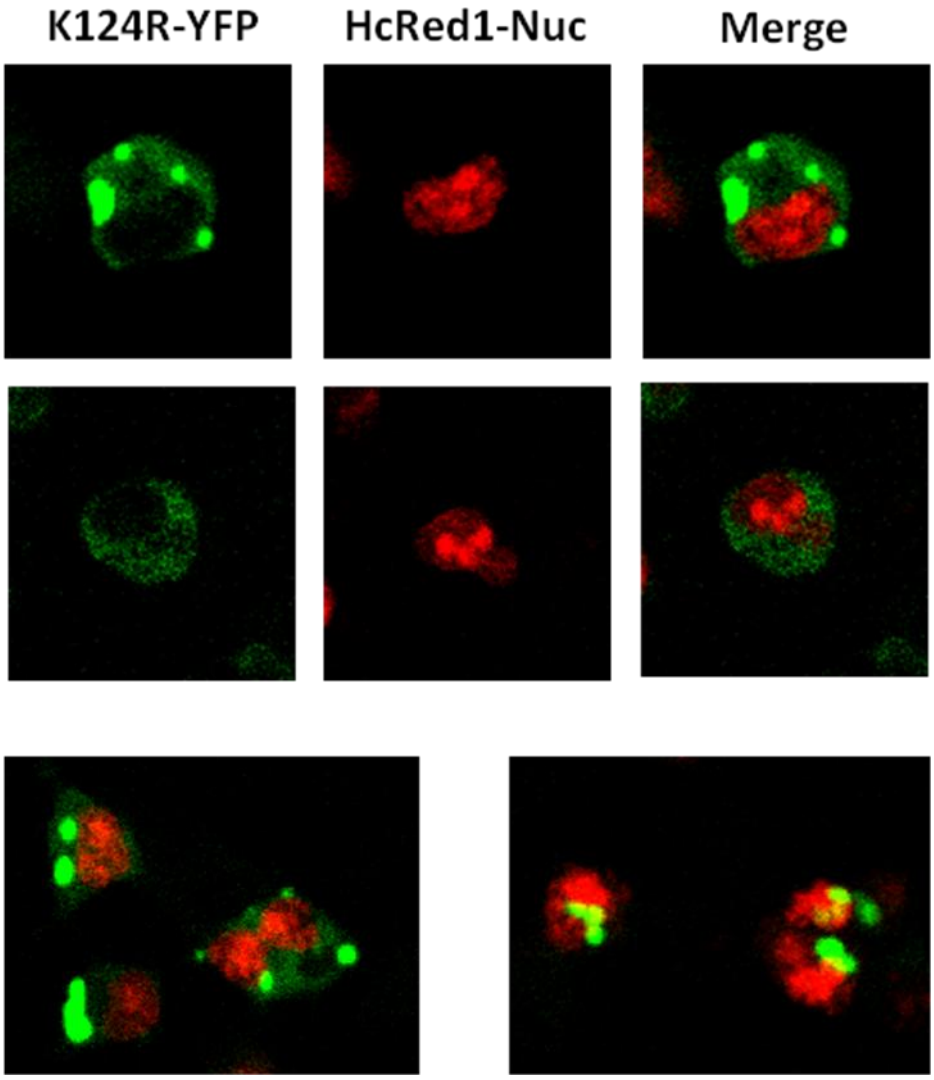


Figure 19. Sub-cellular distributions of TRAF6K124R-YFP.

An expression plasmid (30 ng) coding for TRAF6 K124R-YFP (green) was co-transfected with pHcRed1-Nuc vector (30 ng) into one well of pre-inoculated 293 cells in a 96-well tissue culture plate. Confocal images were taken after 24 hours of transfection. The fluorescent signal of HcRed1-nuc, a nuclear localization marker, is pseudo-colored red.

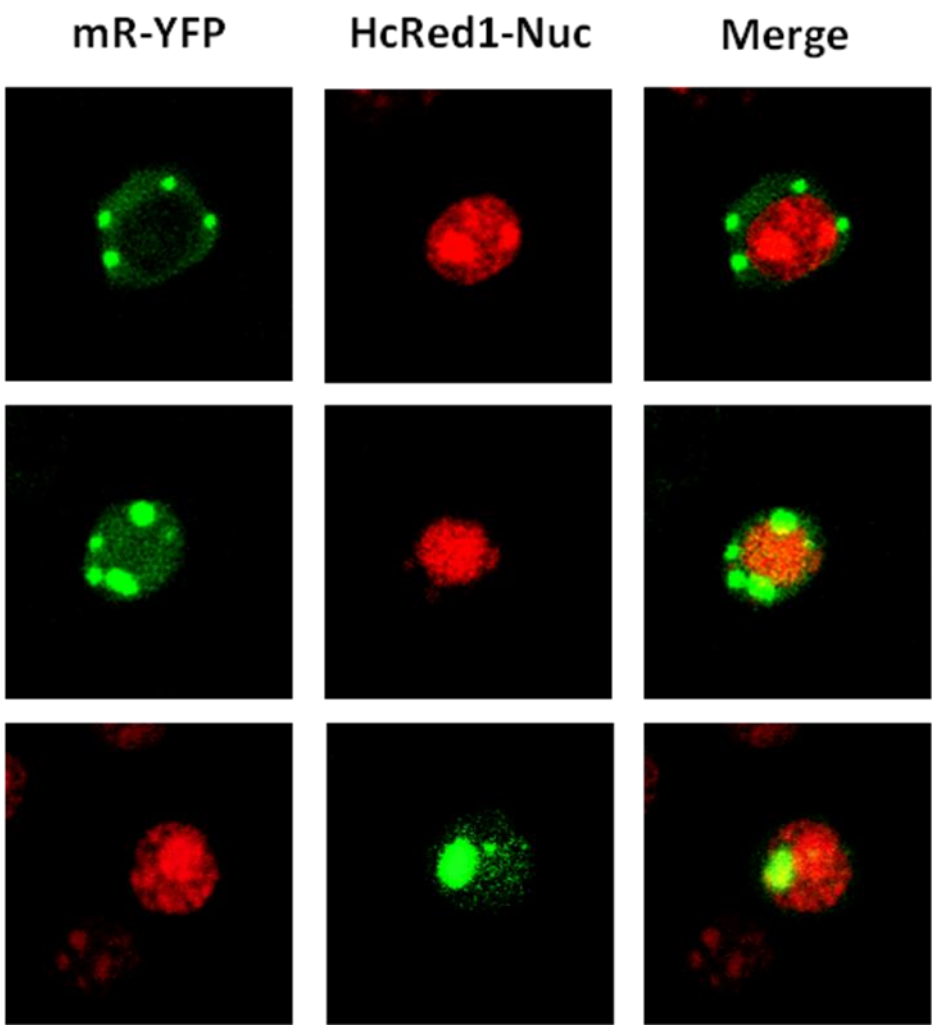


Figure 20. Sub-cellular distributions of TRAF6mR-YFP.

An expression plasmid (30 ng) coding for TRAF6 mR-YFP (green) was co-transfected with pHcRed1-Nuc vector (30 ng) into one well of pre-inoculated 293 cells in a 96-well tissue culture plate. Confocal images were taken after 24 hours of transfection. The fluorescent signal of HcRed1-nuc, a nuclear localization marker, is pseudo-colored red.

To investigate the impact of the RING domain and Zinc fingers on cellular localization of TRAF6, expression plasmids coding for TRAF6ΔRYFP (deletion of

residues 1-124) and TRAF6ΔRZ1YFP (deletion of coding sequence of amino acid residues 1-155) muteins were individually transfected into 293 cells. Intriguingly, both TRAF6ΔRYFP and TRAF6ΔRZ1YFP proteins mainly distribute in the cytoplasm with minimal or no sequestosomal fluorescent signal displayed in transfected cells (Fig. 21 & 22). Therefore, although amino acid residues of 98-100 may not be sufficient for recruitment of E2 ubiquitin conjugating enzyme, the RING domain seems to be necessary for ubiquitination of TRAF6.

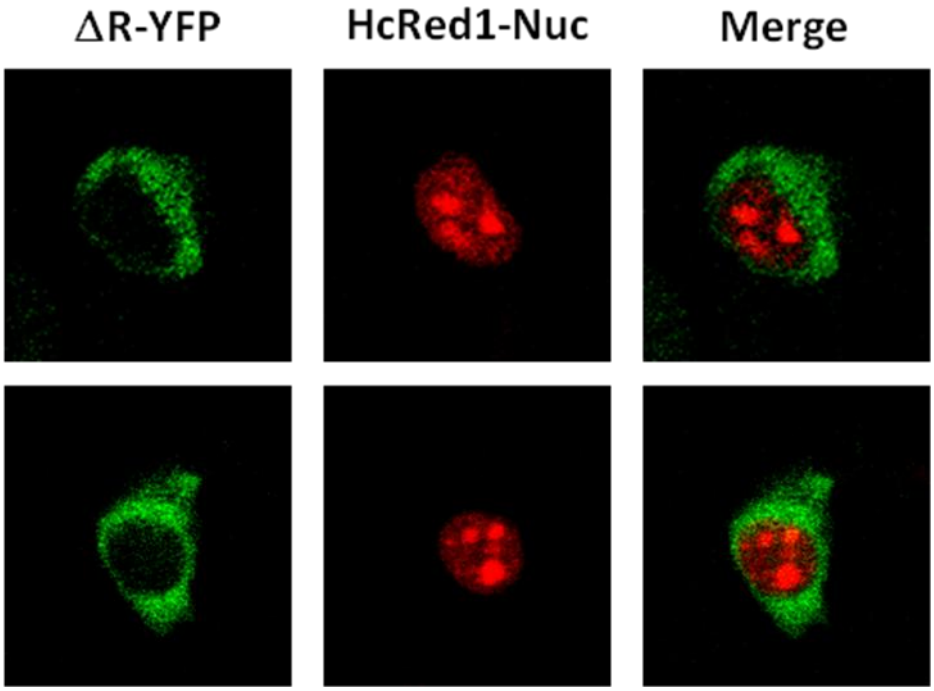


Figure 21. Sub-cellular distributions of TRAF6ΔR-YFP.

An expression plasmid (30 ng) coding for TRAF6ΔR-YFP (green) was co-transfected with pHcRed1-Nuc vector (30 ng) into one well of pre-inoculated 293 cells in a 96-well tissue culture plate. Confocal images were taken after 24 hours of transfection. The fluorescent signal of HcRed1-nuc, a nuclear localization marker, is pseudo-colored red.

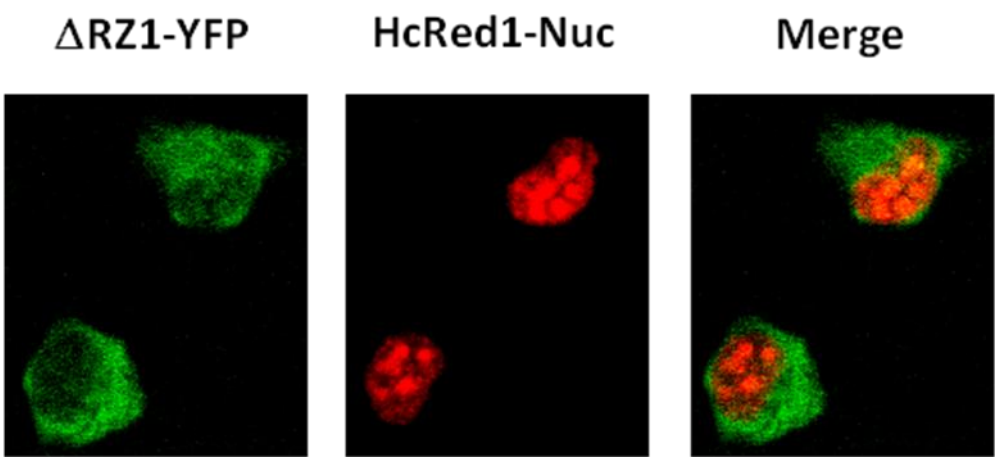


Figure 22. Sub-cellular distributions of TRAF6ΔRZ1-YFP.

An expression plasmid (30 ng) coding for TRAF6ΔRZ1-YFP (green) was co-transfected with pHcRed1-Nuc vector (30 ng) into one well of pre-inoculated 293 cells in a 96-well tissue culture plate. Confocal images were taken after 24 hours of transfection. The fluorescent signal of HcRed1-nuc, a nuclear localization marker, is pseudo-colored red.

Interestingly, in order to gain more insight into the contribution of the first Zinc finger motif which was shown to be important for ubiquitination and activity of TRAF6, we over-expressed TRAF6ΔZ1YFP protein (deletion of first Zinc finger motif, or amino acid residues 125-155) in 293 cells using our standard transfection procedures. As a result, less than 1% of the transfected cells showed strong green fluorescence that smeared throughout the cell, while the majority of cells did not display any detectable fluorescent signal (Fig. 23). This suggests that only a small amount of TRAF6ΔZ1YFP mutein is existed. This was also confirmed by a Western Blot analysis (Fig. 26).

Therefore, deletion of the first Zinc finger motif may induce a conformational instability which results in either a rapid degradation or arrest of translation.

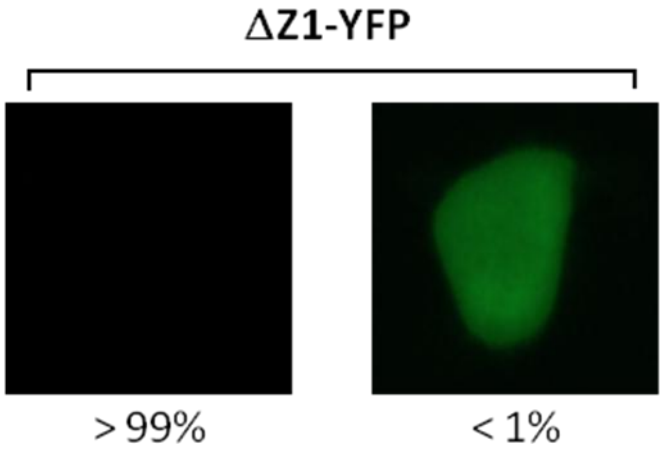


Figure 23. Sub-cellular distributions of TRAF6ΔZ1-YFP.

An expression plasmid (30 ng) coding for TRAF6ΔZ1-YFP (green) was transfected into one well of pre-inoculated 293 cells in a 96-well tissue culture plate. Majority of transfected cells had un-detectable YFP signal (left panel). Less than 1% of cells displayed yellow fluorescence diffusely through whole cell (right panel).

To address the contributions of the C-terminal coiled-coil and MATH domains to cellular localization, we over-expressed the TRAFccMATHYFP mutein and analyzed the fluorescent signal in 293 cells. It displayed similar cellular localization patterns as the TRAF6RZYFP and TRAF6MATHYFP muteins (Fig. 24).

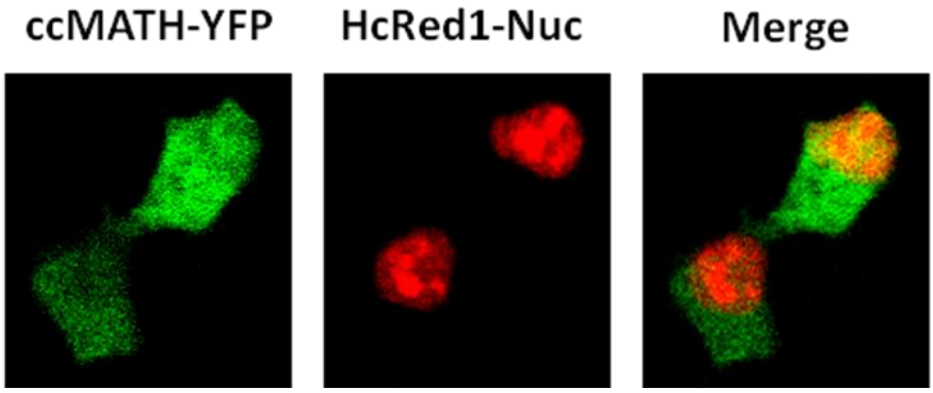


Figure 24. Sub-cellular distribution of TRAF6ccMATH-YFP.

An expression plasmid (30 ng) coding for TRAF6ccMATH-YFP (green) was co-transfected with pHcRed1-Nuc vector (30 ng) into one well of pre-inoculated 293 cells in a 96-well tissue culture plate. Confocal images were taken after 24 hours of transfection. The fluorescent signal of HcRed1-nuc, a nuclear localization marker, is pseudo-colored red.

In summary, cellular distributions of our YFP conjugated wtTRAF6 and muteins can be classified into three typical patterns: sequestosome localization including wtTRAF6-, RZcc-, P3A-, K124R- and mR-YFP; unique cytoplasm speckles including TRAF6Δcc-, ΔR- and ΔRZ1-YFP; whole cell distribution including TRAF6RZ-, MATH- and ccMATH-YFP. Representative confocal microscopic images of each YFP conjugated protein overlaid with the co-expressed HcRed1-Nuc protein have been assembled into a montage in Figure 25.

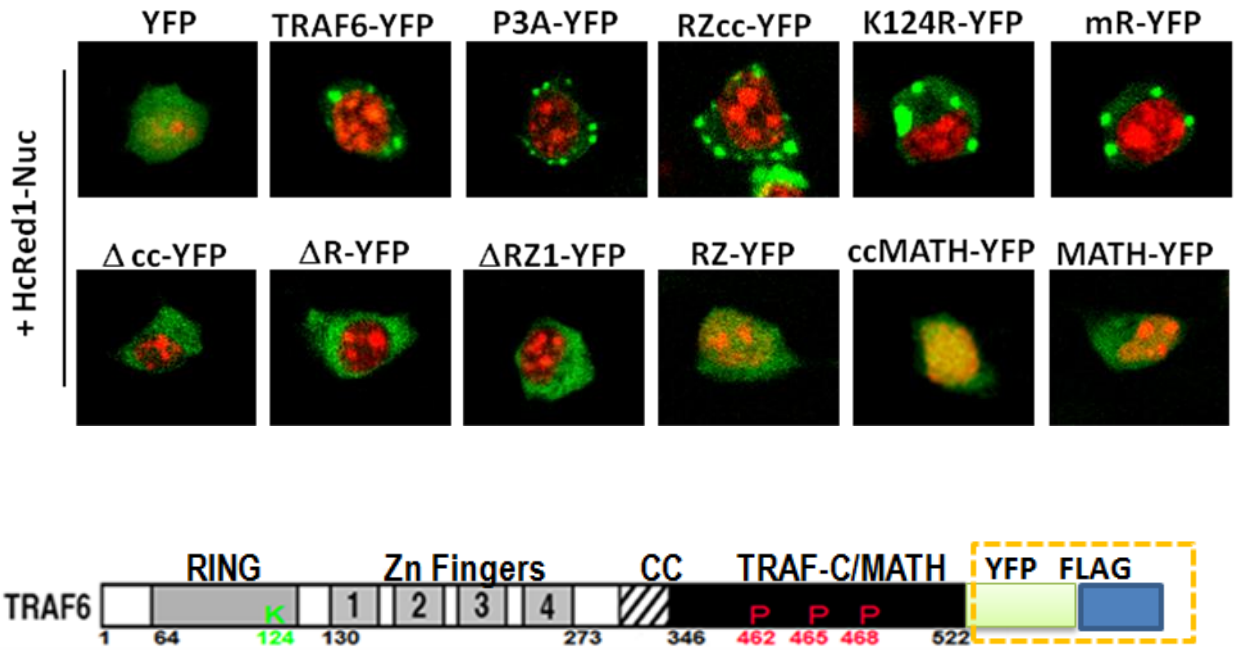


Figure 25. Typical sub-cellular localization pattern of TRAF6 and derived muteins.

Each micrograph represents a typical sub-cellular localization pattern of one ectopically expressed YFP conjugated TRAF6 proteins (green) along with a nuclear localization marker, HcRed1-Nuc (red). As shown, TRAF6-YFP and the P3A-, RZcc-, K124R- and mR-YFP muteins displays typical sequestosomal localization; the Δcc-, ΔR- and ΔRZ1-YFP muteins mainly display cytoplasm localization signal; The RZ-, ccMATH- and MATH-YFP muteins localize throughout whole cells. The bottom panel is a demonstration of TRAF6 structure including different functional domains/motifs.

Sequestosomal localization is correlated to ubiquitination and NF-κB activation

Poly-ubiquitinated TRAF6 has been reported to localize in sequestosomes (Sanz et al., 2000; Seibenhener et al., 2004), though the ubiquitination state of these muteins

have not been collectively investigated. To confirm the correlation of sequestosomal localization and ubiquitination, we over-expressed either TRAF6YFP or an YFP conjugated mutant protein in 293T cells. After 24 hours of transfection, these cells were treated with lysis buffer. Supernatants were immuno-precipitated with anti-FLAG M2-agarose beads. Absorbed proteins were eluted with SDS-PAGE sample buffer and separated in 4-12% SDS-PAGE gradient gel, and then the resolved proteins were transferred onto a PVDF membrane. The membrane was immuno-blotted with either anti-GFP or anti-ubiquitin antibody and visualized by the chemiluminescence substrate. Our results demonstrated that all five YFP conjugated proteins possessing a significant sequestosomal signal were highly ubiquitinated, while the proteins not displaying this localization pattern had no or significantly lower levels of ubiquitination (Fig. 26A). A duplicate membrane blotted with an anti-GFP antibody confirmed that all proteins except the TRAF6ΔRZ1YFP mutein were expressed in similar quantities (Fig. 26B). Furthermore, the molecular sizes of all five ubiquitinated proteins (immune-blotted with anti-GFP antibody) showed good matches with the fastest migrating region in each correlative smear poly-ubiquitinated protein band (immuno-blotted with anti-ubiquitin antibody) (Fig. 26A). A longer exposure of the anti-GFP immune-blotted membrane on X-ray film also displayed some smear signal, which suggests poly-ubiquitination of the proteins (Fig. 26C). Interestingly, we also observed degradation of these proteins. Such degradation seemed to have certain sequential patterns since several muteins displayed similar molecular sizes in their C-termini (fig. 26C).

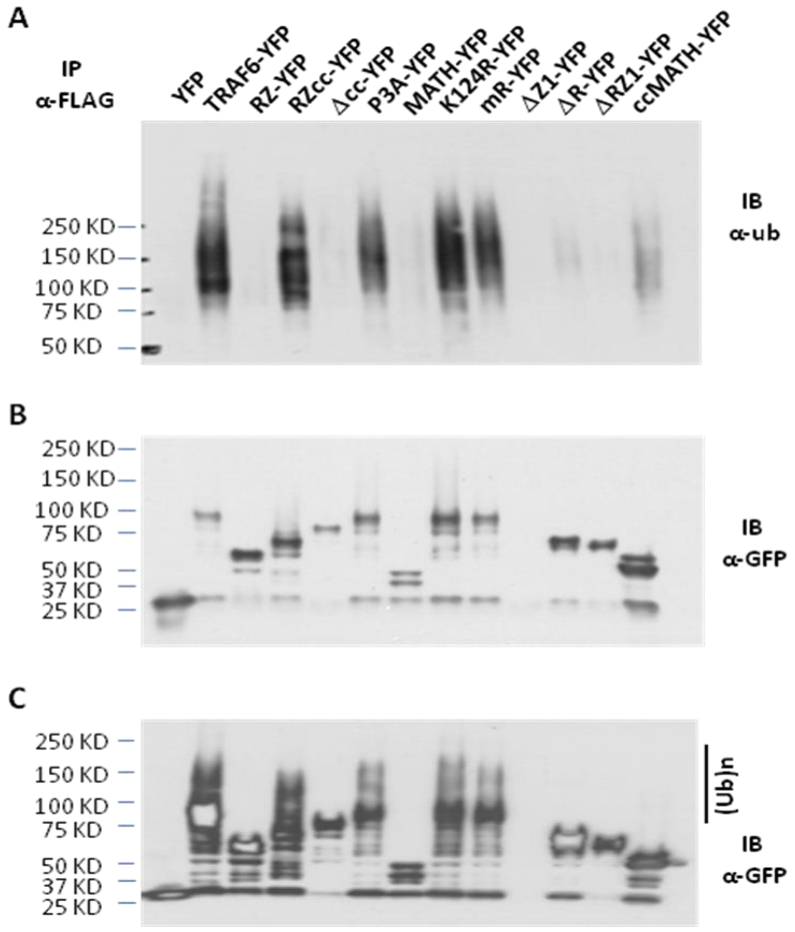
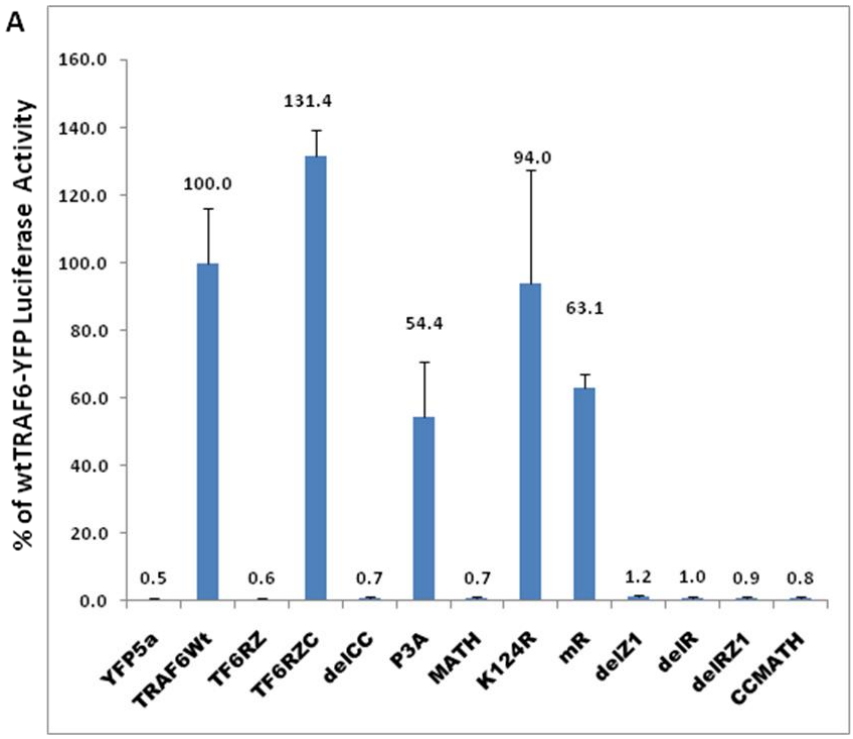


Figure 26. Ubiquitination analysis of wtTRAF6 and derived muteins.

An indicated expression plasmid (1 μg) encoding YFP-FLAG dual-tagged TRAF6 or a derived mutein was transfected into each well containing the 293T cells in 6-well tissue culture plates. Cells were lysed with IP buffer after 24 hours of transfection. Each supernatant containing an indicated FLAG tagged protein was immuno-precipitated with anti-FLAG agarose beads for 4 hours at 4^{o}C. Absorbed proteins were eluted and denatured by 1X SDS-PAGE buffer and 2-mercaptoethanol at 95^{o}C for 5 minutes. Eluted proteins were then resolved in 4-12% gradient SDS-PAGE and transferred onto a PVDF membrane and immuno-blotted with antibodies as indicated. (A). Immuno-blotting with anti-ubiquitin antibody; (B). Immuno-blotting with anti-GFP antibody; (C). A longer exposure image of Immuno-blotting with anti-GFP antibody, which shows positive signal above correlated molecular size of each protein shown in B, confirming ubiquitination of these proteins.

Since ubiquitination of TRAF6 had been reported to be important for its activity, we tested whether there is a correlation between ubiquitination and NF-κB activity in all of the TRAF6 muteins in our collection. Two assays were employed to analyze the NF-κB activity from individual cells that over-expressed each un-tagged and YFP-FLAG dual tagged protein. Figure 27A shows NF-κB activity determined by bulk-cell luciferase assay. The experiments were performed by co-transfecting an indicated expression plasmid coding for either wtTRAF6-YFP-FLAG or a TRAF6 mutein together with a NF-κB luciferase reporter (NF-κB/Luc) in 293 cells. Transfected cells were lysed and assayed for luciferase activity 24 hours post transfection. Our results revealed that all proteins showing significant luciferase activity were also coincidently ubiquitinated (Fig. 26). Similar luciferase activities were also observed when the dual-tagged expression plasmids were replaced by those coding for the corresponding un-tagged TRAF6 proteins (Fig. 27B). These results argue that tags conjugated to the TRAF6 proteins do not significantly affect activities, except for the TRAF6P3A mutein (will be addressed in Discussion Section).

A


% of wtTRAF6-YFP Luciferase Activity
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
0.5
100.0
0.6
131.4
0.7
54.4
0.7
94.0
63.1
1.2
1.0
0.9
0.8
YFP5a
TRAF6Wt
TF6RZ
TF6RZC
delCC
P3A
MATH
K124R
mR
delZ1
delR
delRZ1
CCMATH

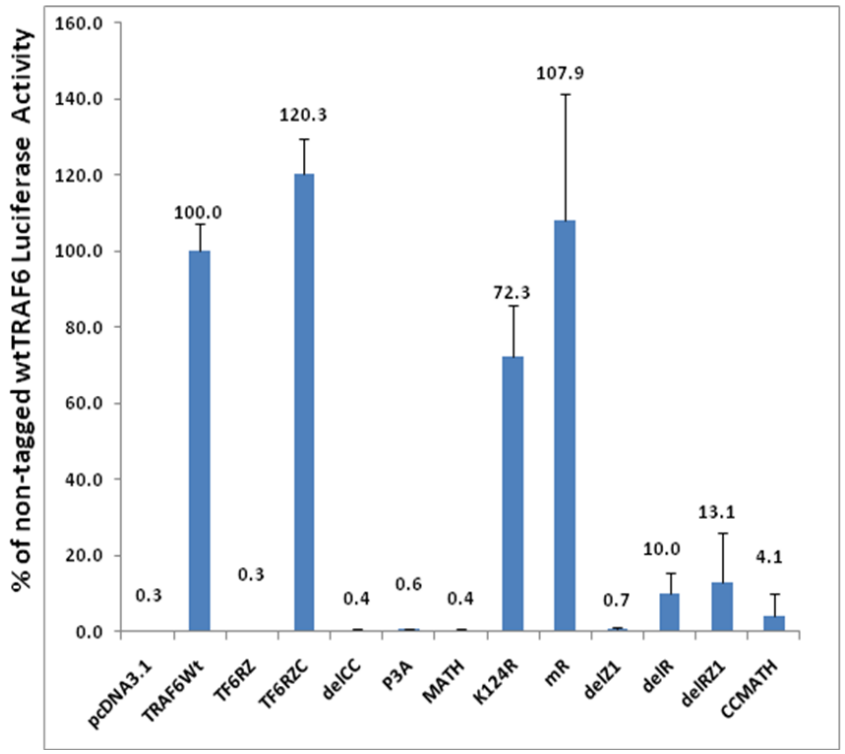

% of non-tagged wtTRAF6 Luciferase Activity
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
0.3
100.0
0.3
120.3
0.4
0.6
0.4
72.3
107.9
0.7
10.0
13.1
4.1
pcDNA3.1
TRAF6Wt
TF6RZ
TF6RZC
delCC
P3A
MATH
K124R
mR
delZ1
delR
delRZ1
CCMATH

Figure 27. NF-κB activation induced by TRAF6 and derived muteins measured in luciferase activity.

Expression plasmids (50 ng) coding for indicated proteins were individually co-transfected along with a luciferase reporter, NF-κB/pGL2, into one well of 293 cells in 24-plates. Luciferase activity was measured 24 hours after transfection and represented as percentage of wild-type TRAF6 activity. The activity of each expressed protein in each experiment was averaged from three independent wells. Results presented in the chart are the average luciferase activity of each protein from three independent experiments. (A). NF-κB activities of dual-tagged TRAF6 and each derived mutein. (B). NF-κB activities of un-tagged TRAF6 and each derived mutein.

In order to analyze NF-κB activation in individual cells that have ectopically expressed TRAF6 proteins, we engineered a live cell NF-κB activity reporter (HcRed1-κB) in which we replaced the luciferase cDNA with a coding sequence of HcRed1 linked to the SV40 antigen nuclear localization signal in the NF-κB luciferase reporter (NF-κB/pGL2) mentioned above (Fig. 10). This approach permits analysis of individual cell NF-κB activity to be correlated with sub-cellular localization of the tagged TRAF6 activators. Consistent with the luciferase results, when this HcRed1-κB reporter plasmid was co-transfected with individual plasmid coding for wtTRAF6 or a mutein, all ectopically expressed YFP tagged TRAF6 proteins that had the sequestosomal localization pattern display strong nuclear pseudo red fluorescence in transfected 293T cells, although a reporter positive cell does not necessary show sequestosomes (Fig. 28). This suggests a connection between ubiquitination and TRAF6 activation. However, sequestosomes are not essentially required for NF-κB activity. Therefore, we speculate that sequestosomes may result from aggregation of poly-ubiquitinated TRAF6 proteins when they accumulate to a certain extent. A similar pattern of NF-κB activation was also observed for un-tagged TRAF6 proteins expressed in 293T cells with the exception of TRAF6P3A mutein shown by the HcRed1-κB reporter (Fig. 28, 29).

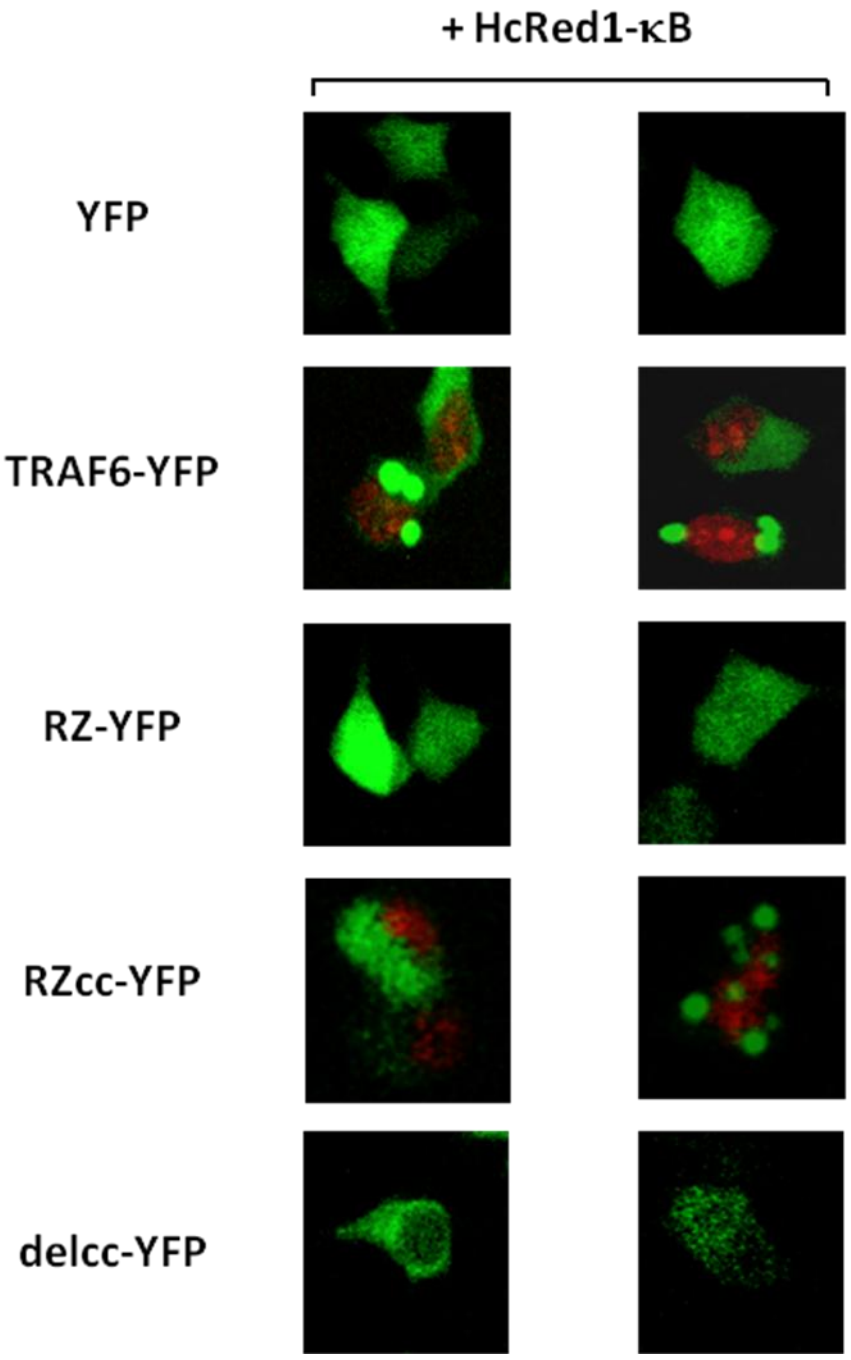


Figure 28A. NF-κB activity of YFP-tagged TRAF6 and derived muteins.

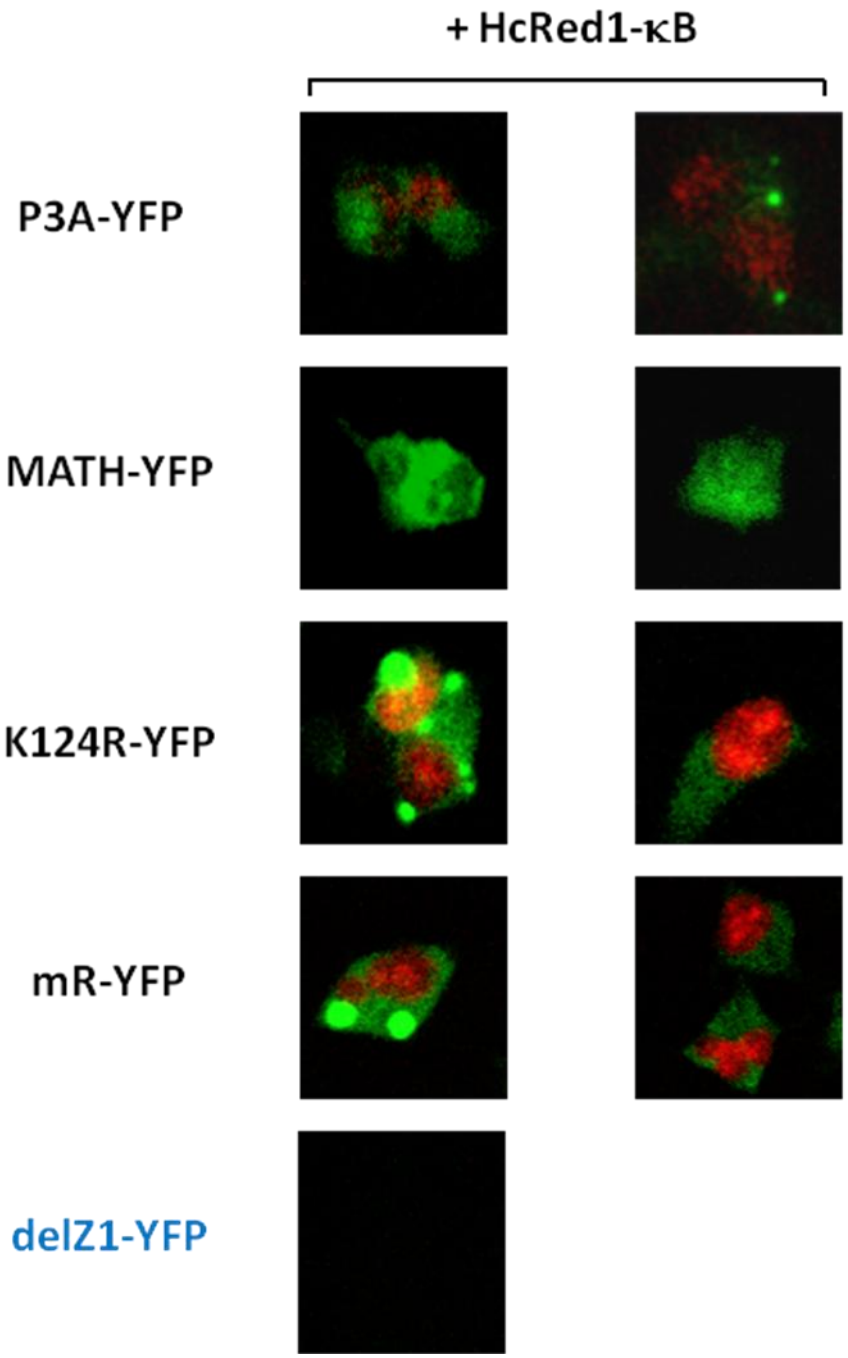


Figure 28B. NF-κB activity of YFP-tagged TRAF6 and derived muteins.

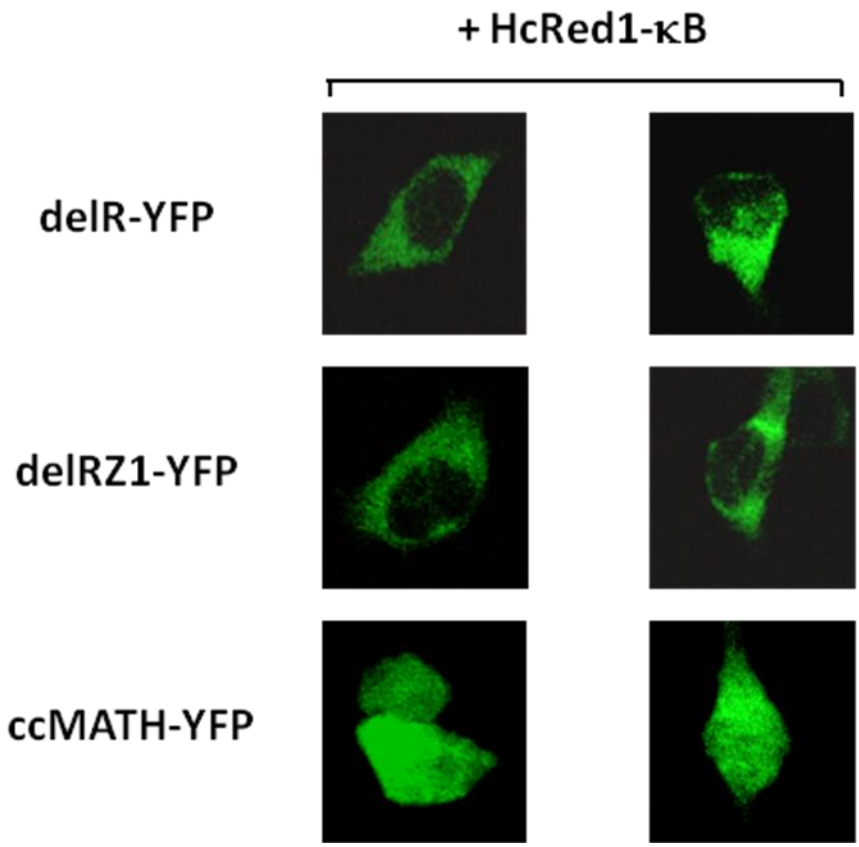


Figure 28C. NF-κB activity of YFP-tagged TRAF6 and derived muteins.

Expression plasmids (30 ng) coding for indicated YFP-tagged proteins were co-transfected with the NF-κB/pGL2 reporter (50 ng) into 293T cells in a 96-well plate. Confocal microscopic images were taken after 24 hours of transfection. The YFP signal is pseudo-colored green and the HcRed1 signal is pseudo-colored red.

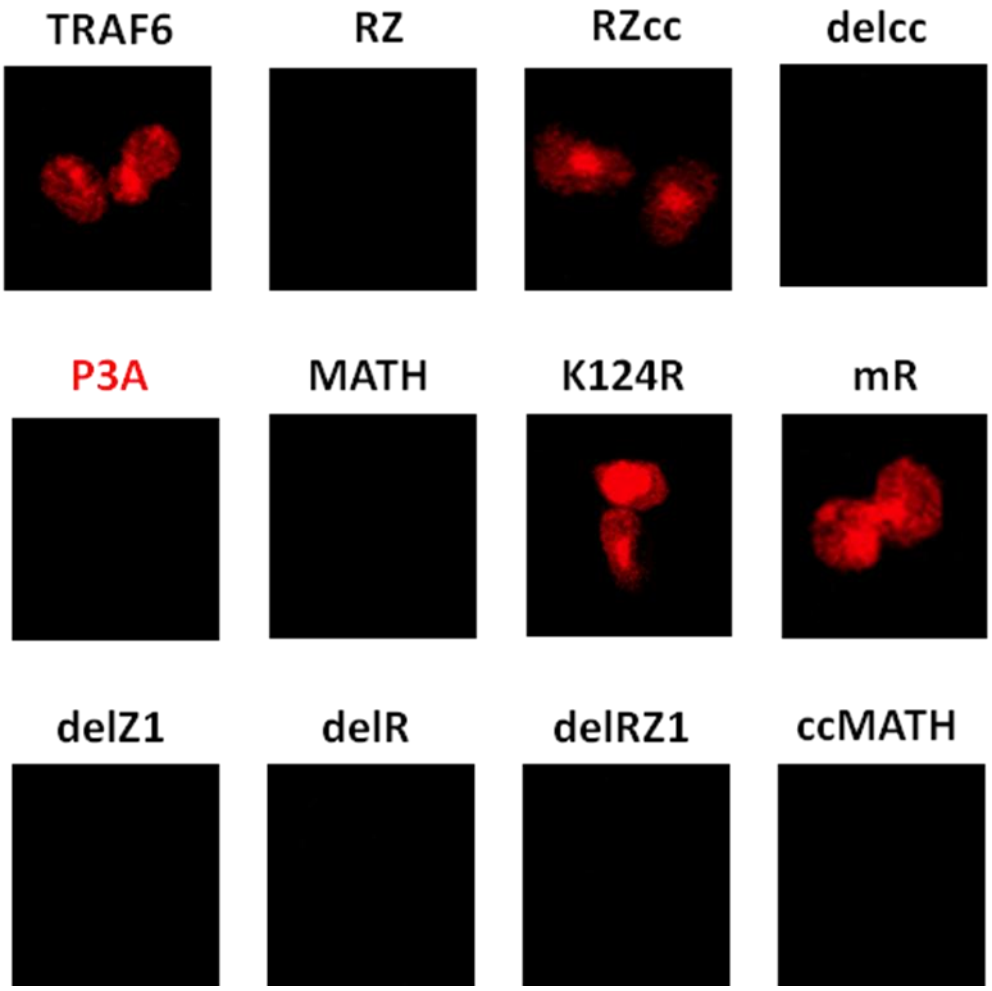


Figure 29. NF-κB activity of un-tagged TRAF6 and derived muteins.

Expression plasmids (30 ng) coding for indicated un-tagged proteins were co-transfected with the NF-κB/pGL2 reporter (50 ng) into 293T cells in a 96-well plate. Confocal microscopic images were taken after 24 hours of transfection. The HcRed1 signal is pseudo-colored red.

We reported that the un-tagged TRAF6P3A mutein was incapable of activating NF-κB and predicted that Pro462, which unlike the two other prolines is conserved in all TRAF-family members, should be buried near the MATH interdomain interaction surface. Consequently, the triple mutation could have compromised the structural integrity of the TRAF trimer, resulting in a nonspecific loss of interaction (Wang et al., 2006). In contrast, the TRAF6P3A-YFP protein displayed compromised but still significant NF-κB activity (Fig. 27A & 28). This suggests that the YFP-tagged TRAF6P3A may stabilize this mutein by helping to maintain its conformational integrity.

In contrast to an earlier report, the TRAF6K124R-YFP mutein is capable of activating NF-κB (Fig. 27-29). The mouse TRAF6K124R mutation was first reported to be defective in ubiquitination. This mutein was also shown to be incapable of regulating NF-κB activation and osteoclastogenesis when it was transduced into a TRAF6 null cell model (Lamothe et al., 2007). Therefore, these results argued that lysine 124 is a specific target for ubiquitination. Since TRAF6 ubiquitination has been disclosed to be a modification by non-degradable K63 linked poly-ubiquitin chains (Deng et al., 2000; Newton et al., 2008; Wang et al., 2001), it also hinted to the signal specificity for such modification. However, these arguments have recently been disputed by studies demonstrating that auto-ubiquitination is dispensable for NF-κB activation (Walsh et al., 2008). In their report, investigators mutated all lysine residues to arginines in the TRAF6 molecule. Intriguingly, this ΔK mutein displayed significantly reduction in ubiquitination, whereas it showed similar properties in TAK1 and NF-κB activation compared to wild-type TRAF6. Furthermore, they also presented contrary results from the TRAF6K124R mutant, in which found no significant reduction in ubiquitination and NF-κB activation.

In the meantime, we have also independently observed that the K124R mutation didn't show any significant compromise compared to wild-type TRAF6 in both ubiquitination and NF-κB activation, and the sub-cellular distributions of the K124R-YFP mutein were similar to that of TRAF6-YFP.

As shown in our results, the TRAF6mR mutein, encoding two substitutions, I98L and D100G, unexpectedly generated wild-type cellular localization along with moderate ubiquitination and NF-κB activities (Fig. 20, 26-29). 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 conjugating enzyme.

In agreement with earlier reports (Wang et al., 2001; Yang et al., 2004), the TRAF6RZcc mutein, with the MATH domain truncation, shows significant NF-κB activity (Fig. 28 & 29). It suggests that the coiled-coil domain is required for TRAF6 activation of NF-κB.

Consistent with the demonstration of the requirement of the coiled-coil domain for TRAF6 activity, both TRAF6RZ and TRAF6Δcc muteins are incapable of ubiquitination and NF-κB activation. Therefore, we confirmed that the coiled-coil domain is important not only for multimerization but also possibly for recruitment of E2 ubiquitin conjugating enzyme, Ubc13/Uev1 by different means (Park et al., 1999; Yang et al., 2004).

The integrity of the RING domain and first Zinc finger of TRAF6 has been shown to be critical for its E3 ubiquitin ligase activity (Lamothe et al., 2008; Yin et al., 2009).

Our results from the TRAF6ΔR and TRAF6ΔRZ1 muteins also demonstrated the importance of these domains (Fig. 26-29).

The dominant-negative effect of ccMATH mutein has been reported in many studies of IL-1/LPS signaling and was described as a competitor for TRAF recruitment by upstream activators (Darnay et al., 1999; Rothe et al., 1995; Ye et al., 2002). Consistent with our previous studies (Yoshida et al., 2004), the TRAF6MATH and ccMATH are inactive for NF-κB activation (Fig. 27-29). Intriguingly, both muteins can significantly inhibit TRAF6 activation of NF-κB when each of them co-expresses wtTRAF6 protein which suggests distinct regulatory mechanisms from upstream activators (more detail will be discussed later).

TRAF6 Ubiquitination is regulated in *trans* through intermolecular interaction

Auto-ubiquitination of TRAF E3 ligases has been proposed to play essential roles for activation of the NF-κB pathway (Chen and Sun, 2009; O'Neill, 2009), but mechanisms for regulating this process are still largely unknown. It has been proposed that many TRAF family members are pre-assembled into multimers (Park et al., 1999; Ye et al., 2002; Ye et al., 1999). Since our data acquired from correlation of ubiquitination and NF-κB activity of different types of TRAF6 muteins has demonstrated that both the RING-Zinc finger and coiled-coil domains are required for ubiquitination and activity, it triggered us to assess whether poly-ubiquitination of TRAF molecules can be regulated inter-molecularly.

To test this hypothesis, we co-expressed un-tagged wild type TRAF6 protein together with each of six YFP-FLAG dual-tagged TRAF6 muteins that are inactive in

auto-ubiquitination and NF-κB activation in 293T cells. In agreement with our hypothesis, sequestosomes appeared in transfected cells co-expressing the TRAF6ΔR-YFP, TRAF6ΔRZ1-YFP, or TRAF6Δcc-YFP proteins which only display cytoplasmic speckles when they are expressed alone in 293T cells (Fig. 30).

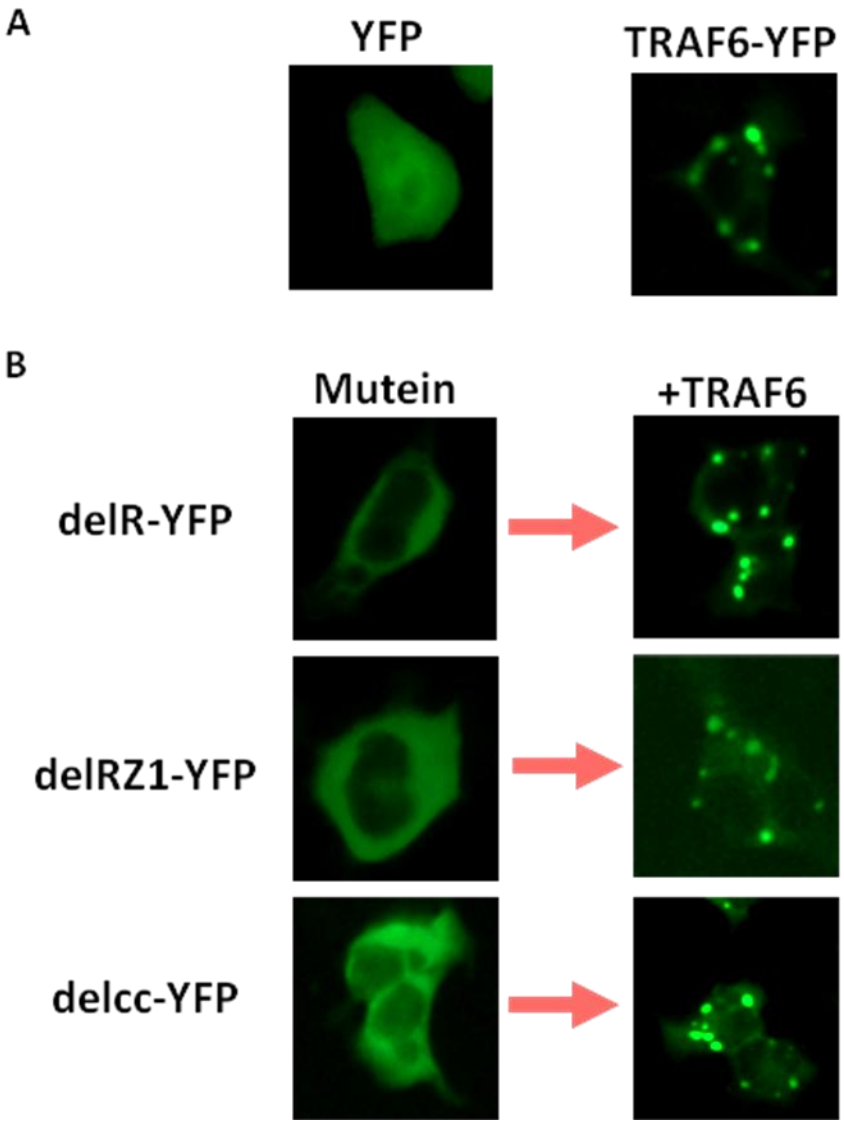


Figure 30. wtTRAF6 rescues sequestosomal localization of ubiquitination deficient muteins.

A. Ectopic expression of YFP or TRAF6-YFP in 293T cells as controls. B. An indicated expression plasmid (1 μg) encoding YFP-FLAG dual-tagged TRAF6 or a derived mutein was transfected either alone or together with the TRAF6pcDNA3.1 plasmid into each well containing 293T cells in 6-well tissue culture plates. Microscopic images were taken after 24 hours of transfection. The YFP signal is pseudo-colored green.

Since a correlation between sequestosomal localization and poly-ubiquitination has been reported, we further analyzed ubiquitination states of the TRAF6 muteins in transfected cells. Cell lysates containing each pair of co-expressed un-tagged wtTRAF6 protein and a YFP-FLAG dual-tagged TRAF6 mutein were immuno-precipitated with anti-FLAG antibody. Each group of immuno-precipitated proteins was then resolved by SDS-PAGE, transferred onto PVDF membranes and immuno-blotted with either anti-ubiquitin or anti-GFP antibody. Fig. 31A shows that all three muteins were poly-ubiquitinated. The lowest molecular size of each heterogeneous protein smear matches the molecular size of the corresponding mutein, TRAF6ΔR-YFP (76 KDa), TRAF6ΔRZ1-YFP (71 KDa), and TRAF6Δcc-YFP (81 KDa), shown in Figure 31B. More interestingly, the over-expressed un-tagged wtTRAF6 (61 KDa) is a dominant positive activator, and it should be robustly poly-ubiquitinated as shown in Figure 26, but there was either little or no detectable signal displayed between 60 KDa and the lowest molecular size of each dual-tagged mutein in all lanes showing ubiquitinated proteins (Fig. 31A). This result suggests that the poly-ubiquitin chains were attached to the indicated muteins. It also argues that ubiquitin modification may disrupt physical interaction between these proteins. Moreover, the coiled-coil domain of TRAF6 has been suggested to be involved in trimerization. The ectopically co-expressed TRAF6 and muteins in the same cell should form hetero-trimers in different combinations. However, the results shown in Figure 31A suggest that these pre-formed hertero-trimers have been disassociated upon ubiquitination.

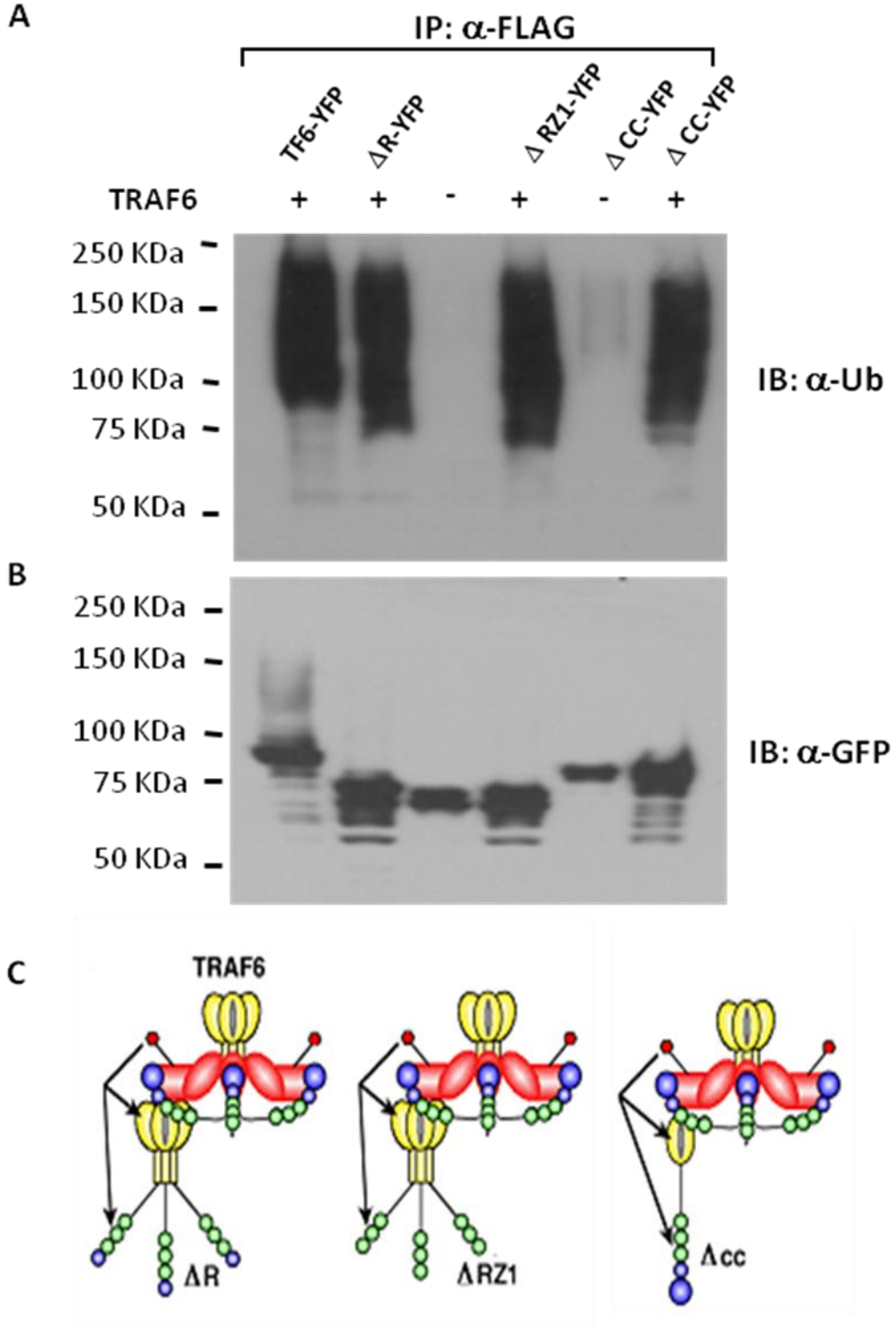

A
IP: α-FLAG
TF6-YFP
ΔR-YFP
Δ RZ1-YFP
Δ CC-YFP
Δ CC-YFP
TRAF6
+ + - + - +
250 KDa
150 KDa
100 KDa
75 KDa
50 KDa
IB: α-Ub
B
250 KDa
150 KDa
100 KDa
75 KDa
50 KDa
IB: α-GFP
C
TRAF6
ΔR
ΔRZ1
Δcc

Figure 31. *Trans*-ubiquitination of TRAF6 muteins regulated by wtTRAF6

Expression plasmids (1 μg) encoding YFP-FLAG dual-tagged TRAF6 or a derived mutein was transfected either alone or together with the TRAF6pcDNA3.1 plasmid (1 μg) into 293T cells in 6-well tissue culture plates. Cells were lysed with IP buffer 24 hours post-transfection. Supernatant containing indicated FLAG tagged proteins were immuno-precipitated with anti-FLAG agarose beads for 4 hours at 4^{o}C. Absorbed proteins were eluted and denatured by 1X SDS-PAGE buffer and 2-mercaptoethanol at 95^{o}C for 5 minutes. Eluted proteins were then resolved in 4-12% gradient SDS-PAGE and transferred onto a PVDF membrane and immuno-blotted with indicated antibodies. (A). Immuno-blotting with the anti-ubiquitin antibody; (B). Immuno-blotting with the anti-GFP antibody; (C). Cartoon demonstrations of *trans*-ubiquitination of indicated muteins regulated by TRAF6.

Unexpectedly, when wtTRAF6 was co-expressed with each of the dual-tagged muteins (TRAF6RZ-YFP, TRAF6ccMATH-YFP, or TRAF6MATH-YFP), the YFP signal was aggregated into large punctuate spots from the original diffuse localization patterns. A significant amount of cells with these punctuate spots co-localized with a constitutively expressed nuclear localizing marker (HcRed-Nuc), which suggests that these muteins may have been modified and translocated into nuclei (Fig. 32).

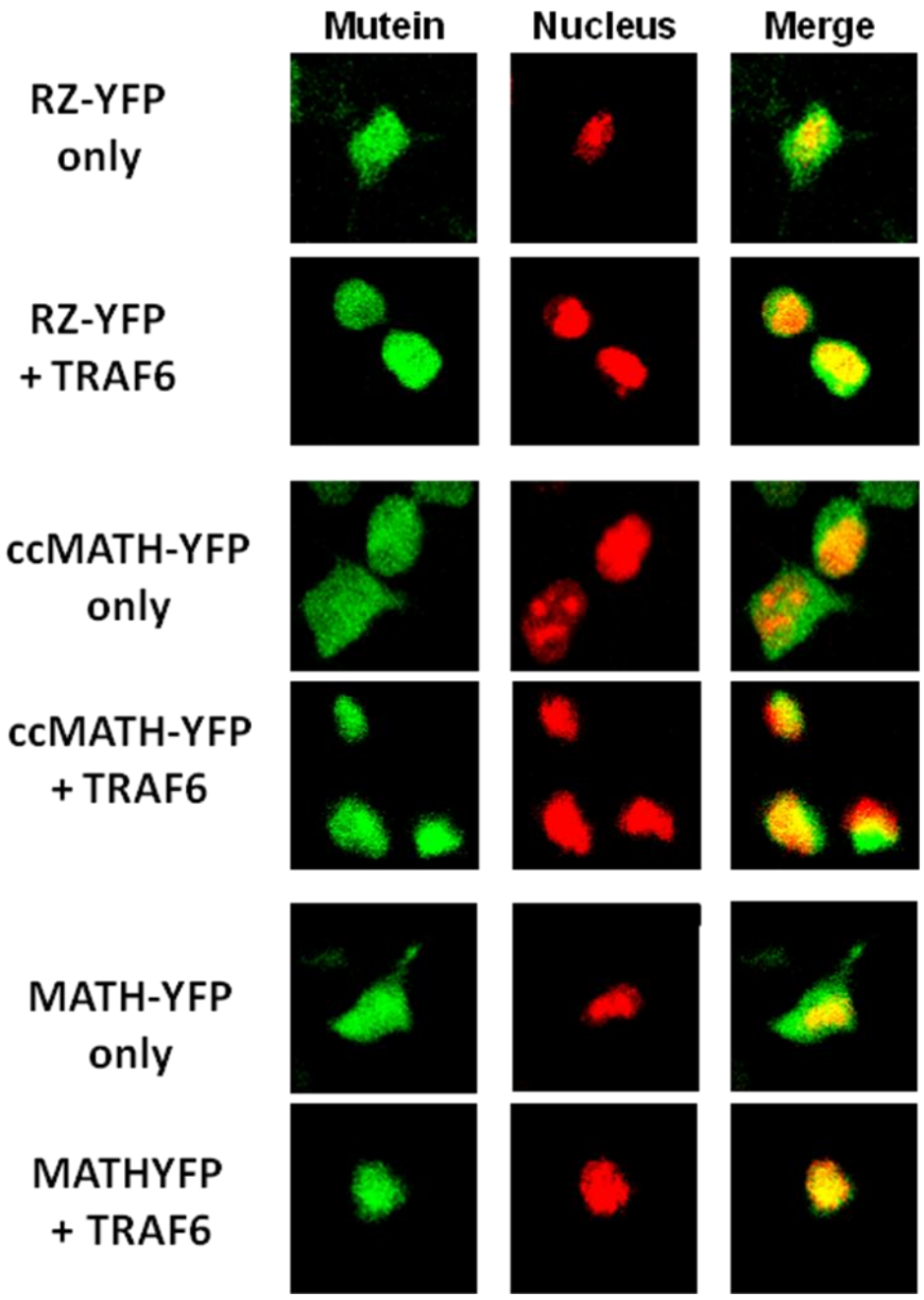


Figure 32. wtTRAF6 induced nuclear translocation of ubiquitination deficient muteins.

Expression plasmid (1 μg) encoding YFP-FLAG dual-tagged TRAF6 muteins was transfected either alone or together with TRAF6pcDNA3.1 plasmid into 293T cells in 6-well tissue culture plates. A nuclear localizing marker (HcRed1-Nuc) was co-expressed in order to label nuclei. Confocal microscopic images were taken after 24 hours of transfection. The YFP signal is pseudo-colored green and HcRed1 signal is pseudo-colored red.

Since we were focusing on the state of ubiquitin modification, we employed similar procedures as those used for analysis of TRAF6ΔR-YFP, TRAF6ΔRZ1-YFP, and TRAF6Δcc-YFP proteins. The left panel in Figure 33 shows poly-ubiquitination of all dual-tagged TRAF6RZ-YFP, TRAF6ccMATH-YFP, and TRAF6MATH-YFP muteins, which were co-expressed with wtTRAF6. Molecular sizes of these tagged muteins shown by immuno-blotting of anti-GFP antibody also confirmed target specificity of poly-ubiquitination (Fig. 33 right panel). These results argue that the poly-ubiquitin modifications may induce protein aggregation, but ubiquitinated proteins may not necessarily display sequestosomes. Furthermore, we have also demonstrated here that poly-ubiquitination of TRAF6 can be an inter-molecular process. In other words, TRAF6 "auto-ubiquitination" occurs in *trans*.

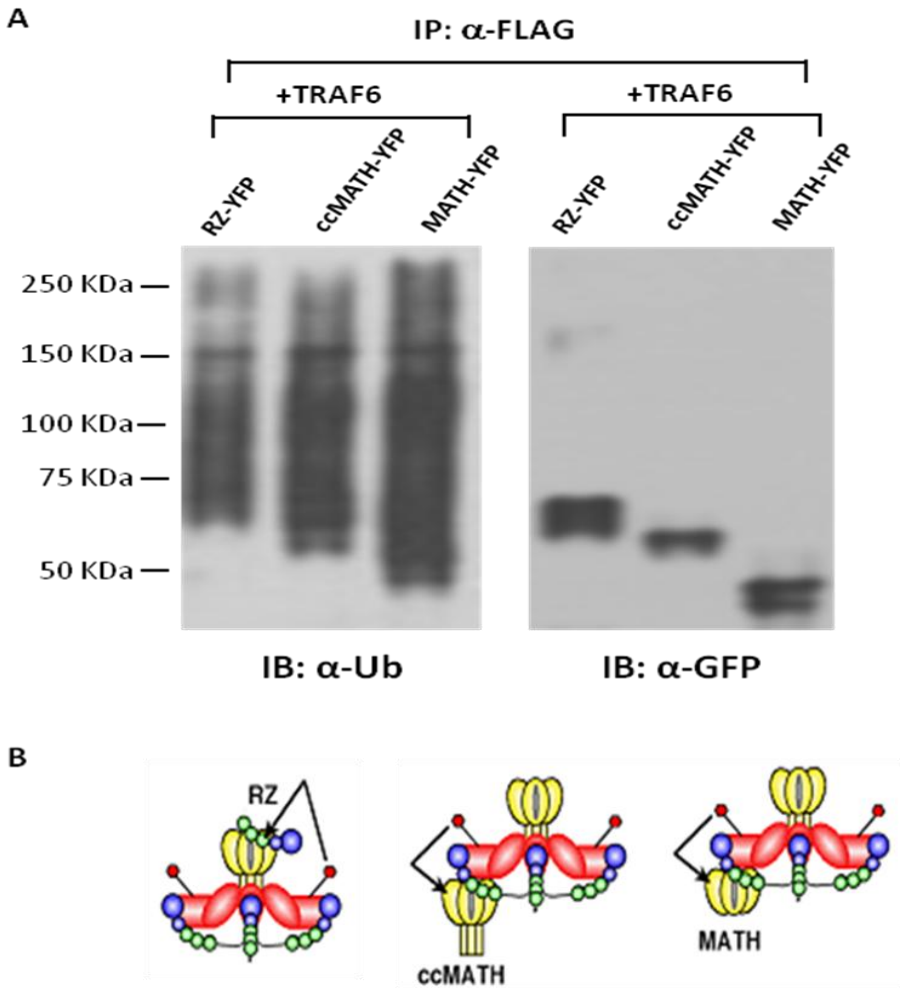


Figure 33.*Trans*-ubiquitination of TRAF6 muteins mediated by wtTRAF6

(A). Expression plasmids (1 μg) encoding YFP-FLAG dual-tagged TRAF6 or a derived mutein was transfected either alone or together with the TRAF6pcDNA3.1 plasmid (1 μg) into 293T cells in 6-well tissue culture plates. Cells were lysed with IP buffer a 24 hours post transfection. Each supernatant containing an indicated FLAG tagged protein was immuno-precipitated with anti-FLAG agarose beads for 4 hours at 4^{o}C. Absorbed proteins were eluted and denatured by 1X SDS-PAGE buffer and 2-mercaptoethanol at 95^{o}C for 5 minutes. Eluted proteins were then resolved in 4-12% gradient SDS-PAGE, transferred onto a PVDF membrane and immuno-blotted with anti-ubiquitin (Left panel) or anti-GFP (Left panel) antibodies; (B). Demonstration of likely interactions responsible for *trans*-ubiquitination presented in (A).

To further explore the regulatory 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. Microscopic analyses revealed that similar large punctate aggregation or sequestosomal fluorescent signals only occurred when one mutein contains the RING-Zinc finger domain and the other the coiled-coil (Fig. 34). As expected, *trans*-ubiquitination was also confirmed in correlative combinations (Fig. 35). This supports a mechanism by which the RING-Zinc fingers collaborate with the coiled-coil domain in the recruitment of the TRAF-specific ubiquitin E2 conjugating enzyme, Ubc13/Uev1A. Since many TRAF family proteins including TRAF6 are thought to be trimerized through their coiled-coil domains (Park et al., 1999; Ye et al., 2002) and each TRAF6 molecule contains a RING-Zinc fingers and a coiled-coil domain, *trans*-ubiquitination may take place between different TRAF6 molecules within a trimer (intra-trimer). However, it may also be accomplished *via* different trimers (inter-trimer).

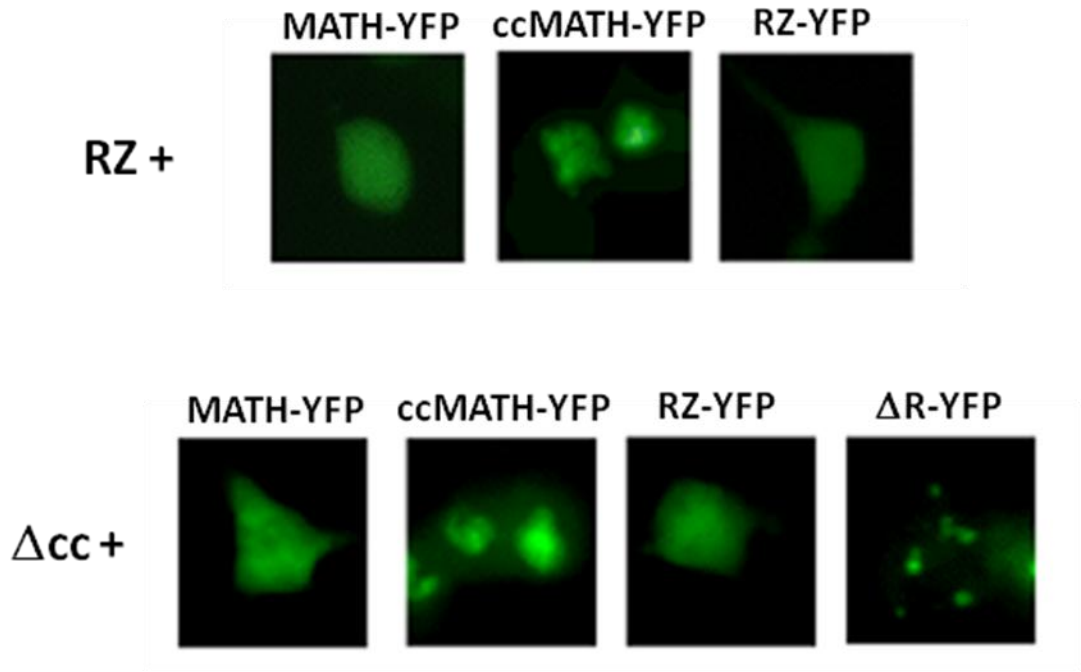
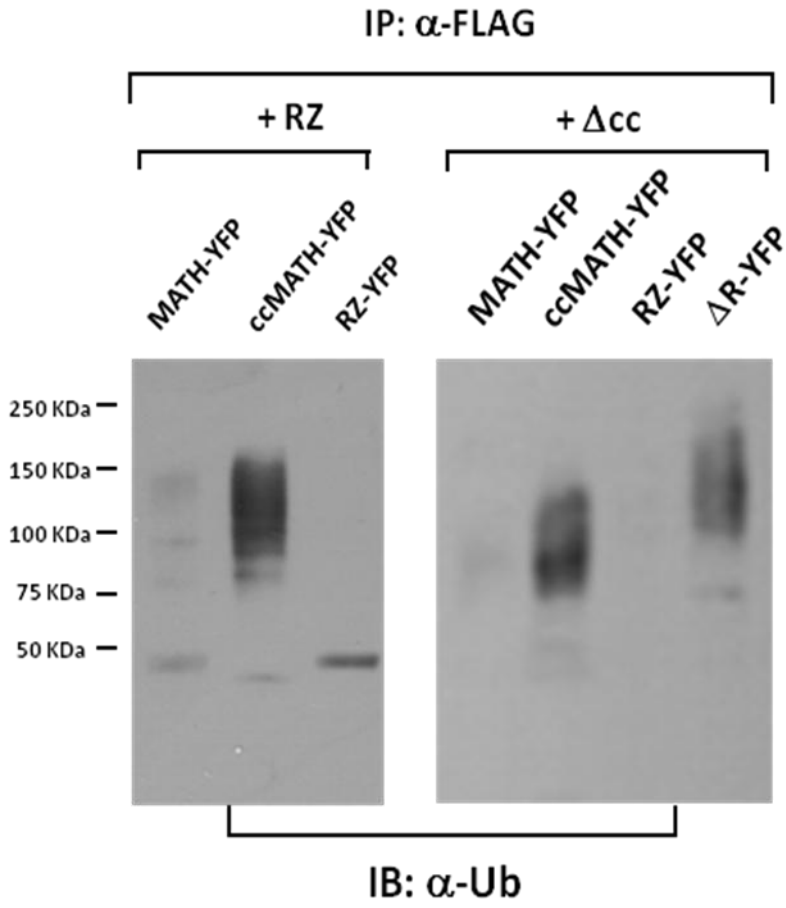


Figure 34. Sub-cellular localization change of TRAF6 muteins induced by *trans*-ubiquitination

Expression plasmid (1 μg) coding for un-tagged RZ or Δcc mutein was co-transfected with a plasmid (1 μg) encoding an indicated YFP-FLAG dual-tagged mutein into 293T cells in 6-well tissue culture plates. Microscopic images were taken after 24 hours of transfection. The YFP signal is pseudo-colored green.

A


IP: α-FLAG
+ RZ
+ Δcc
MATH-YFP
ccMATH-YFP
RZ-YFP
MATH-YFP
ccMATH-YFP
RZ-YFP
ΔR-YFP
250 KDa
150 KDa
100 KDa
75 KDa
50 KDa
IB: α-Ub

B

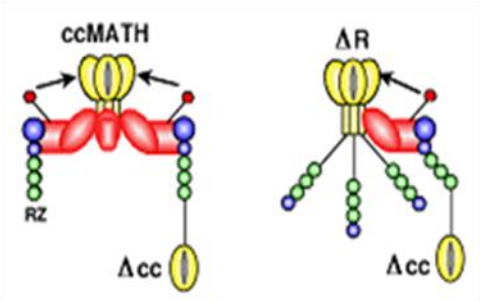

ccMATH
ΔR
RZ
Δcc
Δcc

Figure 35.*Trans*-ubiquitination requires the RING domain from one protein and the coiled-coil from the other.

(A). After taking microscopic images, transfected cells from Fig. 34 were lysed with IP buffer. Each supernatant containing an indicated FLAG tagged protein was immuno-precipitated with anti-FLAG conjugated agarose beads for 4 hours at 4^{o}C. Absorbed proteins were eluted and denatured by 1X SDS-PAGE buffer with 2-mercaptoethanol at 95^{o}C for 5 minutes. Eluted proteins were then resolved in 4-12% gradient SDS-PAGE, transferred onto a PVDF membrane and immuno-blotted with anti-ubiquitin antibody. (B). Demonstration of likely interactions responsible for *trans*-ubiquitination presented in A.

Disconnection between *trans*-ubiquitination and TRAF6 activation of NF-κB

We have shown a correlation between poly-ubiquitination and NF-κB activation in a collection of TRAF6 and derived muteins (Fig. 25-29), although each individual cell showing NF-κB activity may not necessarily display sequestosome structures (Fig. 28). It is of interest to see whether *trans*-ubiquitination can rescue the activity of some inactive muteins. Interestingly, when each of the five FLAG-YFP dual-tagged muteins (ΔR-, Δcc-, RZ-, ccMATH-, and MATH-YFP) demonstrating substantial loss of sequestosomes, ubiquitination, and NF-κB activity (Fig. 25-29) were co-expressed with either wtTRAF6 or an inactive mutein that could mediate *trans*-ubiquitination, a disconnection between ubiquitination and NF-κB activity was observed (Fig. 36). Our results showed that none of the combinations of two inactive muteins (RZ+ccMATH; Δcc+RZ; Δcc+ccMATH; Δcc+ΔR) that are capable of *trans*-ubiquitination produced any significant NF-κB activity. Furthermore, rather than generating additional activity, co-expression of ΔR- Δcc-, or RZ-YFP with wtTRAF6-YFP showed slight inhibition compared to NF-κB activity of wtTRAF6-YFP alone. Strikingly, wtTRAF6-YFP activation of NF-κB was significantly inhibited in the presence of MATH- and ccMATH-YFP (wtTRAF6 + MATH and wtTRAF6 + ccMATH) (Fig. 36). This suggests an inhibitory effect specific for the MATH domain. The dominant-negative effect of ccMATH has been reported in many studies of IL-1/LPS signaling and was previously described as a competitor for TRAF6 recruitment by upstream activators (Darnay et al., 1999; Rothe et al., 1995; Wong et al., 1998). However, our study was independent of

upstream activators and suggests that a distinct mechanism is involved. Therefore, we hypothesized the existence of a potential molecular interaction between the MATH domain and a critical functional domain(s) of TRAF6, which may generate an inhibitory effect.

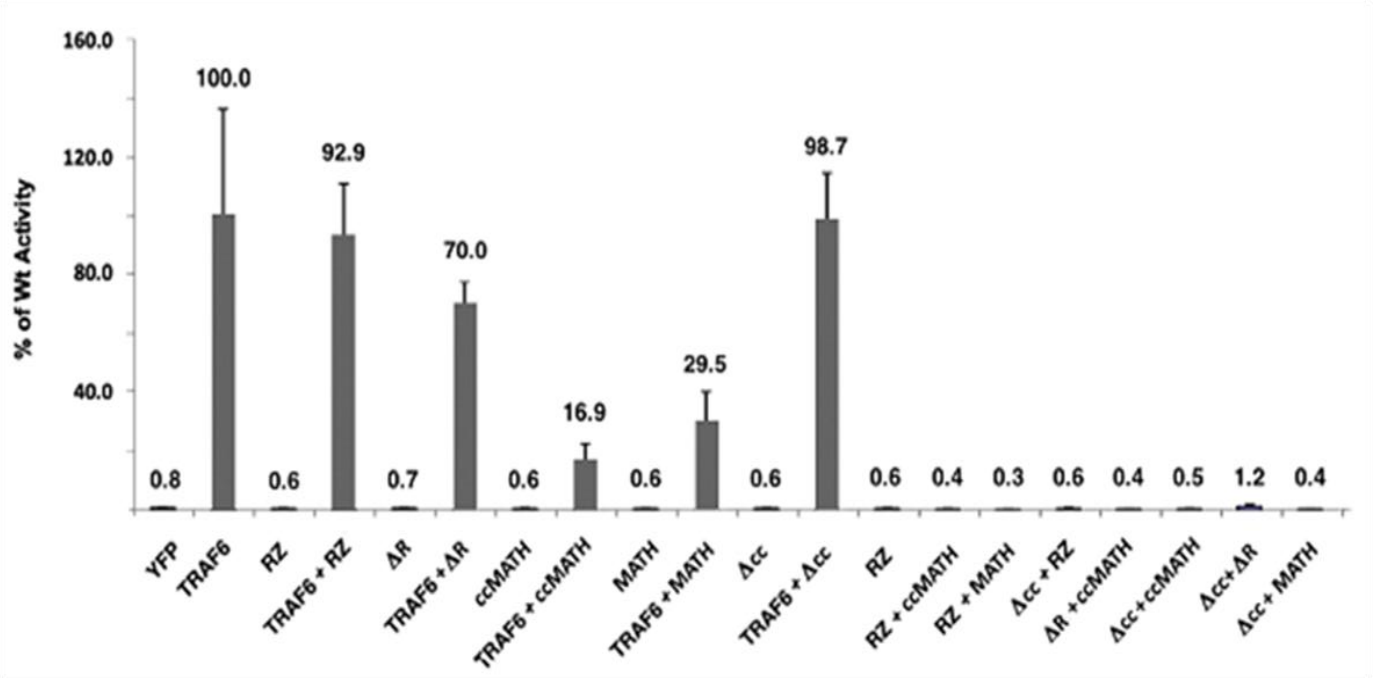


Figure 36. *Trans*-ubiquitination is insufficient for NF-κB activation.

Indicated expression plasmids (50 ng, each) together with 60 ng of NF-κB/pGL2 were co-transfected into 293 cells pre-seeded in 24-well tissue culture plates. Additional 50 ng of pFLAG CMV 5a-YFP plasmid was added to each group with a single plasmid indicated in the chart to make total amount of plasmids in each well to be 160 ng. Luciferase assay was performed after 24 hrs of transfection. Results were average activity of transfected cells from three individual wells.

Physical interaction exists between the N- and C-termini of TRAF6

In order to further pursue the disconnection between *trans*-ubiquitination and NF-κB activation, we individually co-expressed un-tagged wtTRAF6 with three YFP-FLAG dual-tagged proteins, TRAF6-YFP, ccMATH-YFP, and MATH-YFP in 293T cells. As expected, all three of the dual-tagged proteins co-immunoprecipitated with un-tagged wtTRAF6 (Fig. 37).

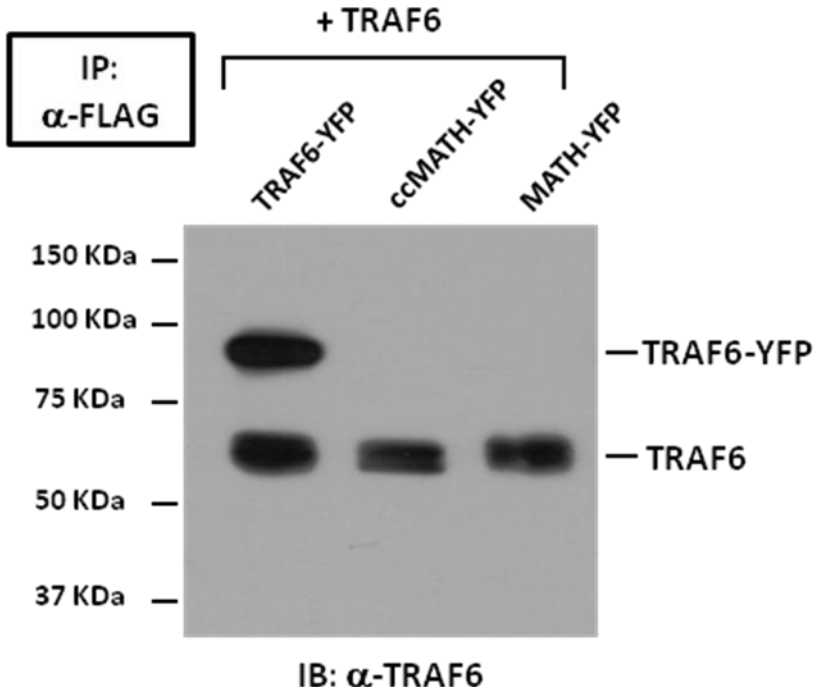


Figure 37. Physical interaction of intact TRAF6 molecule with its MATH domain.

The TRAF6pcDNA3.1 plasmid was co-transfected with an indicated plasmid encoding a YFP-FLAG tagged protein into 293T cells. After 24 hrs of transfection, each cell lysate was immuno-precipitated with anti-FLAG conjugated agarose beads for 4 hours at 4^{o}C. Absorbed proteins were eluted and denatured by 1X SDS-PAGE buffer with 2-mercaptoethanol at 95^{o}C for 5 minutes. Eluted proteins were then resolved in 4-12% gradient SDS-PAGE, transferred onto a PVDF membrane and immuno-blotted with anti-TRAF6 antibody.

This confirms a physical interaction between the MATH domain and the TRAF6 molecule. Since the RING-Zinc fingers of TRAF6 has been implicated in activation of downstream pathways (Baud et al., 1999a), so we speculated that the RING-Zinc domain may be targeted by the MATH domain. To test this hypothesis, the non-tagged RING-Zinc fingers mutein was ectopically expressed individually with each of several YFP-FLAG dual-tagged proteins. The FLAG-tagged proteins were immuno-precipitated with anti-FLAG conjugated agarose beads, followed by immuno-blotting with a TRAF6 anti-RZ antibody. The results revealed that untagged ectopically-expressed RZ mutein was co-immunoprecipitated with these FLAG-tagged proteins with an efficiency that decreased in the order TRAF6-YFP>ccMATH-YFP>MATH-YFP>RZ-YFP (Fig. 38). This implicates an intra-molecular interaction between the RING-Zinc and MATH domains, which may keep TRAF6 in an inactive "closed" conformation. It also argues that co-expression of either MATH or ccMATH with TRAF6 protein will interfere with TRAF6 downstream signaling (Fig. 36) by interacting with and blocking the required RZ domains, as would happen *via* intra-molecular interaction within the intact TRAF6 molecule before activation.

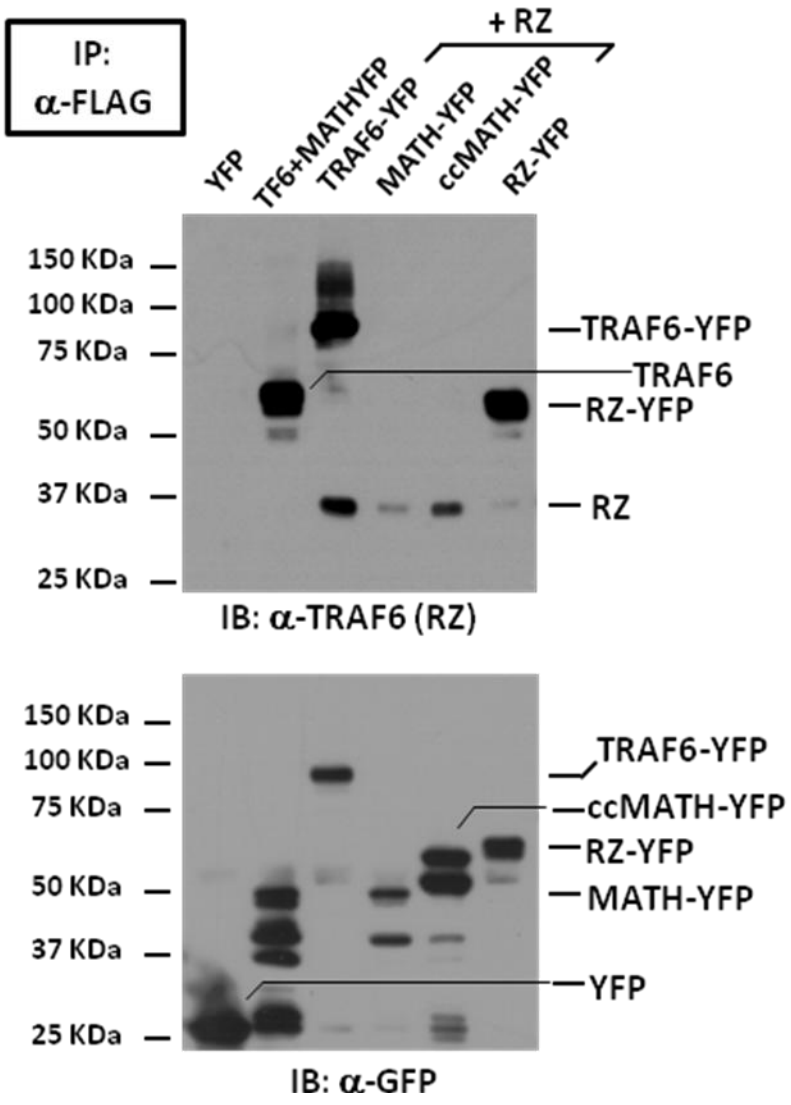


Fig. 38. Physical interaction of TRAF6 functional domains.

Indicated plasmids were transfected into 293T cells. After 24 hrs of transfection, the FLAG-tagged proteins from each cell lysate were immuno-precipitated with anti-FLAG conjugated agarose beads for 4 hours at 4^{o}C, eluted and denatured by 1X SDS-PAGE buffer with 2-mercaptoethanol at 95^{o}C for 5 minutes. Eluted proteins were then resolved in 4-12% gradient SDS-PAGE, transferred onto a PVDF membrane and immuno-blotted with either anti-TRAF6 or anti-GFP antibody.

Ubiquitination activates TRAF6 *via* sustaining its "open" conformation

The correlation of ubiquitination and NF-κB activation in TRAF6 and derived muteins (Fig. 26-29) prompted us to look into the role of ubiquitination in activation of TRAF6. We have shown that wild-type TRAF6 can *trans*-ubiquitinate derived muteins. However, these muteins don't effectively interact with each other after ubiquitination (Fig 31). On the other hand, we also provided evidence that these muteins bind with wtTRAF6 before ubiquitination (Fig. 37 & 38). Taking advantage of such hints, we hypothesized that ubiquitination may interfere with intra-molecular interaction of the RING-Zinc fingers with MATH domains, leaving TRAF6 sustained in an active "open" conformation from its "closed" state. To test this hypothesis, we transfected expression plasmids coding for non-tagged wtTRAF6 and the YFP-FLAG dual-tagged MATH mutein into 293T cells. Twenty-four hours after transfection, cells expressing ectopic TRAF6 proteins were lysed and separated into two aliquots. One aliquot was co-immunoprecipitated with anti-TRAF6 and the other with anti-FLAG antibody. Figure 39 (lanes 1 & 2) shows that anti-TRAF6 and -FLAG antibodies each pulled down a distinct population of poly-ubiquitinated 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 39, lane 3). The absence of the poly-ubiquitinated dual-tagged MATH domain (50 KDa) in the anti-TRAF6 pull-down (Fig. 39, lane 1) suggests that poly-ubiquitinated TRAF6 and the MATH domain don't interact efficiently. Since both MATH and RING-Zinc domains are targeted for ubiquitination (Fig. 33), this implicates TRAF6 poly-ubiquitination in disrupting intra-molecular interaction between the MATH and RING-Zinc domains. Therefore, poly-ubiquitination may help to sustain TRAF6 in an active "open"

conformation.

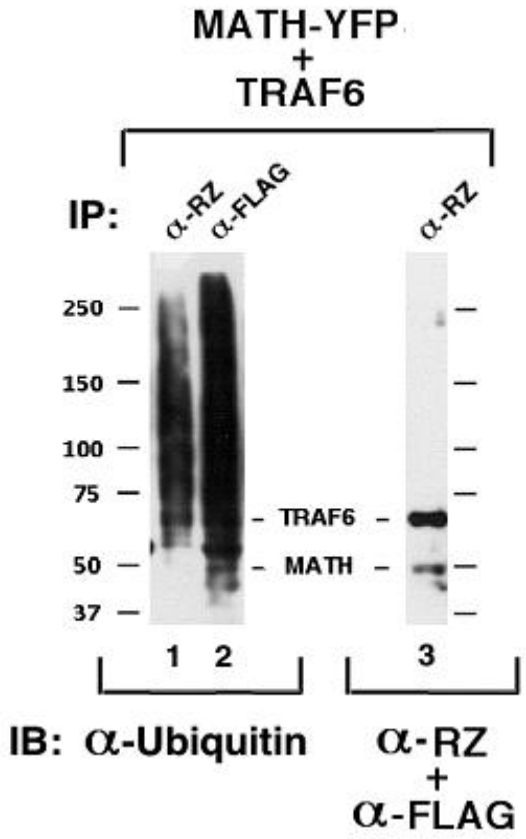


Figure 39. Ubiquitination induced interaction interruption between the RZ and MATH domains.

Expression plasmids coding for MATH-YFP-FLAG and wtTRAF6 proteins were co-transfected into 293T cells. After 24 hrs of transfection, transfected cells were lysed and aliquoted into two equal fractions. One aliquot of cell lysate was incubated with anti-FLAG conjugated agarose, and the other was with anti-TRAF6 and protein A/G agarose for 4 hours with rocking at 4^{o}C. Pull-down proteins were analyzed by Western blot with indicated antibodies.

The "double-edged sword" effects of ubiquitination in TRAF6 activation

We next evaluated whether there is any correlation between ubiquitination levels and NF-κB activity in cells over-expressing TRAF6. 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. This is followed by a dramatic decrease in activity, while sequestosomes continued to significantly increase in size (Fig. 40-41). The decreased NF-κB activity in the presence of super-sized sequestosomes did not appear to be associated either with a generalized mechanism of protein over-expression or cell death, because expression of high levels of either YFP or RZ-YFP did not result in sequestosome formation, a decrease in cell number, or obvious changes in cell morphology (Fig. 42). Since sequestosomes have been characterized as sites of high ubiquitination and proteasome localization, we treated TRAF6-YFP ectopically expressed 293 cells with a specific proteasome inhibitor, MG132. Larger TRAF6-YFP aggregates were displayed at 5 hours of post treatment compared to that in pre-treated cells at the same field (Fig. 43). This observation implies that the proteasome plays a critical role in regulating TRAF6-dependent NF-κB activity. This is consistent with reports that TRAF6 is recycled by deubiquitination (Jensen and Whitehead, 2003; Trompouki et al., 2003), which may occur in the sequestosome. We conjecture that over-expression of TRAF6 results in an increase in ubiquitination and 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.

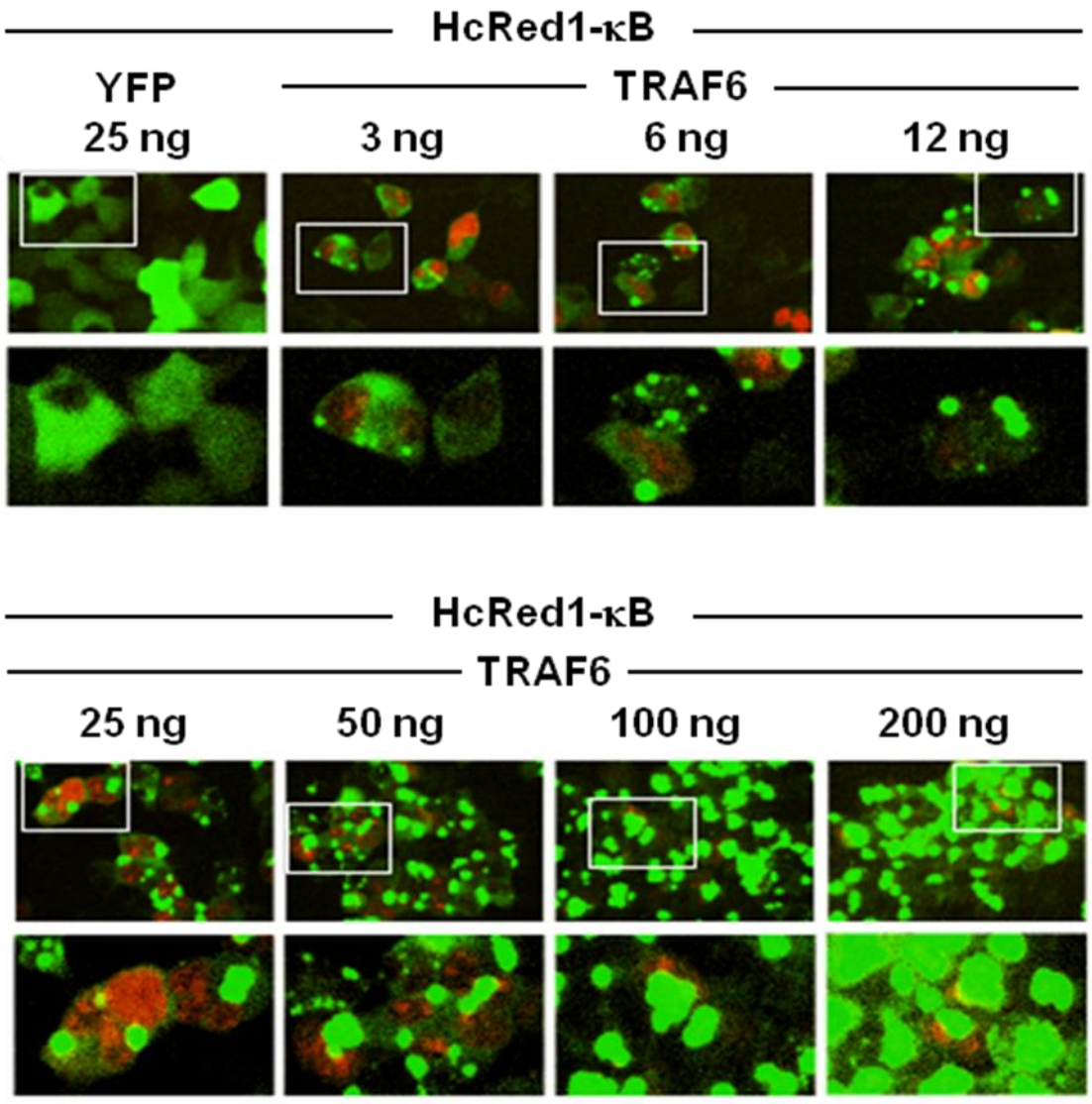


Figure 40. Sequestosome formation and NF-κB activation.

Indicated amounts of a TRAF6-YFP expression plasmid was transfected along with a consistent amount (50 ng) of HcRed1-κB reporter into 293T cells in a 96-well tissue culture plate. Micrographs were taken after 24 hours of transfection. The YFP signal was pseudo-colored green and Hcred1 signal was pseudo-colored red. The upper row shows a field of cells in which a boxed area is magnified in the lower row.

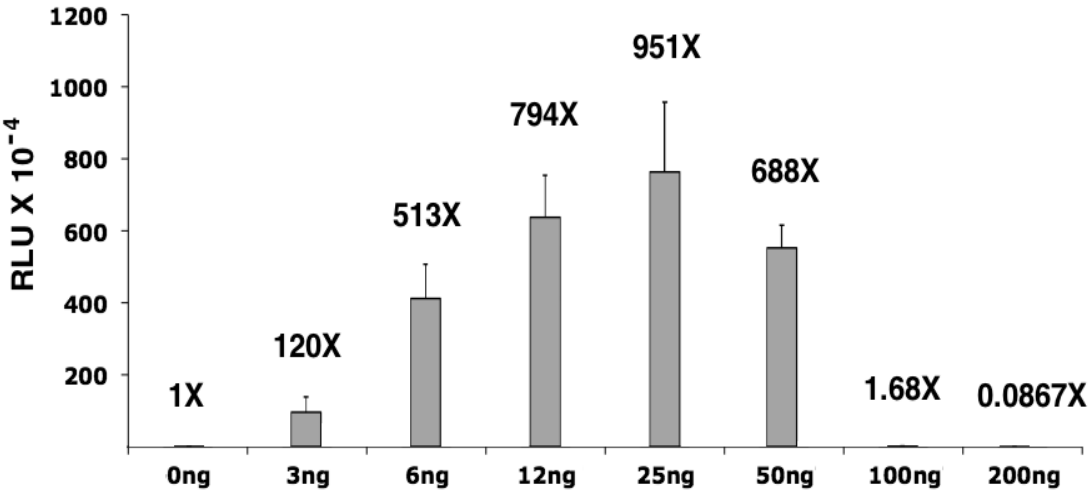


Figure 41. NF-κB activity affected by expression levels of TRAF6.

Indicated amounts of a TRAF6-YFP expression plasmid was transfected along with a consistent amount (20 ng) of NF-κB/pGL2 luciferase reporter into 293T cells in a 96-well tissue culture plate. Luciferase activities from each group of transfected cells were analyzed after 24 hours of transfection. Activity of each bar shown in the chart was the average activity of three independent wells in the same experiment.

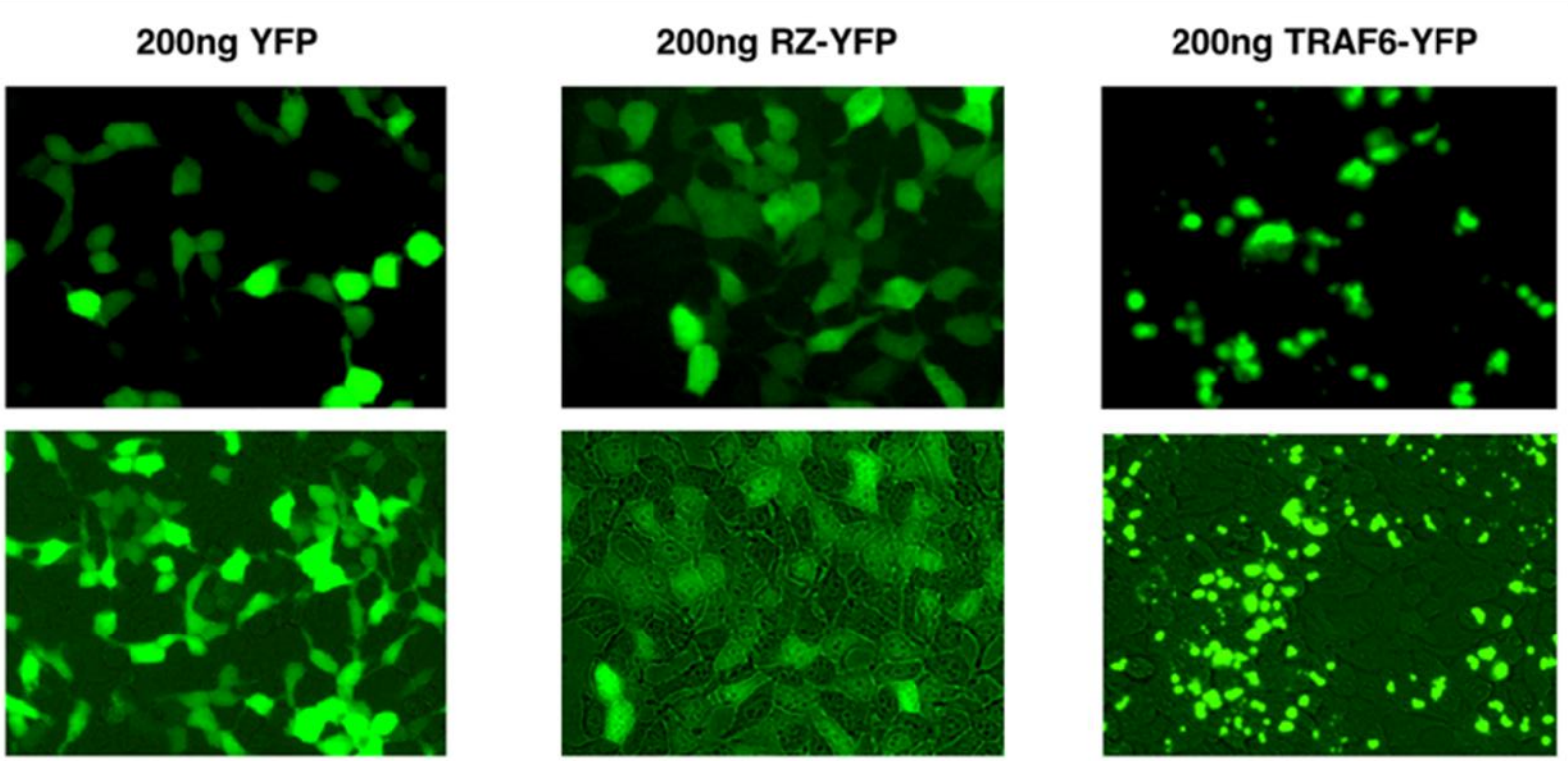


Figure 42. Cell viability and morphology affected by over-expression of TRAF6.
200 ng of each indicated plasmid was transfected into 293T cells in a 96-well tissue culture plate. Micrographs were taken after 24 hours of transfection. There was no significant change observed in cell morphology and density in TRAF6 over-expressed cells compared to that of YFP or TRAF6RZ-YFP.

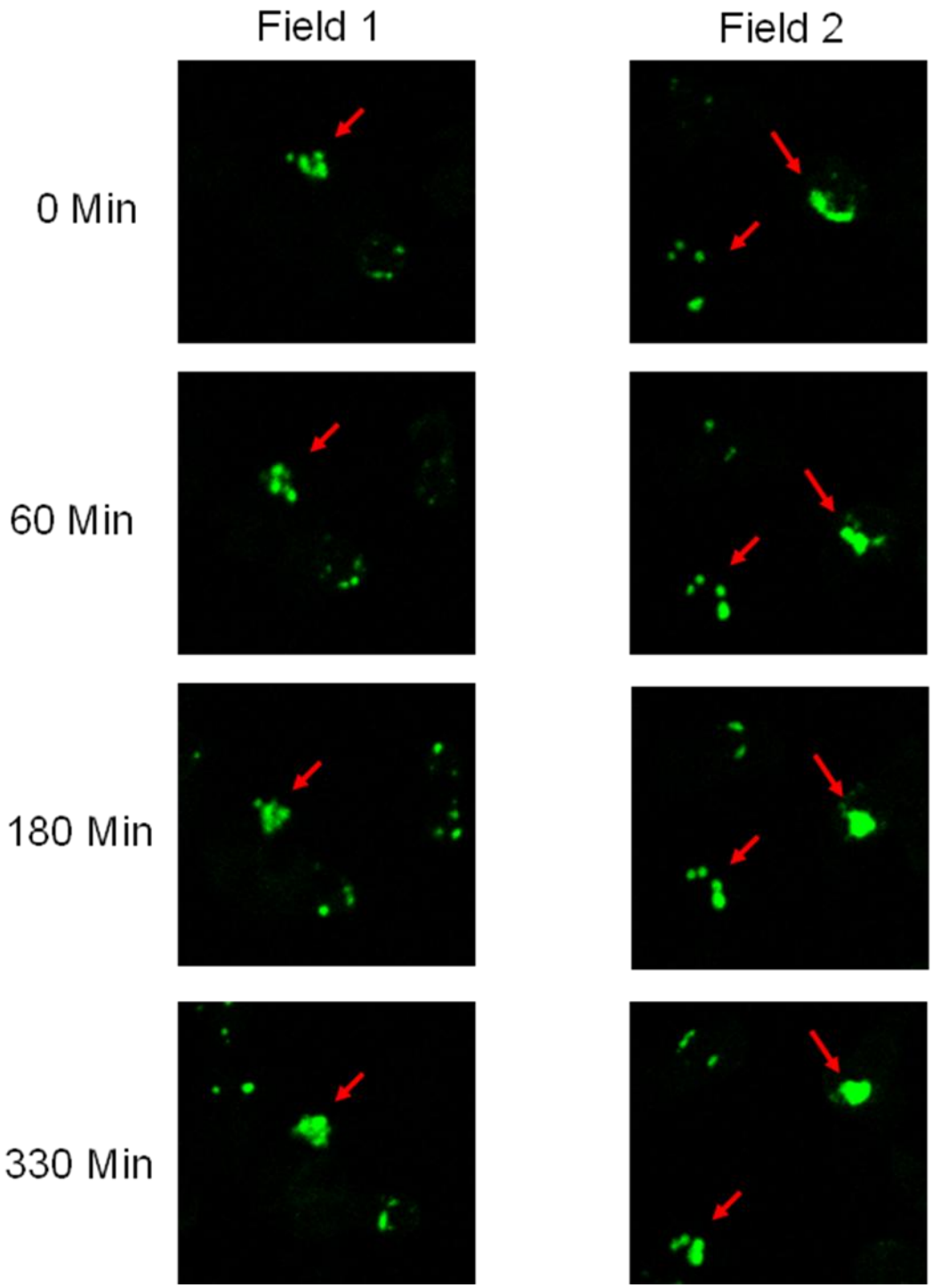

Field 1
Field 2
0 Min
60 Min
180 Min
330 Min

Figure 43. TRAF6 degradation *via* the proteasomal pathway.

An expression plasmid (40 ng) coding for TRAF6-YFP was transfected into 293 cells in a 96-well tissue culture plate with procedures described in Material and Methods. 24 hours after transfection, cells with ectopically expressed TRAF6YFP (pseudo-colored green) were treated with a proteasome inhibitor MG132 in a final concentration of 25 μM. Confocal images were taken at 0 and 5 hours post treatment. As shown in images, larger aggregates of TRAF6YFP protein were observed upon MG132 treatment.

It has been documented that some neurodegenerative diseases, such as Paget's disease, Alzheimer's disease and Parkinson disease, are caused by ubiquitinated protein aggregations within proteasomes (Bence et al., 2001; Ciechanover and Brundin, 2003; Elsasser et al., 2002). Sequestosome 1/P62 was shown to be a TRAF6 binding protein involved in regulation of proteasomal degradation of ubiquitinated proteins (Babu et al., 2005; Moscat et al., 2007; Seibenhener et al., 2004). The expression of P62 is elevated and shown to be aggregated in several neurodegenerative diseases (Wooten et al., 2006). Our observations from the inhibitory effect of TRAF6 on NF-κB activation by excessive ubiquitination prompted us to explore whether P62 co-localizes with TRAF6 in those punctuate sequestosomes. We ectopically over-expressed P62-HcRed1along with either TRAF6-YFP or a derived mutein. Our preliminary studies showed that P62-HcRed1alone displays a very similar sub-cellular localization pattern compared to that of TRAF6-YFP, and co-expression of P62-HcRed with TRAF6-YFP reveals co-localization of both in sequestosomes (Fig.44). Interestingly, sub-cellular co-localization was not observed in co-expression of P62-HcRed with TRAF6RZ-YFP, whereas it was displayed in co-expression of P62-HcRed1 with TRAF6MATH-, TRAF6ccMATH- and TRAF6Δcc-YFP (Fig.44). Therefore, the MATH domain of TRAF6 may have the potential to interact with P62.

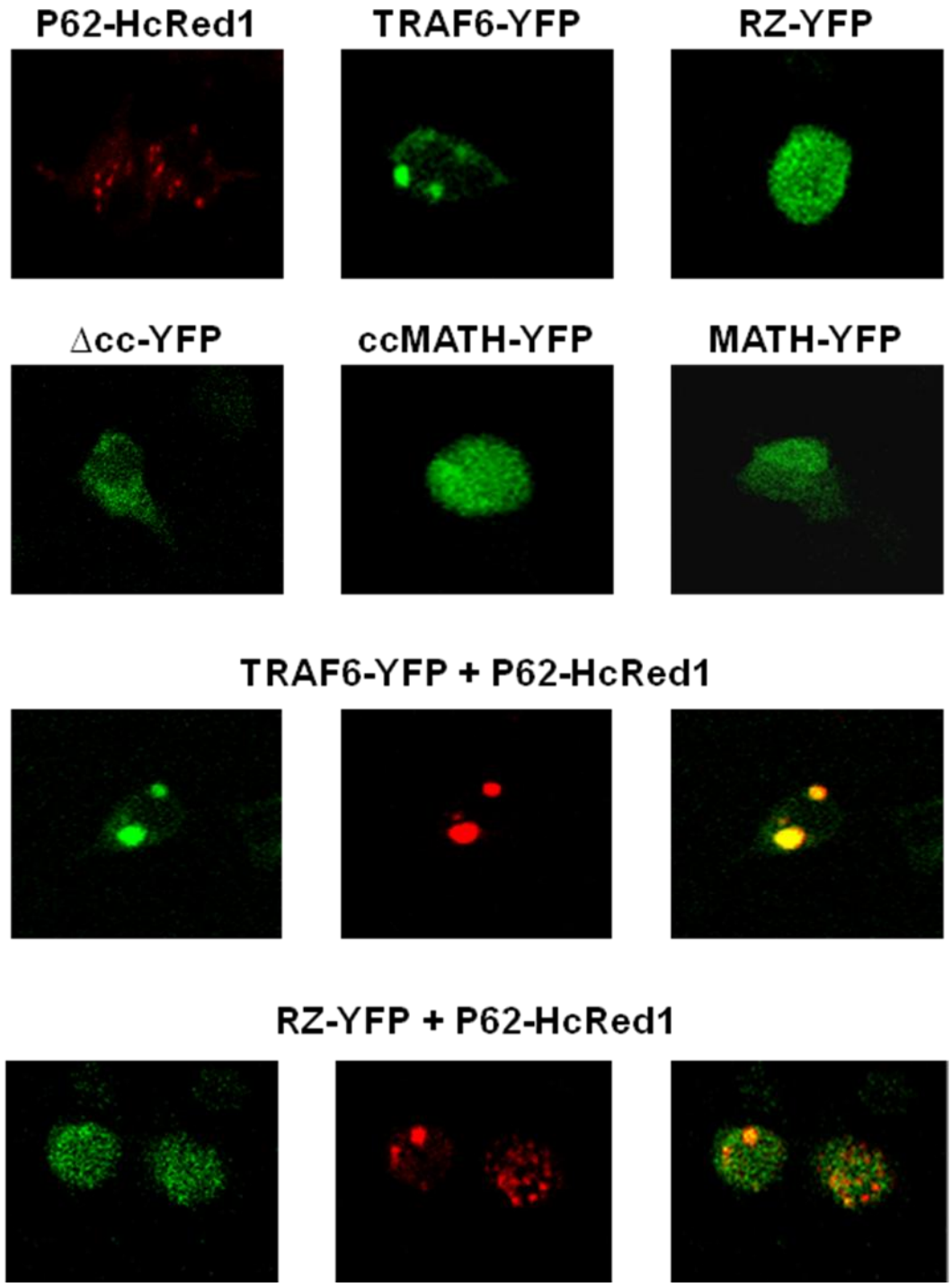


Figure 44. Co-localization of P62 and TRAF6 proteins.

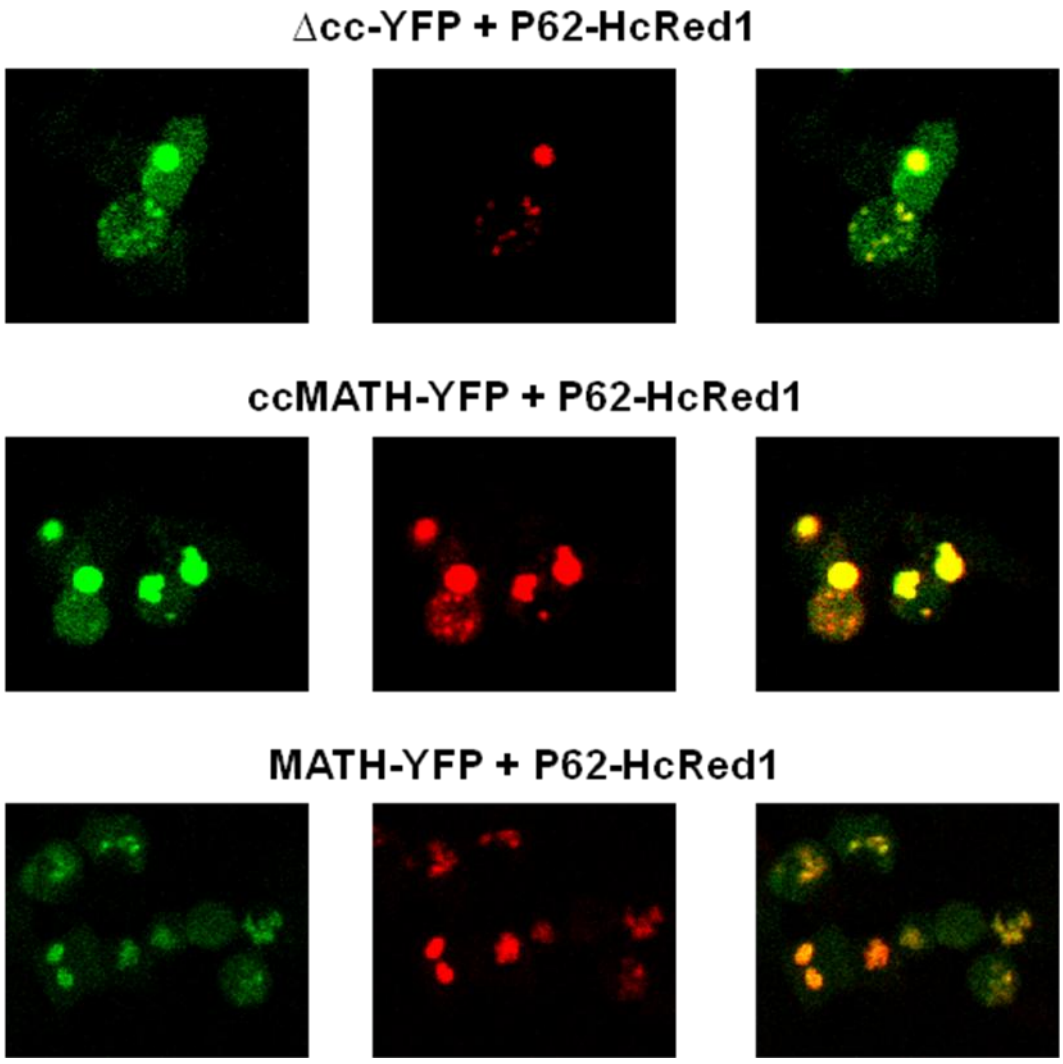


Figure 44. Co-localization of P62 and TRAF6 proteins (continued).

Each indicated expression plasmid (40 ng) was transfected into 293 cells according to procedures described in Materials and Methods. Confocal images of ectopic expressed P62-HcRed1 (red) and TRAF6-YFP proteins (green) were taken 24 hours post transfection. Yellow color is derived overlapping of green and red colors, suggesting co-localization of two fluorescent labeled proteins.

Molecular mechanism of TRAF6 activation

The model proposed in Figure 45 summarizes the experimental results presented above. Prior to stimulation, TRAF6 is maintained in an inactive "closed" state by intra-molecular interaction between the RING-Zinc and MATH domain, similar to that manifested by numerous kinases and other signaling molecules (Huse and Kuriyan, 2002). This is supported by the known X-ray structure data (Ni et al., 2004; Ni et al., 2000; Park et al., 2000; Ye et al., 1999), which argue for an extended trimer, primarily self-associated *via* the coiled-coil domain. In this model, the RING-Zinc domains extend away from the coiled-coil with the potential for a flexible region between the two (Fig. 1). 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 RING-Zinc and coiled-coil domains permitting the association with E2 (Yang et al., 2004; Yin et al., 2009) conjugating enzyme that results in *trans*-ubiquitination. Ubiquitination may serve as a means of providing steric bulk, thus sustaining the active conformation (Fig. 39). Dominant-positive activation by TRAF6 over-expression increases the probability of inter-molecular associations between the RZ and MATH domains, and possibly other RZ (Fig. 37 & 38), disrupting the inhibitory intra-molecular interaction and resulting in *trans*-ubiquitination/activation. However, when poly-ubiquitination of TRAF6 exceeds the recycling capability of the sequestosome, recycling ceases and activity is lost due to the sequestration of inactive TRAF6 (Fig. 40-41).

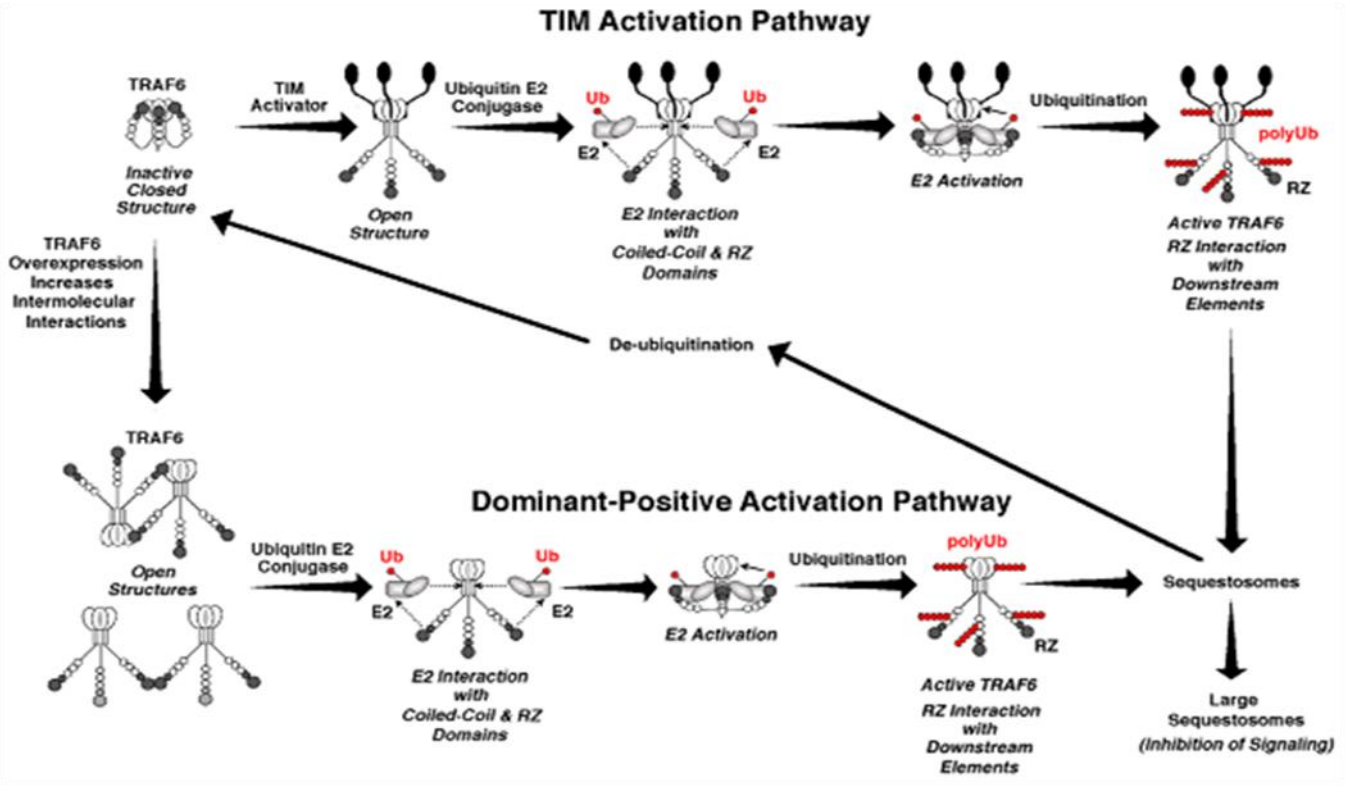


Figure 45. Model of TRAF6 regulation.
The proposed model suggests both the normal (TIM) and ectopic expression (Dominant-Positive) pathways for TRAF6 activation and the role played by ubiquitination to maintain an active, *open*, structure capable of interacting with downstream kinases. Also suggested is the post-activation pathway that results in the formation of large sequestosomes and inactivation, which may be related to proteasome activity.

DISCUSSION

TRAF family molecules mediate numerous signal transduction pathways, which are emerging as one of the most interesting topics in the area of signal transduction regulation (Chang and Dong, 2009; Dinarello, 1999; Wajant et al., 2001; Wu and Arron, 2003). In this study, we have engineered a collection of expression constructs coding for TRAF6 and muteins representing different functional domains based on information from the literature and our previous data. This allows us to systematically investigate the contribution of different functional domains and motifs in sub-cellular localization, ubiquitination and NF-κB activation of TRAF6 protein. By employing biochemical and microscopic assays, I have reached the goals set out in my specific aims, and discovered a novel mechanism for TRAF6 regulation.

Sub-cellular localization of TRAF6 is controlled by different domains and poly-ubiquitination

Sub-cellular localization of a molecule is usually linked to a potential function in certain biological regulatory processes. TRAF family molecules have been suggested to translocate from the cytoplasm to the plasma membrane, sequestosome, and nucleus upon stimulation from different signaling events. It was reported that TRAF2 and TRAF3 can translocate from the cytoplasm to plasma membrane raft microdomains of B cells upon CD40 engagement with anti-CD40 antibody (Hostager et al., 2000). Another study showed that CD40 formed an immune synapse complex containing adaptor molecules TRAF2 and TRAF3, ubiquitin-conjugating enzyme Ubc13, cellular inhibitor of apoptosis

proteins 1 and 2 (c-IAP1/2), IkappaB kinase regulatory subunit IKKγ (also called NEMO), and mitogen-activated protein kinase (MAPK) kinase kinase MEKK1 upon anti-CD40 antibody ligation. Furthermore, these kinases were not activated unless the multi-component signaling complex was translocated from CD40 to the cytoplasm upon c-IAP1/2-induced degradation of TRAF3. The authors hypothesized that this two-stage signaling mechanism may apply to other innate immune receptors, accounting for spatial and temporal separation of MAPK and IKK signaling (Matsuzawa et al., 2008). Recently, TRAF2 plasma membrane translocation to raft microdomains was also reported in CD40 positive helper T cell (Th40), a T cell sub-population that is significantly expanded in aggressive autoimmunity. Such an event provides survival signals to Th40 (Vaitaitis and Wagner, 2008). Membrane raft localization of TRAF6 was first implicated in RANK signaling (Ha et al., 2003b). Ha *et al* showed that RANK ligand (RANKL) induced TRAF6 lipid rafts translocation in RAW264.7 cells and osteoclasts analyzed by triton X-100 fractionation. When cells were pre-treated with a lipid raft disruption reagent, methyl-β-cyclodextrin (MCD), TRAF6 was greatly reduced in the detergent insoluble fraction, and RANK/RANKL signaling was also disrupted. These results suggest that lipid rafts serve as a platform for TRAF6 localization and function. Studies involving TRAF6 regulation of CD40 B lymphocyte functions has led to the discovery of two regulation modes, in which TRAF6 can either translocate to the plasma membrane and directly interact with CD40 or remains in the cytoplasm to transmit CD40 signaling through un-defined bridging regulator(s) (Rowland et al., 2007; Xie et al., 2008). Interestingly, stable TRAF6 membrane localization was not observed in IL-1R signaling. It was proposed that TRAF6 may be transiently recruited to the plasma membrane and

engage with other signaling regulators. Subsequently, the complex containing TRAF6-IRAK-TAK1-TABs would rapidly disassociate from the plasma membrane and stay in the cytoplasm, where TRAF6 activates TAK1 leading to activation of the MAPK and NF-κB pathways (Li and Qin, 2005; Qian et al., 2001; Takaesu et al., 2001). We also did not observe evident plasma membrane localization when functionally dominant-positive TRAF6-YFP protein was over-expressed in 293 cells. This may be due to lack of membrane receptors such as CD40 and RANK in 293 cells (Ha et al., 2003a). Our results showed that the YFP labeled wild-type TRAF6 was mainly displayed as cytoplasmic speckles and sequestosomes, with a small number of cells exhibiting nuclear localization. (Fig. 13). It should be noted that the TRAF6RZ-YFP, TRAF6ccMATH-YFP, and TRAF6cMATH-YFP muteins localize throughout the entire cell, suggesting that the isolated N- and C-termini of TRAF6 alone can move into the nucleus with higher efficiency than the intact TRAF6 molecule. We cannot rule out any apparent effect of the coiled-coil domain in nuclear targeting, because the ccMATH-YFP mutein showed nuclear localization while RZcc-YFP displayed patterns like that of wtTRAF6-YFP. Similar results were observed in cellular distribution of TRAF4 (Glauner et al., 2002). In that study, researchers reported that the TRAF4 molecule mainly localized to the cytoplasm, while the RING-Zinc fingers and TRAF domains (a fragment similar to our ccMATH) were pre-dominantly in the nucleus. However, the N-TRAF domain (analogous to the coiled-coil domain in TRAF6) was distributed in the cytoplasm. Taking the potential intra-molecular interaction of TRAF6 into consideration, we speculate that intra-molecular interaction between the N- and C-termini produces a steric conformation and blocks nuclear localization. If this is true, TRAF6 localized to nuclei may be

ubiquitinated or modified by other means, such as sumoylation (Gill, 2003, 2005; Pham et al., 2008). Significant increase in nuclear localization of the RZ-, MATH- and ccMATH-YFP mediated by *trans*-ubiquitination may support this reasoning (Fig. 32). It is of interest to understand the mechanism of TRAF6 nuclear localization, since several studies have disclosed that TRA6 might act as a transcriptional regulator. For instance, Bai *et al* showed that TRAF6 localizes in the nuclei of osteoclasts, but not in the nuclei of their bone marrow macrophage precursors. Moreover, RANK ligand (RANKL) stimulation significantly increases intra-nuclear abundance of TRAF6 in osteoclasts. Further mechanistic studies suggested that TRAF6 nuclear localization requires FHL2, where TRAF6 recognizes FHL2 and RUNX1, and the three molecules form a DNA-binding complex that recognizes and transactivates the RUNX1 response element in the *fhl2* promoter (Bai et al., 2008). However, TRAF6 sumoylation displays inhibitory effects on gene transcription. Pham *et al* reported that sumoylated TRAF6 can move into nuclei, but acts as a negative regulator of c-Myb gene transcription (Pham et al., 2008).

Sequestosomal localization of TRAF6 has been linked to its ubiquitin modification (Sanz et al., 2000; Seibenhener et al., 2004). Our observations also showed a good agreement to this linkage. The wild-type TRAF6-YFP and several muteins (TRAF6mR-, TRAF6K124R-, TARF6P3A-, and TRAF6RZcc-YFP) displaying sequestosomes were demonstrated to be poly-ubiquitinated. In contrast, all muteins (TRAF6RZ-, TRAF6MATH-, TRAF6ccMATH-, TRAF6delcc-, TRAF6delR, TRAF6delZ1, and TRAF6delRZ1-YFP) that didn't display sequestosomal localization only showed minimal levels of ubiquitination (Fig. 25-26). When ubiquitination of TRAF6delcc-, TRAF6delR, and TRAF6delRZ1-YFP was rescued by co-expression of

either TRAF6 or another mutein that could induce their *trans*-ubiquitination, sequestosomal localization of these muteins re-occurred (Fig. 31-35). However, we also demonstrated that ubiquitination may not be a necessary result for sequestosomal localization. The nuclear translocation of RZ-, MATH-, and ccMATH-YFP muteins after *trans*-ubiquitination has provided convincing evidence for this argument (Fig. 32-33).

Ubiquitination sites on TRAF6

It was suggested that lysine 124 is a specific ubiquitination site for mouse TRAF6. A mutein carrying a point mutation for this lysine residue (K124R) showed significant loss of ubiquitination, NF-κB activity, and osteoclastogenesis compared to wild-type TRAF6 (Lamothe et al., 2007). However, these results were challenged by another group which showed no significant difference between wtTRAF6 and the TRAF6K12R mutein in ubiquitination and NF-κB activation (Walsh et al., 2008). A similar discrepancy was observed for the human TRAF6K124R mutein in our results. We do not observe any significant difference in sub-cellular localization patterns, ubiquitination and NF-κB activity between wtTRAF6 and the TRAF6K124R proteins (Fig. 25-39). Furthermore, we have provided evidence that both the N-terminus (RING-Zinc fingers) and C-terminus (MATH domain) of TRAF6 can be targeted for ubiquitination (Fig. 33). It was proposed that the K63 linked poly-ubiquitin chain(s) attached to TRAF6 or a target protein might cooperate with its modified substrate and serve as specific signals for downstream regulators. Therefore, K63 linked ubiquitination may behave like the phosphorylation of many proteins that require site-specific modification (Lamothe et al., 2007). Our data has raised questions pertaining to the site

specificity of K-63 linked poly-ubiquitination and argued that such modification may not be sufficient for NF-κB activation. In supporting the second part of this reasoning, we showed that none of the muteins (TRAF6RZ-, TRAF6MATH-, TRAF6ccMATH-, TRAF6delcc-, TRAF6delR, and TRAF6delRZ1-YFP) deficient in ubiquitination and NF-κB activation are capable of NF-κB activation upon induction of ubiquitination (Fig. 36). Another group used a genetic approach to mutate all 23 lysine residues of TRAF6 (TRAF6ΔK), resulting in abolishment of poly-ubiquitination. Intriguingly, there was no significant change when comparing this mutein to wtTRAF6 in activation of NF-κB and osteoclastogenesis (Walsh et al., 2008). Although diminished in ubiquitination, TRAF6ΔK also displayed similar properties to wtTRAF6 in binding with and activating TAK1, an immediate down-stream kinase effector of TRAF6. However, two E3 ligase inactive muteins (TRAF6C70 and TRAF6C85A/H87A) showed stronger binding affinity with TAK1, but were both incapable of activating TAK1. These implicated that E3 ligase activity of TRAF6, not ubiquitin modification, is required for activation of TAK1, NF-κB, and osteoclastogenesis. Their results also challenged an earlier report regarding TRAF6 mediation of TAK1 activation, since it was believed that TRAF6 activates TAK1 through mediation of TAB1-TAB2/3, in which the K63-linked poly-ubiquitin chains on TRAF6 bind to the ubiquitin binding domain (UBD) of TAB2/3 and forms a complex of TRAF6-TABs-TAK1 (Kanayama et al., 2004).

Controversies in functions of poly-ubiquitin chains for NF-κB activation

A recent publication showed that free poly-ubiquitin chains that were not attached

to any protein (also called unanchored poly-ubiquitin chains) were capable of activating the NF-κB pathway (Xia et al., 2009). In this paper, authors conducted *in vitro* ubiquitination reactions by adding in different combinations of components. They declared that a classical combination of components which included TRAF6, E1, E2 (Ubc13/Uev1A), ubiquitin, and ATP could only synthesize unanchored K63 linked poly-ubiquitin chains, whereas TRAF6 itself was not ubiquitinated. These *in vitro* synthesized poly-ubiquitin chains could then directly activate TAK1. Similar types, if not the same, of unanchored poly-ubiquitin chains were also implicated to be produced in IL-1β stimulated 293 cells. These *in vivo* synthesized poly-ubiquitin chains seemed to be involved in IKKγ (NEMO) ubiquitination/activation and IKBα phosphorylation. The K63 linked poly-ubiquitin chains produced *in vitro* by TRAF6 were first demonstrated by the same research group (Deng et al., 2000). But the K63 linked specific poly-ubiquitination of TRAF6 in both *in vitro* and *in vivo* conditions was also confirmed later (Wang et al., 2001). Aiming at getting insight into Ubc13/Uev1A and TRAF6 mediated K63 linked poly-ubiquitination, Petroski *et al* from another research group, immobilized TRAF6 on Sepharose prior to *in vitro* incubation with the subunits of Ubc13-Uev1A in the presence of E1, ATP, and ubiquitin. Their results showed that unanchored poly-ubiquitin chains were dominantly synthesized, but TRAF6 itself was also significantly poly-ubiquitinated (Petroski et al., 2007). Putting aside the discrepancy regarding whether TRAF6 ubiquitination can be induced by Ubc13-Uev1A *in vitro*, if unanchored poly-ubiquitin chains can indeed directly activate TAK1, it suggests that the poly-ubiquitin chains anchored to the inactive muteins by *trans*-ubiquitination in our *in vivo* experiments may have different properties from the above mentioned unanchored poly-

ubiquitin chains. Furthermore, it also implies that the poly-ubiquitin chains attached to wtTRAF6 and the active muteins (TRAF6RZcc, TAF6mR and TRAF6K124R, *etc*.) are different from that on the inactive muteins.

Our other observations also suggest additional challenge to existing paradise relating to the biological function of unanchored poly-ubiquitin chains. For instance, if IL-1 stimulation could produce unanchored poly-ubiquitin chains for NF-κB activation, could ectopic over-expression of TRAF6 also generate the same types of ubiquitin chains, since TRAF6 is the unique member in the TRAF family able to mediate IL1R signaling (Cao et al., 1996; Ye et al., 2002). However, similar to the observation of the dominant negative effect of the MATH domain of TRAF molecules in many signaling events, in which the MATH domain was proposed to compete with endogenous TRAF in binding with upstream activators (Darnay et al., 1999; Rothe et al., 1995; Wong et al., 1998; Yoshida et al., 2004), we demonstrated that the MATH domain of TRAF6 could inhibit TRAF6 activation of NF-κB likely by binding to the RING-Zinc domain of TRAF6 and interfering with its downstream function. The gap of regulation between the MATH domain dominant negative effect and the unanchored poly-ubiquitin chains dominant positive effect in the NF-κB pathway needs to be filled, unless the MATH domain can also target and block these ubiquitin chains for activation of the NF-κB pathway. But this seems not to be the case. Ectopic over-expression of TRAF6RZcc-YFP displays similar sub-cellular localization, ubiquitination, and NF-κB activity as that of wtTRAF-YFP. However, when we co-expressed equal amounts of TRAF6RZcc with either the MATH or ccMATH mutein in 293 cells, the MATH or ccMATH mutein had minimal inhibitory effect to TRAF6RZcc activation of NF-κB (data not shown). If the

NF-κB pathway is directly activated by the unanchored poly-ubiquitin chains rather than by the TRAF6 or TRAF6RZcc molecule, co-expression of the MATH domain should produce similar inhibitory effects to wtTRAF6 and TRAF6RZcc activation of NF-κB. A possible explanation for the incapability of the MATH domain inhibiting TRAF6RZcc is that *trans*-ubiquitination results in disruption of interaction between the MATH and RING-Zinc domains in RZcc. In contrast, the wtTRAF6 molecule contains an intra-molecular MATH domain, while the TRAF6RZcc does not, which can generate a large difference in interaction and inhibitory efficiency due to the effect of anchimeric assistance.

Another report showed that the ubiquitin ligase complex LUBAC can induce conjugation of head to tail-linked linear poly-ubiquitin chains onto Lysine 285 in the CC2-LZ domain of NEMO and results in activation of the canonical NF-κB pathway (Tokunaga et al., 2009), but controversially, the linear poly-ubiquitin chains generated *in vitro* seemed not to be sufficient for activating TAK1 and IKK (Xia et al., 2009).

It has been shown that poly-ubiquitin chains can be recognized by proteins containing an ubiquitin binding domain (UBD) with some specificity. Such ubiquitin "code" recognition *via* UBD bearing signaling proteins has been suggested as a novel signaling event (Chen, 2005; Chen and Sun, 2009; Hurley et al., 2006; O'Neill, 2009). For instance, sequestosome 1/P62, TAB family members and NEMO, *etc*. in the TRAF6 signaling network seem to fall in this category (Kanayama et al., 2004; Lo et al., 2009; Moscat et al., 2007). Intriguingly, many of these poly-ubiquitin binding proteins are also capable of ubiquitination (Ishitani et al., 2003; Tang et al., 2003). The roles of ubiquitin modification on target proteins and the signal specificity of those anchored and

unanchored poly-ubiquitin chains are yet to be manifested. The complexity in both ubiquitin linkages and length of the chains render a great challenge to the study. It requires more advanced purification and detection means in order to sort out existing controversies.

Roles of ubiquitination in TRAF6 activation of NF-κB

Many reports have suggested that ubiquitination modification of TRAF6 is critical for its activation. Endogenous TRAF6 in most cell types only has minimal levels of ubiquitination modification in unstimulated conditions. Ubiquitination of TRAF6 can be triggered in two ways, but the regulatory mechanism is unknown. One trigger is from upstream activators initiated at cell surface receptors. These include IL-1RI (Deng et al., 2000; Jensen and Whitehead, 2003; Wang et al., 2001), TLRs (Kaisho and Akira, 2006), TCR (Sun et al., 2004), RANK (Lomaga et al., 1999), and TGFβ receptor I (TGβRI) (Sorrentino et al., 2008). The other is from ectopic expression of regulators inside cells, such as P62 (Seibenhener et al., 2004) and TAB2 (Kishida et al., 2005). Intriguingly, over-expression of TRAF6 can also trigger its auto-ubiquitination (Fig. 26). To explore the regulatory mechanism controlling such "auto-ubiquitination", we have engineered a collection of mutant constructs encoding different functional domains of the TRAF6 molecule. Our data show that ubiquitination of TRAF6 or an effective mutein requires the RING-Zinc fingers and coiled-coil domain (Fig. 26). This is in agreement with results from others (Wang et al., 2001; Yang et al., 2004). Furthermore, we have demonstrated that TRAF6 ubiquitination can be collaboratively regulated *in trans* by one mutein containing a RING-Zinc domain and the other with the coiled-coil (Fig. 35).

It was suggested that the RING domain of TRAF6 could interact with E2 ubiquitin conjugating enzyme Ubc13 by means of yeast two hybrid (Wooff et al., 2004), nuclear magnetic resonance (Markin et al., 2008), and X-ray crystallography (Yin et al., 2009). Yin *et al* showed that Ubc13 interacted with the RING and first zinc finger (RZ1) of TRAF6. Rather than forming trimers, the RZ1 peptides were dimeric in both the crystal and solution. In the RZ1-Ubc13 complex, residues Glu69, Pro71, Ile72, Leu74, Met75, Ala101, and Pro106 in the RZ1 form major contact with Ubc13. Among these residues, Ile72 and Leu74 were found to be completely buried at the interface of the RZ1-Ubc13 complex (Yin et al., 2009). But these stories seem to be incomplete since "auto-ubiquitination" and NF-κB activation could not be induced by over-expression of the RING-Zinc fingers (Fig. 26-29) and the dimeric gyrase B conjugated RING-Zinc fingers (RZ-GyrB) (Wang et al., 2001) in contrast to that of wtTRAF6 and TRAF6RZcc. Though we found inter-molecular interaction between the RING-Zinc domains of TRAF6, their binding affinity was significantly lower than that of the RING-Zinc and MATH domains interaction (Fig. 38). These observations suggest that the RING-Zinc fingers alone may not be sufficient for E2 recruitment to trigger ubiquitination reactions. The coiled-coil domain was shown to directly interact with Ubc13 (Yang et al., 2004). Taking these two facts together, our data support the model that ubiquitination of TRAF6 needs collaboration between the RING-Zinc and coiled-coil domains.

Although signaling specificity of poly-ubiquitin chains is controversial and subject to more defined investigation, as we have discussed earlier, our *in vivo* studies concerning ubiquitination modification of TRAF6 and its NF-κB activity clearly implicate the linkage of both. We also demonstrated potential intra-molecular interaction

between the RING-Zinc and MATH domains and their interaction disruption upon ubiquitination (Fig. 37-39). Therefore, it is reasonable to hypothesize that the intra-molecular interaction of the RING-Zinc and MATH domains "locks" up the TRAF6 molecule in an inactive closed conformation, while disruption of the intra-molecular interaction by upstream activators or over-expression of itself turns TRAF6 into an active open state allowing access of Ubc13/Uev1 complex. Recruitment of Ubc13/Uev1 to the coiled-coil and RING-Zinc domains triggers *trans*-ubiquitination of TRAF6, which generates steric barriers disrupting interaction between the RING-Zinc and MATH domain and sustains TRAF6 in an open conformation (Fig. 45).

On the other hand, our results from *in vivo trans*-ubiquitination *via* two inactive muteins showed that such events were insufficient for NF-κB activation. At least two conclusions could be drawn. First, the anchored poly-ubiquitin chains, or possibly unanchored poly-ubiquitin chains (if there are any) do not provide downstream signaling specificity in the NF-κB pathway. Second, sufficient recruitment of E2 (Ubc13/Uev1) and E3 ubiquitin ligase activity are equally important for activation of a downstream activator(s). In other words, the dominant positive effect of TRAF6 or TRAF6RZcc results from possessing E3 ubiquitin ligase activity and being able to proximally recruit E2 (Ubc13/Uev1), which allows them to directly activate downstream signaling regulators through ubiquitination modification of these molecules. In supporting this argument, poly-ubiquitination of TAB2 and TAB3 were shown to be directly mediated by TRAF6 (Ishitani et al., 2003); K63 linked ubiquitination and activation of TAK1 at lysine 34 in TGFβ signaling was regulated by TRAF6 (Sorrentino et al., 2008). More recently, Yang *et al* reported that TRAF6 is a direct E3 ubiquitin ligase for Akt k63

linked poly-ubiquitination. Such modification is critical for Akt plasma membrane translocation and activation (Yang et al., 2009). In this study, they found that two lysine residues (K8 and K14) located inside the PH domain of Akt were targeted for TRAF6 directed ubiquitination; ubiquitination of Akt was required for its phosphorylation and membrane recruitment; ubiquitination of Akt contributed to both the survival and oncogenic signaling pathways (Restuccia and Hemmings, 2009).

Finally, we employed a live cell NF-κB reporter (HcRed1-κB) to analyze correlation between sub-cellular distribution of TRAF6 and NF-κB activity in individual cells. Our data uncovered that a significant NF-κB activity was coincidently correlated to cells showing cytoplasmic speckles and/or small sequestosomes, whereas excessive ubiquitination of TRAF6 caused by large amount of ectopic over-expression resulted in significant increase in numbers and sizes of sequestosomes and reduction of NF-κB activity (Fig. 40-41). Therefore, ubiquitin modification may be a double-edged sword in regulation of TRAF6 (Fig. 45). This observation is consistent with our previous report (Wang et al., 2006), in which we investigated a correlation between sub-cellular localization of TRAF6 and NF-κB activity. For this we ectopically expressed TRAF6-YFP along with different proteins that could interact with TRAF6. It turned out that co-expression of TRAF6-YFP with IRAK1 did not display a significant change in TRAF6 localization and NF-κB activity; co-expression of TRAF6-YFP along with MyD88 greatly reduced sequestosomal distribution of TRAF6 but significantly increased NF-κB activity. However, co-expression of TRAF6-YFP with an IRAK2 mutein generated very large sequestosomes along with significant loss of NF-κB activity (Wang et al., 2006). In agreement with these observations, we also demonstrated that larger sequestosomal

TRAF6-YFP aggregates were formed upon proteasome inhibition by MG132, suggesting involvement of the proteasomal pathway (Fig. 43).

Taken together, TRAF6 ubiquitination may take place in the cytoplasm, resulting in the activation of downstream signaling pathways. The sequestosomes may facilitate de-ubiquitination and recycling of TRAF6 (Jensen and Whitehead, 2003). However, if accumulation of ubiquitinated TRAF6 saturates the capability of effective de-ubiquitination, very large sequetosomes will be formed due to the aggregation of involved protein complexes (Seibenhener et al., 2007), ceasing TRAF6 recycling and TRAF6 downstream signaling.

There is significant structural homology among TRAF family members, which implicates similarities in their biological functions (Bradley and Pober, 2001; Chung et al., 2002; Lee and Lee, 2002). Moreover, many TRAF molecules can interact with each other and form hetero-complexes (Arch et al., 1998; Bradley and Pober, 2001; Wajant et al., 2001). These suggest that TRAF6 *trans*-ubiquitination and our hypothesized regulatory mode for TRAF6 activation may be globally, or at least partially, applied to other TRAF family members.

Co-localization of TRAF6 and Sequestosome 1/P62

It has been reported that Sequestosome 1/P62 contains a TRAF6 and a K63 linked poly-ubiquitin binding site in its ZZ finger and UBA domain, as well as multiple protein-protein interaction motifs. Therefore, P62 is also considered as a scaffold (Geetha and Wooten, 2002; Seibenhener et al., 2004; Wooten et al., 2005). The N-terminus of P62

protein can interact with the proteasome subunit component, in which P62 shuttles interacting proteins to the proteasome for degradation (Janse et al., 2004; Seibenhener et al., 2004). P62 can also regulate poly-ubiquitination of TRAF6 in nerve growth factor (NGF) induced activation of the NF-κB pathway (Wooten et al., 2005). However, the site of TRAF6 that interacts with P62 is still unidentified. We employed fluorescent microscopic techniques and co-expressed a HcRed1fluorescent protein tagged P62 with YFP tagged TRAF6 and several different muteins. Our preliminary studies suggest that the MATH domain of TRAF6 may be involved in physical interaction between TRAF6 and P62 (Fig. 44). Interestingly, all co-localization signals displayed similar sequestosome like punctuate spots. Consequently, the ubiquitination status of these proteins will be analyzed in our future studies.

P62 has been reported to be involved in the regulation of many neurodegenerative pathological maladies such as Alzheimer's disease, Parkinson's disease, and Paget's disease of bone (Layfield and Searle, 2008). Involvement of TRAF6 in the context of expression and ubiquitination levels for such P62 mediated pathological events are of interest for future pursuit.

CONCLUSIONS

Accumulating evidence argues that TRAF6 plays critical roles in many signaling networks. The biological processes involved in TRAF6 regulation include innate and adaptive immunity, inflammatory disease, bone development and disease, neuronal cell development, cancer cell progression, and metastasis. Acting as a RING domain-containing E3 ubiquitin ligase, TRAF6 mediates specific lysine 63 linked poly-ubiquitination modification to several target molecules, as well as to itself. Aiming at understanding the molecular regulatory mechanism of TRAF6 activation, we have engineered a series of expression plasmids coding for different functional domains of the TRAF6 molecule. Each coding sequence was inserted into an un-tagged and YFP-FLAG dual tagged expression plasmid. The YFP-FLAG tags conjugated to the C-terminus of each protein provided us advantages in biochemical and microscopic analysis, while each untagged protein was used to ensure consistency in biological activities compared with its dual-tagged partner.

In this work, we have demonstrated correlations among TRAF6 sub-cellular localization, ubiquitination, and NF-κB activity; the roles of different functional domains such as the RING-Zinc, coiled-coil, and MATH; and the impact of ubiquitination on TRAF6 and derived muteins. Finally, a model representing TRAF6 activation regulation has been suggested. Results obtained from current studies are summarized as follows:

1. TRAF6 is mainly localized in the cytoplasm, displayed as tiny speckles and punctate sequestosomes. The number and size of sequestosomes increases in parallel with ectopic expression of TRAF6. There is also some nuclear

localization (about 5%) observed (Fig. 13). The modification and biological function of nuclear-localized TRAF6 will be of interest in our future studies.

2. Although ectopically expressed TRAF6-YFP is mainly localized in the cytoplasm, its N- and C-termini (TRAF6RZ-, ccMATH and MATH-YFP) are distributed throughout the entire cell. *Trans*-ubiquitination of these muteins induces a significant increase in their nuclear localization (Fig. 25, 32 & 33). These observations suggest that intra-molecular interaction of TRAF6 may prevent it from the entering the nucleus.
3. TRAF6 and all muteins displaying sequestosomal localization patterns are demonstrated to be ubiquitinated (Fig. 25 & 26). Interestingly, sequestosomal localization can be rescued for some ubiquitination deficient muteins upon *trans*-ubiquitination modification (Fig. 30 & 31). These implicate sequestosomes as sites for ubiquitinated TRAF6 to localize. However, we also show that ubiquitination of a TRAF6 mutein (such as TRAF6RZ-, ccMATH- and MATH-YFP, *etc*.) doesn't necessarily translocate into sequestosomes (Fig. 32 & 33).
4. We are the first to demonstrate that TRAF6 "auto-ubiquitination" can be mediated *in trans*, in which the RING-Zinc fingers from one molecule coordinates with the coiled-coil domain from another molecule for regulating the process (Fig. 34 & 35). Therefore, our data argue that, besides functioning in multimerization, the coiled-coil domain of TRAF6 is also critical for effective recruitment of E2 (Ubc13/Uev1A) ubiquitin conjugating enzyme.
5. TRAF6 poly-ubiquitination can occur on both its N- and C-termini (Fig. 33). This argues that there is no single site specificity for such modification on TRAF6.

6. There is a good correspondence between ubiquitination and NF-κB activity in TRAF6 and several active muteins (Fig. 25-29), but *trans*-ubiquitination of inactive muteins is insufficient for NF-κB activation (Fig. 36). This draws concerns regarding biochemical specificities of the poly-ubiquitin chains which have been implicated to play a role as signaling regulators. Furthermore, significant inhibition of TRAF6 activation by co-expression of the MATH domain (Fig. 36) highlights the discrepancy between our results and literature information in this regard.
7. Investigation of molecular interaction between different functional domains of TRAF6 has revealed that the RING-Zinc fingers can bind to each other weakly. However, a stronger binding affinity exists between the RING-Zinc and MATH domains (Fig. 38). Therefore, we propose that a potential intra-molecular interaction between the N- and C-termini of TRAF6 keeps this molecule in an inactive closed conformation under unstimulated conditions. However, such "auto"-inhibition can be counteracted through *trans*-ubiquitination, due to disruption of the interaction between the N- and C-termini of TRAF6 (Fig. 39). Moreover, our data also implicate that the proposed TRAF6 trimer derived from the crystal structures may be disassociated upon *trans*-ubiquitination (Fig. 31 & associated text on page 73).
8. Ubiquitin modification of TRAF6 is a double-edged sword. It is required for TRAF6 activation. However, excessive ubiquitination may result in an increase in sequestosome localization and inhibition of TRAF6 activity. Since inhibition of proteasome function (by MG132) also results in formation of larger

sequestosomes, this implies that proteasomes play important roles in regulating TRAF6-dependent NF-κB activity (Fig. 40-43).

9. Our preliminary studies showed that TRAF6 is co-localized with Sequestosome 1/P62, a scaffold regulating proteasomal degradation of ubiquitinated proteins, and suggest that the MATH domain of TRAF6 may be involved in interaction of TRAF6 with P62 (Fig. 44).
10. Based upon the manifestation of potential intra-molecular interaction and *trans*-ubiquitination, a regulatory model presenting the molecular mechanism of TRAF6 activation regulation has been proposed as shown in Figure 45.
11. Finally, a table showing schematic cartoons of TRAF6 and the various muteins summarizes sub-cellular distribution, *trans*-ubiquitination, and NF-κB activity as observed for our studies (Fig. 46).

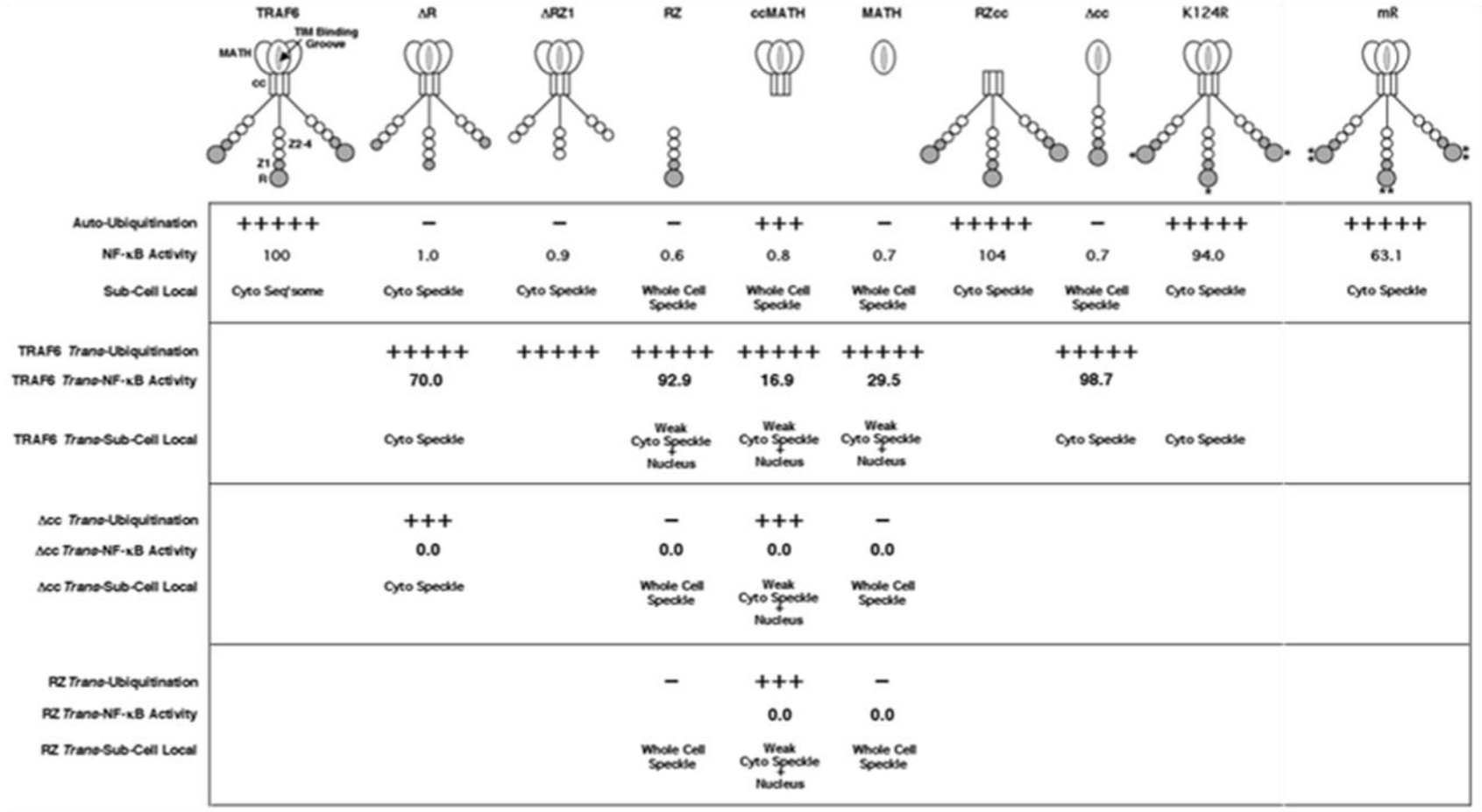


Figure 46. Summary of TRAF6 and derived muteins for sub-celluar localization, ubiquitination and NF-κB activity

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