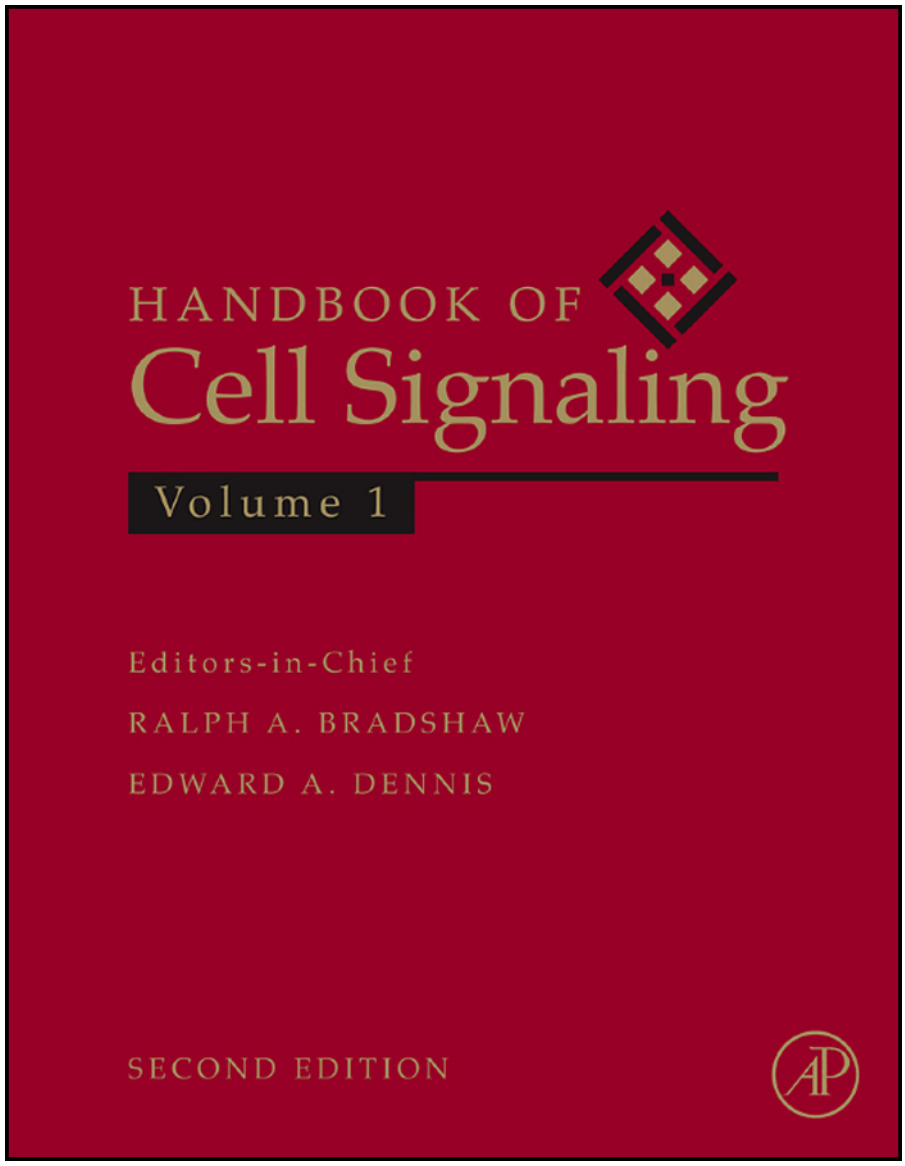

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Section D

Membrane Proximal Events

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Chapter 49

Tumor Necrosis Factor Receptor-Associated Factors in Immune Receptor Signal Transduction

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INTRODUCTION

It is crucial for cells to detect extracellular and intracellular stresses and act correspondingly. Sophisticated receptor families have been developed to sense both extracellular and intracellular stress signals, such as pathogen-associated molecular patterns (PAMPs). While Toll-like receptors (TLRs) sense extracellular PAMPs, the NOD-like receptors (NLRs) and the more recently identified double-strand RNA (dsRNA) receptors sense intracellular PAMPs, eliciting signal transduction for immune and inflammatory responses as part of the innate immunity against pathogens [1]. Cytokine secretion and induction of specific adaptive immunity ensue to complete the complex network of host defense. Tumor necrosis factor (TNF) receptor-associated factors (TRAFs) are signaling proteins that participate in many aspects of the signal transduction of these immune receptors, including receptors for TNF and related cytokines, the IL-1 receptor, TLRs, NLRs, dsRNA receptors, the T cell receptors (TCR), and the B cell receptors (BCR) [1, 2]. TRAF activation eventually leads to activation of transcription factors such as NFκB and AP-1 (Figure 49.1).

DISCOVERY OF TRAF PROTEINS

The first two and founding members of the TRAF family, TRAF1 and TRAF2, were discovered in 1994 via yeast two-hybrid using the cytoplasmic region of TNF-R2 [3] as the bait. From then on, five more human TRAF family members have been identified and characterized and shown to participate in signal transduction beyond the TNF receptor superfamily (Table 49.1). Unlike many cell surface receptors, TNF receptors (TNFRs) do not possess any catalytic activity in their cytoplasmic tails, and rely on TRAFs and other intracellular proteins for signal transduction. TRAFs mainly reside in cytosolic fractions, which are consistent with their functions as receptor-proximal signaling proteins. However, they can be found in membrane fractions when overexpressed. TRAF4 has been found in nucleus, but its nuclear function remains unknown.

BIOLOGICAL FUNCTIONS OF TRAF PROTEINS

Mouse genetic studies have revealed that TRAF proteins regulate innate and adaptive immunity, embryonic development, stress responses, and bone metabolism through the modulation of cell survival, proliferation, differentiation, and cell death [4, 5]. TRAF homologs have been found in lower eukaryotes, including *Danio rerio* [6], *Drosophila melanogaster* [7–9], *C. elegans* [10], and *Dictyostelium discoideum* [11]. In drosophila, TRAFs are essential for dorsoventral polarization and innate host defense [12, 13].

The biological functions of TRAF proteins appear to be mediated through activation of transcription factors such as NFκB and AP-1. In fact, almost all NFκB activation is mediated by TRAFs except that involved in DNA damage responses [14]. The interceding steps between TRAF activation and transcription factor activation involve kinase cascades such as the IκB kinases (IKKs) and mitogen-activated protein kinases (MAPKs), including c-Jun N-terminal kinase (JNK) and p38. TRAF proteins can play either a positive or a negative role in NFκB activation.

TRAF proteins can interact with each other and with numerous receptors, kinases, and signaling proteins from the immune pathways they participate in [10]. Therefore,

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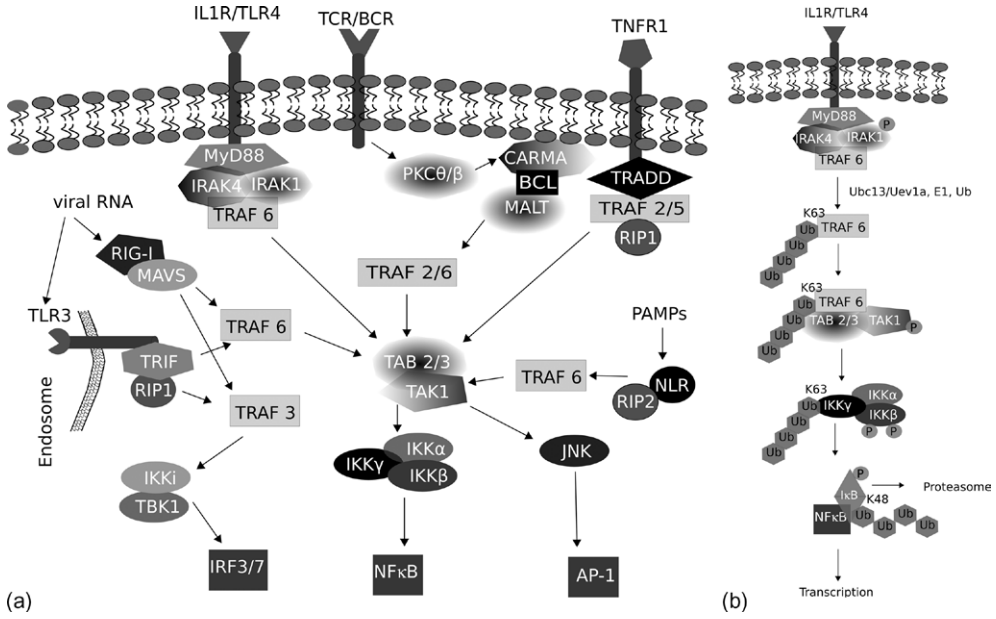

FIGURE 49.1 TRAF-mediated signaling pathways.
(a) An overall view of the pathways that TRAFs engage in. (b) TRAFs, in particular TRAF6, mediate Lys63-linked polyubiquitination and NF-κB signaling.

TABLE 49.1 Discovery of human TRAF proteins

	Identification methods	Reference
TRAF1	Yeast two-hybrid (TNF-R2)	3
TRAF2	Yeast two-hybrid (TNF-R2)	3
TRAF3	Yeast two-hybrid (CD40)	11, 56, 57
TRAF4	Screening of a cDNA library from breast cancer-derived metastatic lymph nodes	58
TRAF5	Yeast two-hybrid (CD40)	59
	Degenerate oligonucleotide PCR (conserved residues in the TRAF domain of TRAF1, 2 and 3)	60
TRAF6	EST database search (TRAF-C domain of TRAF2) Yeast two-hybrid (CD40)	29 61
TRAF7	EST database search (TRAF-like RING) TAP/LC-MS/MS	62 63

In parentheses are the baits used in yeast two-hybrid screens, templates used in expressed sequence tag (EST) database search or primers used for degenerate PCR.

tight regulation of the myriad of interactions is required for precise and specific signaling. Which TRAF protein is activated and which downstream pathway is elicited upon its activation are dependent on both the nature of the stimuli and the cell types [15].

DOMAIN ORGANIZATIONS AND STRUCTURES OF TRAFs

The N-terminal part of TRAFs 2–7 consists of one really interesting new gene (RING) type zinc binding domain followed by several CCHC zinc fingers (Figure 49.2). TRAF1 lacks the most N-terminal RING domain, but retains one predicted CCHC zinc finger. With the exception of TRAF7, all TRAF proteins share the characteristic TRAF domain, or merpin and TRAF homology (MATH) domain at their C-termini, which mediates interaction with receptors and adaptor proteins (Figure 49.2). Instead of a TRAF domain, TRAF7 has seven WD40 repeats that are also well known to mediate protein–protein interactions.

The N-Terminal RING Domain and Zinc Fingers

The RING domain is a special type of zinc binding domain. The consensus RING sequence is $CX_2CX_{(9-39)}CX_{(1-3)}HX_{(2-3)}C/HX_2CX_{(4-48)}CX_2C$ with eight cysteines and histidines in a "cross-brace" topology to coordinate two zinc ions [16]. RING domains are present in many E3 ubiquitin–protein ligases to mediate the interaction between E2 ubiquitin conjugating enzymes and their substrates. Zinc fingers such as the CCHC type in TRAFs, on the other hand, were first identified in transcription factors [17]. They wind themselves on one zinc ion, and often occur in tandem. Today, the zinc finger family has expanded rapidly to mediate protein–DNA, protein–RNA, protein–protein and protein–lipid interactions [18–20]. They have even been

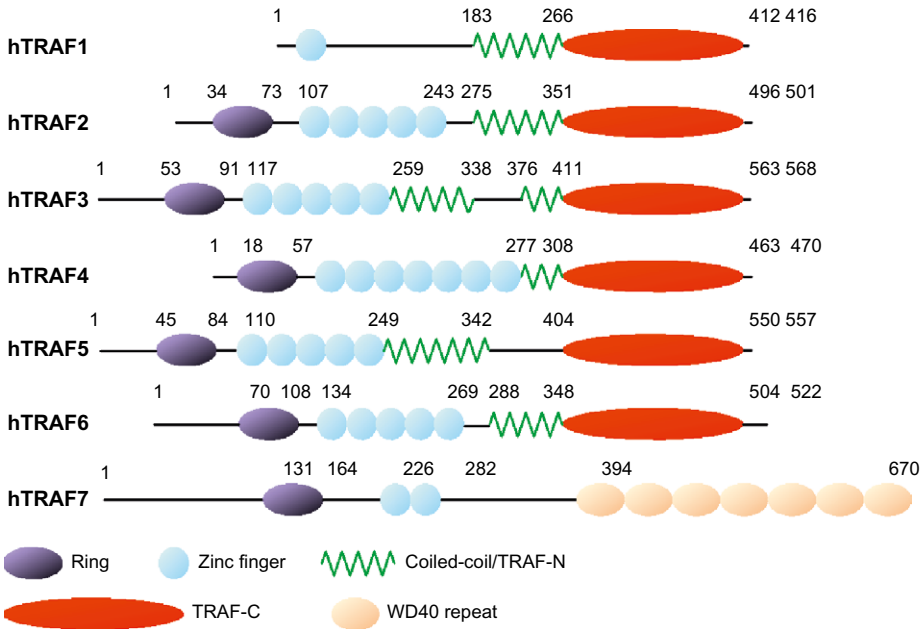

FIGURE 49.2 Domain Organizations of human TRAF1–7.

exploited by protein engineering to create artificial zinc fingers with novel binding specificity [21].

The C-Terminal TRAF Domain

The C-terminal TRAF domain can be further divided into TRAF-N and TRAF-C domains. TRAF-N domain is a coiled-coil region that mediates homo- and hetero-oligomerization among TRAF family members. TRAF-C domain is responsible for physical association with upstream receptors and adaptor proteins. Crystal structures of TRAF domains from different TRAF proteins have shown that they form mushroom-shaped trimers with the TRAF-C domain as the "head" of the mushroom and the bundled coiled-coils as the "stalk" [22–24] (Figure 49.3). Both TRAF-N and TRAF-C domains contribute to the trimer interface; however, without the coiled-coil TRAF-N domain, the TRAF-C domain alone exists as a monomer in solution [25]. Each TRAF-C domain adopts a unique β-sandwich topology composed of eight anti-parallel β strands, which is similar to the conformation observed in merpin metalloproteases and the drosophila Siah protein.

X-ray crystallographic studies have also revealed the interaction details of TRAFs with peptides derived from receptors [22–24] and with the adaptor protein TRADD (TNF receptor associated protein with a death domain) [26] (Figure 49.3). One peptide molecule binds exclusively to one TRAF protomer. The receptor-derived peptides bind to a shallow surface groove on the TRAF-C domains, but the binding details differ in different TRAF-peptide pairs. In particular, TRAF6 has a peptide binding specificity that is distinct from TRAF2 and TRAF3. TRAF trimerization enhances the otherwise weak interactions between TRAF proteins and the peptides via avidity [27]. TRAF2–TRADD interaction is quite distinctive from TRAF–peptide interactions [26]. It is much stronger and can effectively compete with TRAF2–receptor complexes, which may ensure recruitment of anti-apoptotic cIAPs to the TNFR1 signaling complex to suppress apoptosis.

THE UNIQUE TRAF6

Evolutionarily, TRAF6 is probably the most ancient mammalian TRAF and most resembles the primordial TRAF protein, while the divergence of TRAFs 1, 2, 3 and 5 from TRAF4 and among themselves is the result of recent gene duplications [28]. Consistently, the TRAF-C domain homology suggests that TRAF6 is most distant from other TRAFs [29]. Along with TRAF2, 3, and 5, TRAF6 physically associates with and mediates signaling from TNFR superfamily members, including CD40, RANK, and TACI [10]. However, TRAF6 distinguishes itself by binding to a different motif on TNF receptors [30].

Moreover, TRAF6 is the major transducer of IL-1 receptor/TLR signaling. TRAF6 was first identified as a mediator of IL-1 signaling [29]. Ligation of IL-1 to IL-1R first recruits myeloid differentiation primary response gene 88 (MyD88) via the interaction between the Toll/interleukin receptor domains (TIRs) present in both IL-1R cytoplasmic tail and MyD88 (Figure 49.1). MyD88 then recruits IL-1 receptor-associated kinase 1 (IRAK1) and its homolog IRAK4 via death domain (DD)–DD interactions.

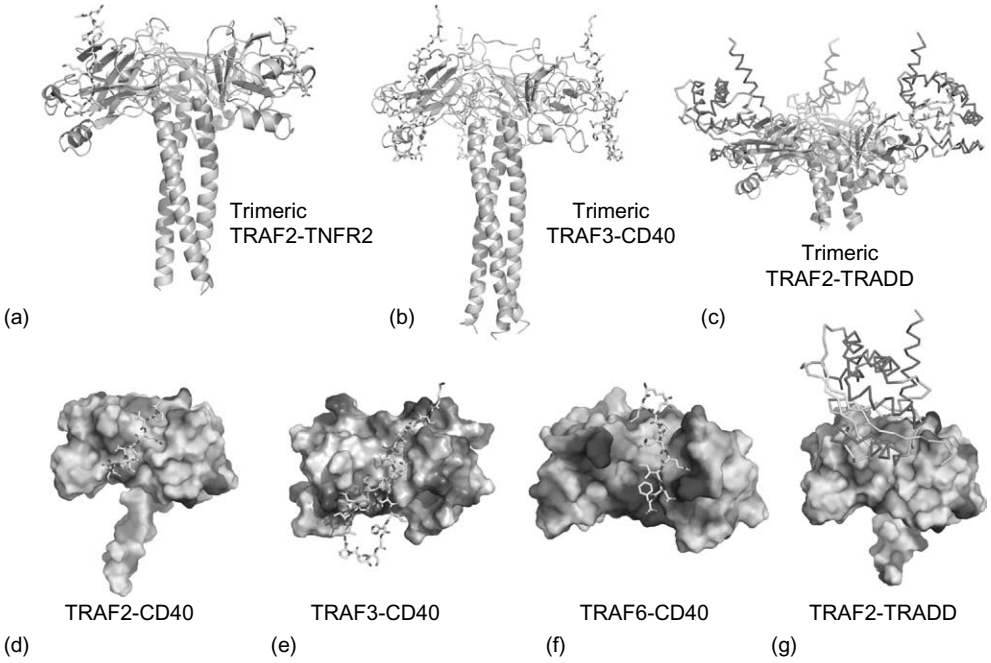

FIGURE 49.3 Structural studies of the TRAF domains and their complexes.
(a) Trimeric TRAF domain structure of TRAF2 (ribbon) in complex with the TNFR2 peptide (stick model); (b) trimeric TRAF domain structure of TRAF3 (ribbon) in complex with the CD40 peptide (stick model); (c) trimeric TRAF domain structure of TRAF2 (ribbon) in complex with the N-terminal domain of TRADD (Cα trace); (d) surface electrostatic diagram of TRAF2 shown in complex with the stick model of the TRAF2/3/5 binding peptide of CD40; (e) surface electrostatic diagram of TRAF3 shown in complex with the stick model of the TRAF2/3/5-binding peptide of CD40; (f) surface electrostatic diagram of TRAF6 shown in complex with the stick model of the TRAF6 binding peptide of CD40; (g) surface electrostatic diagram of TRAF2 shown in complex with the Cα trace of the N-terminal domain of TRADD.

The kinase activity of IRAK4 is required for IL-1 signaling, while that of IRAK1 is not required. When recruited to the receptor proximal complex, IRAK4 phosphorylates IRAK1 and TRAF6 is recruited to this complex via IRAK1 [24]. Recent studies have shown that while TRAF6 mediates NFκB and MAP kinase activation by these receptors, TRAF3 may be involved in interferon production upon TLR activation [31, 32]. For T cell receptor and B cell receptor mediated NFκB activation, TRAF6 acts downstream of the CARMA1–Bcl10–MALT1 (CBM) complex [33]. In addition, the role of TRAF6 in signaling of the dsRNA sensor RIG-I and NLRs such as Nod1 and Nod2 has been implicated.

TRAF SIGNALING AND LYS63 LINKED POLYUBIQUITINATION

Biochemical fractionation and *in vitro* reconstitution have revealed that MAP kinase and NFκB activation by TRAF6 requires a novel form of polyubiquitination [34, 35]. Ubiquitination is one of the most prevalent posttranslational modifications that is accomplished in three steps by ATP-dependent attachment of ubiquitin (Ub) via thioester bond to Ub activating enzyme (E1), transfer of Ub from E1 to the active site Cys of Ub conjugating enzyme (E2), and transfer of Ub from E2 active site to Lys residues of substrates with the aid of a Ub ligase (E3). TRAF6 is a RING-type E3 that ubiquitinates via the Lys63 linkage instead of the Lys48 linkage for proteasomal degradation.

On activation by the relevant signaling pathways upon ligand stimulation, TRAF6 promotes Lys63-linked polyubiquitination of itself and downstream signaling proteins, a process that requires a heterodimeric E2 of Ubc13 and the ubiquitin E2 variant (Uev) known as Uev1A [36]. The Lys63-linked polyubiquitin chains function as anchors to recruit the transforming growth factor (TGF)-β-activated kinase 1 (TAK1) complex consisting of TAK1, the TAK1 binding protein 1 (TAB1), and TAB2 or TAB3. Both TAB2 and TAB3 contain ubiquitin binding motifs, which mediate the interaction between the TAK1 complex and ubiquitinated TRAF6 [37]. Activated TAK1 then phosphorylates and activates IκB kinase β (IKKβ), a catalytic subunit of the IKK complex. Phosphorylation of IκB by the IKK complex leads to its ubiquitination via the Lys48 linkage, and its destruction by the 26S proteasome. Rid of its inhibitor, the previously cytosol-trapped NFκB is free to translocate to the nucleus, induce transcriptional activation of various genes, and launch a battery of immune responses to encounter the stress signals. NFκB essential modulator (NEMO) or IKKγ, the regulatory subunit of the IKK complex, is also a substrate of TRAF6 ubiquitin ligase activity. TAK1 also directly phosphorylates MAP kinases, leading to activation of AP-1 transcription factors.

For other TRAF family members, Lys63-linked polyubiquitination also appears to be the common mechanism of their downstream action. It has been shown that TNF activated endogenous TAK1, and the kinase-negative TAK1 acted as a dominant negative inhibitor against TNF induced NFκB activation [38]. More convincingly, siRNA-mediated knockdown showed that TAK1 is critical for TNF-induced activation of the NFκB pathway [39]. It was shown that TNF-induced IKK activation is mediated through the TRAF2, TRAF5, and TAK1 signaling pathway [40]. Similarly, the role of the TAB2-related protein TAB3 in TNFR signaling has been uncovered (41–43). The role of Lys63 ubiquitination in TRAF2 and TRAF5 signaling has also been inferred from the negative regulation by the Lys63 linkage specific de-ubiquitinating enzyme CYLD [44–46].

REGULATION OF TRAF SIGNALING

TRAF signaling is tightly regulated by many mechanisms; in particular, oligomerization and ubiquitination. These regulatory mechanisms ensure that TRAFs are activated upon ligand stimulation and turned off at the appropriate times. Oligomerization appears to be the common theme of TRAF6 activation in all known signaling pathways: oligomerization by TNFRs, by TIR signaling complexes, and by the CBM complex. The C-terminal TRAF domain mediates its trimerization. Forced dimerization by fused dimerizing domain also activates TRAF6 [47]. A cytosolic TRAF-interacting protein known as TIFA, with a forkhead-associated (FHA) domain, can enhance TRAF6 oligomerization and activation [48].

Several de-ubiquitinating and ubiquitinating enzymes, such as A20 and CYLD, have been shown to provide feedback inhibition of TRAF-mediated NFκB activation. A20 was originally characterized as an early response gene to TNF stimulation [49], and possesses dual ubiquitin editing functions [50]. While the N-terminal domain of A20 is a de-ubiquitinating enzyme (DUB) for Lys63-linked polyubiquitination of signaling mediators such as TRAF6 and RIP, its C-terminal domain is a ubiquitin ligase (E3) for Lys48-linked degradative polyubiquitination of the same substrates [50–54]. CYLD is Lys63-specific de-ubiquitinating enzyme, whose mutations are the underlying causes of familial cylindromatosis, with predisposition to tumors of skin appendages called cylindromas [44–46, 54, 55].

SUMMARY AND PERSPECTIVES

Since the identification of the first two TRAF family members in 1994, it has become clear that different TRAFs exhibit specific biological functions. The membrane-proximal events for initiating differential TRAF signal transduction have been relatively well established from the wealth of structural and functional studies. The biggest challenge now lies ahead – to further elucidate the molecular mechanisms of the E3 activities of the different TRAF family members. While the E3 activity of TRAF6 has been demonstrated both *in vitro* and in cells, relatively less is known regarding the E3 activity of other TRAFs and their specificity. Therefore, this aspect of TRAF signaling will remain an important focus of investigation for a wide range of biological interests.

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