

# TRAF6, a molecular bridge spanning adaptive immunity, innate immunity and osteoimmunology

Hao Wu<sup>1\*</sup> and Joseph R. Arron<sup>2</sup>

## Summary

Tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6) is a crucial signaling molecule regulating a diverse array of physiological processes, including adaptive immunity, innate immunity, bone metabolism and the development of several tissues including lymph nodes, mammary glands, skin and the central nervous system. It is a member of a group of six closely related TRAF proteins, which serve as adapter molecules, coupling the TNF receptor (TNFR) superfamily to intracellular signaling events. Among the TRAF proteins, TRAF6 is unique in that, in addition to mediating TNFR family signaling, it is also essential for signaling downstream of an unrelated family of receptors, the interleukin-1 (IL-1)

receptor/Toll-like receptor (IL-1R/TLR) superfamily. Gene targeting experiments have identified several indispensable physiological functions of TRAF6, and structural and biochemical studies have revealed the potential mechanisms of its action. By virtue of its many signaling roles, TRAF6 represents an important target in the regulation of many disease processes, including immunity, inflammation and osteoporosis. *BioEssays* 25:1096–1105, 2003. © 2003 Wiley Periodicals, Inc.

## Introduction

The tumor necrosis factor (TNF) receptor associated factors (TRAFs) were first identified as two intracellular proteins, TRAF1 and TRAF2, associated with TNF-R2,<sup>(1)</sup> a member of the TNF receptor (TNFR) superfamily. There are currently six mammalian TRAFs (TRAF1-6), which have emerged as important proximal signal transducers for the TNFR superfamily.<sup>(2–4)</sup> In addition, the most recently identified TRAF family member, TRAF6, plays critical roles in the signal transduction of the interleukin-1 (IL-1) receptor/Toll-like receptor (IL-1R/TLR) superfamily.<sup>(5,6)</sup> By linking the activation of these receptors to downstream signaling events, culminating in the regulation of gene transcription, TRAFs exert indispensable functions in a wide array of physiological and pathological processes, in particular various aspects of adaptive and innate immunity, inflammation and tissue homeostasis.

Many of the biological effects of TRAF signaling are mediated by the activation of kinases such as the I $\kappa$ B kinase (IKK) and mitogen-activated protein (MAP) kinases, which in turn modulate the transcriptional activities of the NF- $\kappa$ B and AP-1 families, respectively. IKK is a hetero-trimeric enzyme comprising two kinase subunits, IKK $\alpha$  and IKK $\beta$ , and a regulatory subunit, IKK $\gamma$ /NEMO.<sup>(7)</sup> Upon activation, IKK phosphorylates the inhibitor of NF- $\kappa$ B, I $\kappa$ B, resulting in its degradation. This releases NF- $\kappa$ B, enabling it to translocate to the nucleus and activate transcription.<sup>(8)</sup> MAP kinases are Ser/Thr kinases that include JNKs/SAPKs, ERKs and p38s.<sup>(9)</sup> They are at the downstream end of a three-tiered system consisting of MAP kinase kinases (MAP2Ks) and MAP kinase kinase kinases (MAP3Ks). Direct phosphorylation and transcriptional activation of AP-1 components by MAP kinases lead to the stimulation of AP-1 activity.<sup>(10)</sup> While NF- $\kappa$ B is known to promote the

<sup>1</sup>Department of Biochemistry, Weill Medical College of Cornell University, New York.

<sup>2</sup>Tri-Institutional MD-PhD Program, Weill Medical College of Cornell University, New York.

Current affiliation for Dr. Arron: Department of Pathology, Stanford University School of Medicine, Stanford, CA

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\*Correspondence to: Hao Wu, Department of Biochemistry, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10021. E-mail: haowu@med.cornell.edu

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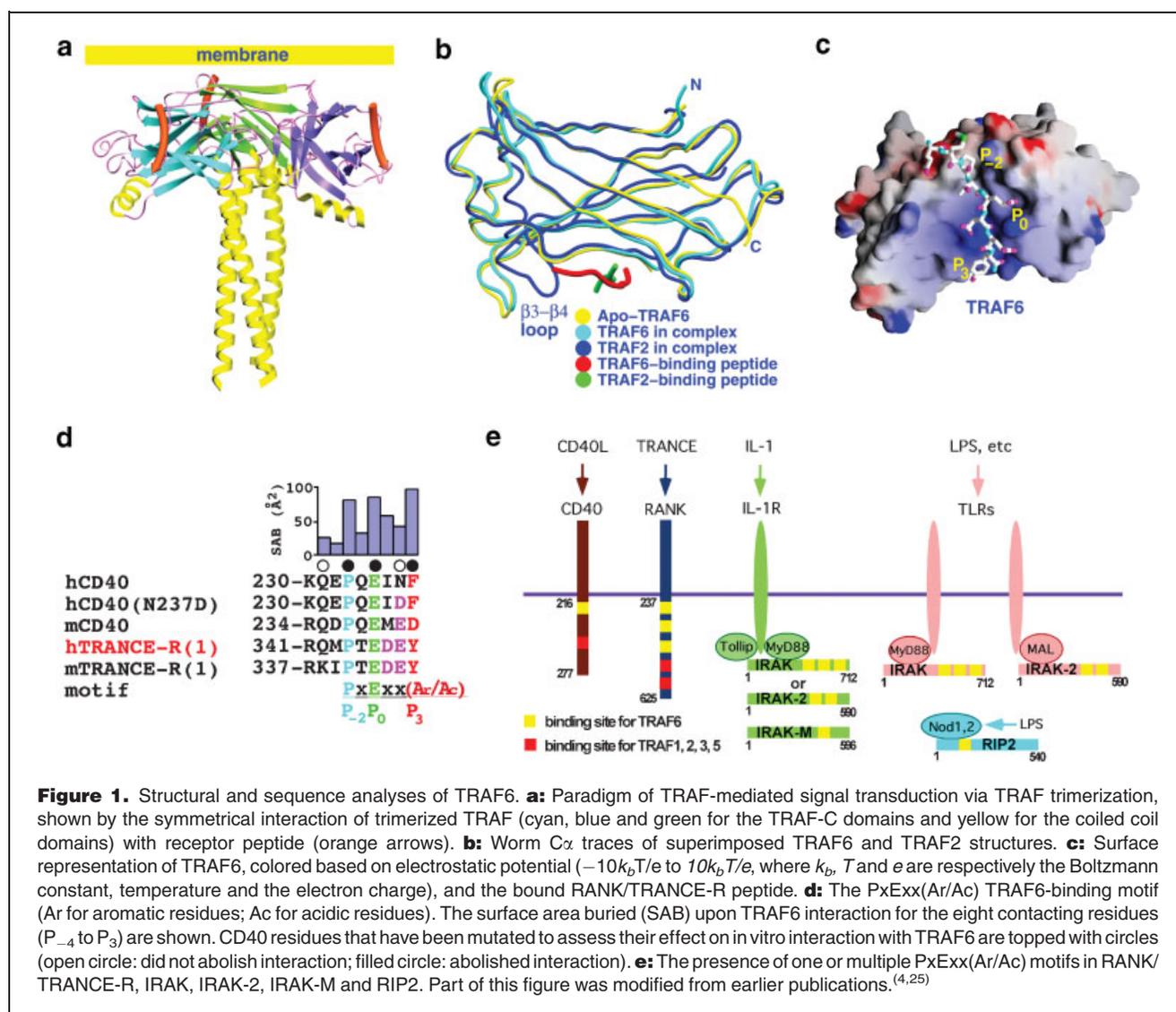
Abbreviations: TNF, tumor necrosis factor; TNFR, TNF receptor; TRAF, TNF receptor associated factor; IL-1, interleukin-1; IL-1R, IL-1 receptor; TLR, Toll-like receptor; I $\kappa$ B, inhibitor of NF- $\kappa$ B; IKK, I $\kappa$ B kinase; MAPK, mitogen-activated protein kinase; MAP2K, MAP kinase kinase; MAP3K, MAP kinase kinase kinase; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; RANK, receptor activator of NF- $\kappa$ B; TRANCE, TNF-related activation-induced cytokine; LT $\beta$ R, lymphotoxin  $\beta$  receptor; LPS, lipopolysaccharides; TIR, Toll/IL-1 receptor domain; MyD88, myeloid differentiation protein 88; IRAK, IL-1 receptor associated kinase; Mal, MyD88 adapter-like protein; TIRAP, TIR domain containing adapter protein; EBV, Epstein-Barr virus; HCV, hepatitis C virus; TAK1, transforming growth factor  $\beta$ -associated kinase 1; TAB1, TAK1-binding protein 1; TAB2, TAK1-binding protein 2.

expression of genes involved in inflammatory responses and protection from apoptosis,<sup>(11)</sup> the stimulation of AP-1 activity by MAP kinases may elicit stress responses and promote both cell survival and cell death.<sup>(12)</sup>

TRAFs comprise an N-terminal zinc-binding domain, specifically a RING finger followed by several zinc fingers, and a C-terminal TRAF domain, consisting of a coiled-coil domain known as the TRAF-N domain and a highly conserved TRAF-C domain.<sup>(1,13)</sup> The N-terminal domain is essential for the activation of downstream signaling cascades, and deletion of this domain renders it dominant-negative for signaling.<sup>(14)</sup> Structural studies have shown that the C-terminal TRAF domain adopts a mushroom-like shape with the “stalk” as the coiled-coil TRAF-N domain and the “head” as the TRAF-C domain<sup>(13)</sup> (Fig. 1a). This TRAF domain permits self-association and interactions with receptors and other signaling

proteins. TRAFs have been identified in other multicellular organisms such as *Drosophila* (dTRAF1-3), *Caenorhabditis elegans* and *Dictyostelium discoideum* with a high degree of evolutionary conservation.<sup>(4)</sup>

TRAF-mediated signal transduction is initiated by trimeric TNF family ligands that induce receptor oligomerization and/or conformational changes to produce signaling competent receptors.<sup>(15)</sup> This appears to result in the trimerization and recruitment of TRAFs through avidity-based affinity enhancement,<sup>(16)</sup> which subsequently activates intracellular signaling pathways. Artificial oligomerization of TRAF2 and TRAF6 has been shown to activate effector kinases and gene induction.<sup>(17)</sup> Although trimerization per se may be sufficient for signaling by some receptors, it is likely that the formation of higher-ordered complexes comprising multiple receptor-TRAF trimers localized in one area of the cell membrane upon



ligand engagement may play an essential role and/or enhance signaling for other receptors.

This review will focus on the biology and signaling mechanism of TRAF6, the most recently discovered mammalian TRAF family member. For general comments on other TRAFs, please see recent reviews on the subject.<sup>(2–4)</sup>

### TRAF6 is a unique TRAF family member

TRAF6 was independently cloned by a search against a DNA database for TRAF2-like sequences followed by cDNA library screening<sup>(18)</sup> and by a yeast two-hybrid screen using CD40 as bait.<sup>(19)</sup> Unlike other TRAFs, which only mediate signaling from the TNFR superfamily, TRAF6 also participates in the signal transduction from the IL-1R/TLR superfamily. The importance of TRAF6 in signal transduction outside the TNFR superfamily was first shown by its participation in IL-1 signaling<sup>(18)</sup> and subsequently by its involvement in TLR signaling.<sup>(20)</sup> Interestingly, TRAF6 exhibits close homology to dTRAF2, which has been implicated as the intracellular adapter for the *Drosophila* Toll receptor involved in anti-microbial responses and in dorsal–ventral patterning.<sup>(21)</sup> Evolutionary analyses showed that TRAF6 is one of the most divergent mammalian TRAFs in both sequence homology in the TRAF-C domain and its gene structure.<sup>(4)</sup>

The unique biological function of TRAF6 is largely a product of its distinct specificity for upstream receptors and signaling proteins, which is determined by its unique TRAF-C domain. While TRAF1, TRAF2, TRAF3, and TRAF5 exhibit similar receptor-binding specificity,<sup>(22)</sup> TRAF6 recognizes completely different binding sites on members of the TNFR superfamily, such as CD40 and RANK (also known as TRANCE-R).<sup>(23,24)</sup> Structural studies of TRAF6 in complex with CD40 and RANK peptides revealed striking differences between receptor recognition by TRAF6 and TRAF2<sup>(25)</sup> (Fig. 1b,c). The bound receptor peptides on the surface of TRAF6 showed a 40° difference in the peptide directions relative to TRAF2-binding peptides. Structure-based sequence alignment suggested that TRAF6 recognizes a conserved Pro-X-Glu-X-X-(aromatic/acidic residue) motif (Fig. 1d). Moreover, further sequence inspection showed that signaling proteins IRAK,<sup>(26)</sup> IRAK-2<sup>(27)</sup> and IRAK-M<sup>(28)</sup> in the IL-1R/TLR pathways contain one or multiple copies of the TRAF6-binding motif, providing a structural basis for the participation of TRAF6 in these pathways (Fig. 1e). IRAK-4, the most recently identified IRAK-like protein,<sup>(29)</sup> does not appear to contain TRAF6-binding motifs, suggesting that it interacts with TRAF6 indirectly, possibly through hetero-oligomerization with other IRAKs.

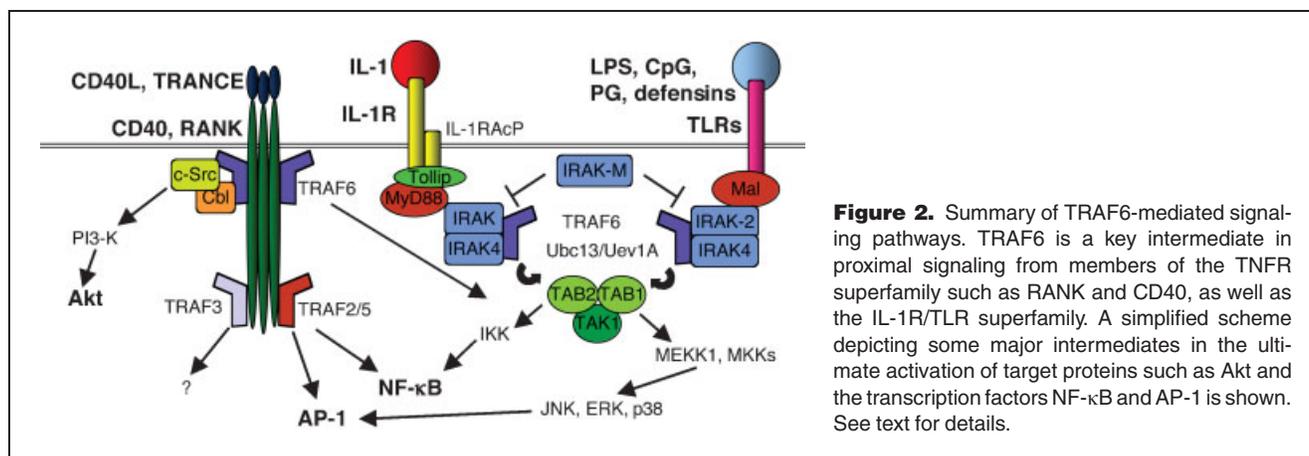
A qualitative difference may also be expected between TRAF6- and TRAF2-mediated downstream biological effects. Like TRAF2, TRAF6 activates the NF- $\kappa$ B and AP-1 transcription factors. However, it does so through different downstream signaling complexes and is therefore regulated by different signaling contexts. For example, TRAF2 appears to

cooperate with RIP to directly activate IKK.<sup>(30,31)</sup> In contrast, the activation of IKK by TRAF6 appears to involve the assembly of a large signaling complex containing ubiquitin ligases, TAK1 and TABs, for which nondegradative polyubiquitination may be required.<sup>(32,33)</sup> The RING domain of TRAF6 is required for this signaling event, likely by acting as an E3 ubiquitin ligase. In addition, TRAF6 can also activate Src family nonreceptor tyrosine kinases such as c-Src,<sup>(34)</sup> imparting additional diversity to TRAF6 signaling.

### Non-redundant role of TRAF6 in the signal transduction of members of the TNFR superfamily

The TNFR superfamily is classified based on extensive homology of extracellular regions containing conserved cysteine-rich repeats.<sup>(35)</sup> The intracellular regions of these receptors, however, do not share significant sequence homology, but are often characterized by the presence of TRAF-binding sites. Like other TRAFs, TRAF6 can directly interact with and participate in the signal transduction of members of this receptor superfamily. The two best-characterized TRAF6-interacting receptors are CD40 and RANK, both of which play important roles in the generation of antigen-specific adaptive immunity. CD40 is crucial for the maturation and survival of B cells and dendritic cells. RANK is essential for osteoclast differentiation, maturation, and survival and plays an important role in dendritic cell biology. Both CD40 and RANK can recruit TRAF1, TRAF2, TRAF3, TRAF5 and TRAF6 to their cytoplasmic tails. While TRAF1, TRAF2, TRAF3, and TRAF5 interact with the same conserved binding sites on these receptors, TRAF6 interacts with binding sites distinct from those of the other TRAFs<sup>(23,24)</sup> (Fig. 2). More importantly, TRAF6-binding sites on these receptors appear to exert specific and non-redundant biological roles.

In CD40 signaling, TRAF6 can either mediate distinct effector functions or cooperate with TRAF2 for certain downstream events. For example, TRAF6 appears to dominantly mediate p38 MAP kinase activation,<sup>(36)</sup> is important for CD40-induced IL-6 and immunoglobulin (Ig) secretion and B7-1 up-regulation,<sup>(37)</sup> and controls affinity maturation and plasma cell survival.<sup>(38)</sup> Defective CD40 signaling is observed in TRAF6-deficient cells.<sup>(39)</sup> In renal epithelial cells, TRAF6 is crucial for the production of IL-8 and chemokine MCP-1 upon CD40 ligation.<sup>(40)</sup> In contrast, both TRAF2- and TRAF6-binding sites appear to be required for optimal NF- $\gamma$ B and JNK activation<sup>(36)</sup> and transcriptional induction of germline Ig-C $\gamma$ 1 and Ig- $\epsilon$  promoters, an obligatory step in Ig class switching in B cells.<sup>(41)</sup> In another study, either the TRAF6- or TRAF2-binding site of CD40 can induce significant extrafollicular B cell differentiation and Ig class switching, but germinal center formation requires both TRAF2 and TRAF6.<sup>(42)</sup> TRAF2, but most likely not TRAF6, may down-modulate CD40 signaling by regulating CD40 membrane trafficking.<sup>(43)</sup> Given the discrepant findings



**Figure 2.** Summary of TRAF6-mediated signaling pathways. TRAF6 is a key intermediate in proximal signaling from members of the TNFR superfamily such as RANK and CD40, as well as the IL-1R/TLR superfamily. A simplified scheme depicting some major intermediates in the ultimate activation of target proteins such as Akt and the transcription factors NF- $\kappa$ B and AP-1 is shown. See text for details.

between some of these studies, it is likely that TRAF2 and TRAF6 play cell-type-specific roles in CD40 signaling.

TRAF6 appears to be the dominant adapter for RANK, at least in its osteoclast-related functions, as TRAF6 knock-out mice display severe osteopetrosis (abnormal thickening of the bone).<sup>(39,44)</sup> Of the two independently reported TRAF6 deletions, one completely lacks osteoclasts,<sup>(44)</sup> while the other has osteoclasts that are incapable of resorbing bone.<sup>(39)</sup> In both cases, this complete lack of osteoclast function likely accounts to a large extent for the runt phenotype, lack of tooth eruption, extramedullary hematopoiesis and early death (within 2 weeks) after birth. In another study, the interaction of RANK with TRAF6 is absolutely required for the proper formation of cytoskeletal structures and functional resorptive activity of osteoclasts.<sup>(45)</sup> TRAF6<sup>-/-</sup> mice also have deficiencies in mammary gland development as a result of impairment in RANK signaling and in lymph node organogenesis, possibly also through a RANK-dependent pathway downstream of LT $\beta$ R.<sup>(44)</sup> RANK on dendritic cells responds to TRANCE (also known as RANKL), the cognate ligand for RANK, on activated T cells, resulting in increased dendritic cell activation and survival.<sup>(46)</sup> TRANCE–RANK interactions are necessary for T-cell-mediated clearance of certain viral pathogens. Since TRANCE is expressed on activated CD8<sup>+</sup> in addition to CD4<sup>+</sup> T cells, while CD40L is only expressed on CD4<sup>+</sup> T cells, it is likely to play other non-redundant roles in T cell–dendritic cell communication.<sup>(47)</sup>

TRAF6 has been implicated in signaling by several other members of the TNF receptor superfamily. A recently identified member of the TNFR superfamily, XEDAR, depends on TRAF6 for signaling, as TRAF6<sup>-/-</sup> mice displayed hypohidrotic ectodermal dysplasia, with deficiencies in the development of epidermal appendices such as guard hair follicles, sweat glands and several types of sebaceous glands.<sup>(48)</sup> The p75 neurotrophin receptor appears to directly interact with TRAF6 and induce NF- $\kappa$ B activation.<sup>(49)</sup> Interestingly, IRAK has been implicated as a conserved component in p75-

mediated NF- $\kappa$ B activation.<sup>(50)</sup> TRAF6 also likely participates in signaling by BCMA, a member of the TNFR family that is expressed only on B-lymphocytes.<sup>(51)</sup>

### The dominant role of TRAF6 in IL-1R/TLR signaling

The IL-1R/TLR superfamily plays critical roles in innate immunity to infection and injury.<sup>(6)</sup> While the IL-1Rs consist of receptors for IL-1 and IL-18, TLRs are a family of receptors that share homology to *Drosophila* Toll and recognize molecular patterns associated with pathogens. Examples of ligands for TLRs include bacterial lipopolysaccharides (LPS), lipoproteins, peptidoglycan, CpG DNA, flagellin, and heat-shock proteins.<sup>(52)</sup> These receptors are characterized by the presence of an intracellular protein interaction module known as the Toll/IL-1 Receptor (TIR) domain.<sup>(52)</sup>

The signal transduction pathway for the IL-1R/TLRs was first established for IL-1. IL-1 binds to IL-1R, which is associated with an accessory protein (IL-1RAcP), inducing the formation of an intracellular signaling complex that includes the TIR-domain protein MyD88<sup>(20,53)</sup> and Tollip.<sup>(54)</sup> This is then followed by the recruitment of Ser/Thr kinases IRAKs (IRAK, IRAK-2 and IRAK-M).<sup>(26–28)</sup> While many studies have established that some TLRs activate NF- $\kappa$ B through similar IL-1 signaling mediators, other TLRs such as TLR4 recruit a TIR domain containing adapter Mal (also known as TIRAP), followed by IRAK-2.<sup>(55,56)</sup> In either case, IRAKs in turn dissociate from the receptor complex, and associate with TRAF6 to elicit signaling<sup>(18)</sup> (Fig. 2). Recently, IRAK-4,<sup>(29)</sup> an IRAK molecule closely related to the *Drosophila* Pelle protein, was shown to be indispensable for responses to IL-1 and ligands that stimulate various TLRs.<sup>(57)</sup> On the other hand, IRAK-M appears to be a negative regulator, as targeted deletion of IRAK-M leads to enhanced TLR signaling.<sup>(58)</sup>

The critical biological role of TRAF6 in IL-1R/TLR signaling has been demonstrated by the targeted deletion of TRAF6.<sup>(39,44)</sup> In the absence of TRAF6, IL-1 treatment failed

to induce T cell proliferation,<sup>(44)</sup> and LPS stimulated proliferation of B cells was dramatically reduced.<sup>(39)</sup> In addition, TRAF6 is required for IL-1- and LPS-induced NF- $\kappa$ B activation and IL-1-mediated JNK activation.<sup>(39)</sup>

TRAF6 also appears to mediate the signal transduction of several pathogens, pathogenic proteins and receptors beyond the TNFR and IL-1R/TLR superfamily. In TRAF6-knockout fibroblasts, LMP1 signaling to p38 MAP kinase is severely affected.<sup>(59)</sup> Additionally, the activation of TRAF6 (and TRAF5) by LMP1 appears to negatively control the latent replication origin of EBV through a p38-dependent pathway.<sup>(60)</sup> Similarly, TRAF6 appears to participate in NF- $\kappa$ B activation by the Hepatitis C virus (HCV) core protein,<sup>(61)</sup> JNK activation by the equine herpesvirus protein E-10<sup>(62)</sup> and NF- $\kappa$ B activation in gastric cancer cells by *H. pylori*.<sup>(63)</sup> In addition, it has been suggested that TRAF6 participates in the signal transduction of the intracellular protein RIP2,<sup>(64)</sup> proinflammatory cytokine IL-17 and the integrin Mac-1.<sup>(65,66)</sup>

In addition to the well-documented role of TRAF6 in cell survival and inflammation, targeted deletion of TRAF6 led to increased frequency of neural-tube-closure failure and exencephaly.<sup>(67)</sup> This suggests a novel and prominent role of TRAF6 in the regional control of programmed cell death within the developing central nervous system, possibly through a JNK-dependent pathway. In keeping with this hypothesis, it has been reported that LPS-induced endothelial cell death is realized through TRAF6-mediated JNK activation.<sup>(68)</sup>

### Mechanisms of TRAF6 downstream signaling

Signal amplification by TRAF6 involves the activation of multiple kinase cascades including the I $\kappa$ B Kinase (IKK), MAP kinases, and Src-family tyrosine kinases. The N-terminal zinc-binding domain of TRAF6, especially the RING domain, appears to mediate these downstream signaling events. Recent studies have provided insights into some potential molecular mechanisms of these signaling events.

The activation of both IKK and MAP kinases by TRAF6 appears to involve the MAP3K TAK1,<sup>(33,69)</sup> which is linked to TRAF6 in the IL-1 and RANK signaling pathways via adapter proteins such as TAB2<sup>(70)</sup> (Fig. 2). IRAK, the signaling protein upstream of TRAF6 in the IL-1 pathway, appears to play an important role in the assembly of the TAK1 activation complex by bringing TAB2 from the membrane to TRAF6.<sup>(71,72)</sup> In IRAK-deficient cells, TAB2 translocation and its association with TRAF6 are abolished.<sup>(71)</sup> A three-step mechanism has been proposed for this process. First, phosphorylated IRAK recruits TRAF6 to the receptor complex. IRAK then brings TRAF6 to the pre-associated complex of TAK1–TAB1–TAB2 on the membrane to form the complex of IRAK–TRAF6–TAK1–TAB1–TAB2. This is then followed by the phosphorylation of TAK1 and TAB2, the dissociation of IRAK, the translocation of the TRAF6–TAK1–TAB1–TAB2 complex to the cytosol and the activation of IKK and MAP kinases.<sup>(73)</sup>

Recent findings have demonstrated the role of polyubiquitination in TRAF6-mediated TAK1 activation and the activation of IKK and MAP kinases.<sup>(32,33)</sup> Using in vitro reconstitution, it was shown that the RING domain protein TRAF6, in conjunction with ubiquitin-conjugating enzyme Ubc13 and the Ubc-like protein Uev1A, mediates a novel form of polyubiquitination involving Lys-63 of ubiquitin. This is different from the well-characterized degradative pathway of polyubiquitination involving Lys-48 of ubiquitin, which can also be mediated by RING domain-containing E3 ubiquitin ligases such as Cbl family proteins.<sup>(74)</sup> TRAF6-mediated Lys-63-linked polyubiquitination does not lead to degradation of target proteins. Rather, it is indispensable for the activation of TAK1, which in turn activates IKK in the NF- $\kappa$ B pathway and phosphorylates MKK6 in the JNK-p38 kinase pathway.

However, there are several unknown aspects of the mechanism of TRAF6-mediated IKK and MAP kinase activation. First, some experiments suggest that the function of RING domains of TRAFs in JNK activation is to induce TRAF raft localization. For example, in the case of TRAF2, if raft translocation is artificially induced, the RING domain becomes dispensable for the activation of JNK but not NF- $\kappa$ B.<sup>(43)</sup> Induced raft translocation of TRAF3 has also been correlated with its acquired ability to activate JNK.<sup>(75)</sup> In contrast, raft translocation may not be required for TRAF6 signaling, which is further supported by the observation that TRAF6 forms cytoplasmic complexes with IRAKs.<sup>(72)</sup> Second, it was shown that for IL-1 and LPS signaling pathways, the RING finger and first zinc finger domains of TRAF6 are likely not required for NF- $\kappa$ B activation but are required for full activation of MAP kinases.<sup>(76)</sup> In addition, it appears that different regions of IRAK are required for IL-1-induced NF- $\kappa$ B and JNK activation, suggesting a divergence of these pathways at the level of IRAK.<sup>(77)</sup>

Several other signaling proteins have also been implicated in inducing and/or modulating TRAF6-mediated NF- $\kappa$ B and MAP kinase activation. ECSIT, a protein conserved between *Drosophila* and mammals, appears to regulate MEKK-1 processing and NF- $\kappa$ B activation in IL-1R/TLR pathways.<sup>(78)</sup> Pellino is another evolutionarily conserved protein family involved in Toll signaling in *Drosophila* and IL-1 signaling in mammals by interacting with Pelle and IRAK–IRAK4–TRAF6 complex, respectively.<sup>(79,80)</sup> Ablation of Pellino 1 or Pellino 2 using either an antisense construct or siRNA showed that Pellino is crucial for IL-1 or LPS-induced activation of NF- $\kappa$ B and IL-8 gene expression.<sup>(79,80)</sup> TRAF6-mediated activation of NF- $\kappa$ B also appears to be regulated by small G proteins such as Ras and Rac1, possibly by associating with the IRAK–TRAF6–TAK1 components.<sup>(81)</sup> In addition, the cytokine-inducible zinc finger protein A20 and A20-like proteins inhibit IL-1 induced NF- $\kappa$ B activation by interacting with TRAF6.<sup>(82)</sup>

Unlike other TRAFs, TRAF6 can activate the Src family of tyrosine kinases, leading to activation of the anti-apoptotic

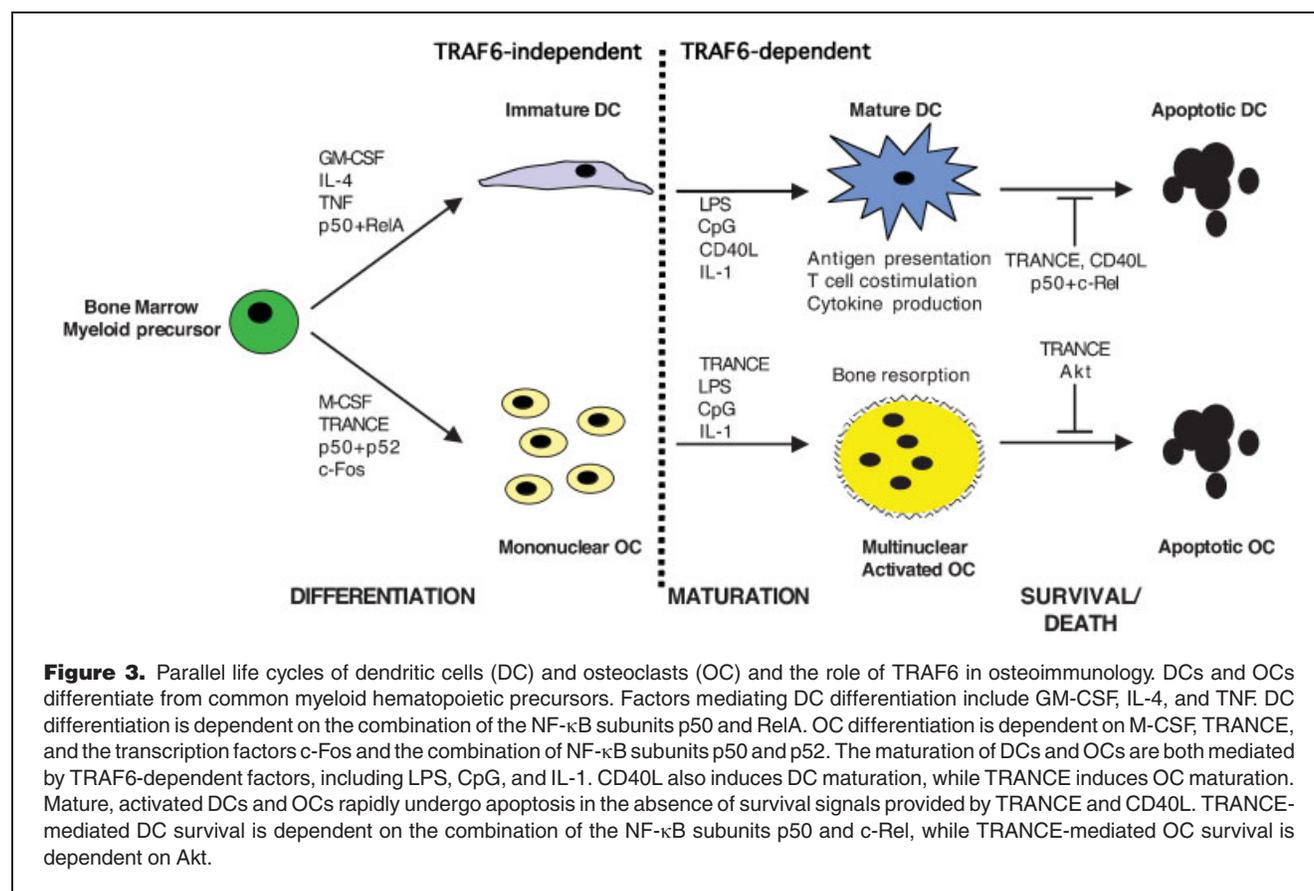
kinase Akt via a PI3-K-dependent pathway.<sup>(34)</sup> In osteoclasts, the activation of c-Src appears to be the mechanism whereby TRANCE and IL-1 induce membrane ruffling and actin ring formation necessary for bone resorption.<sup>(83)</sup> In the absence of c-Src, activation of osteoclasts is severely impaired.<sup>(84)</sup> In addition, c-Src-mediated activation of the survival kinase Akt serves to prolong the lifespan of activated osteoclasts.<sup>(34)</sup> In dendritic cells, although c-Src is activated, the absence of c-Src does not exhibit a dramatic phenotype, possibly due to the presence of other Src family members in these cells (J.R.A. and Y. Choi, unpublished data). In nasal fibroblasts, it has been shown that IL-1-induced chemokine production involves the association of TRAF6 with another Src family member, Syk.<sup>(85)</sup> Interestingly, Cbl family scaffolding proteins, which often downregulate Src signaling, play a positive regulatory role in RANK and CD40-mediated Akt activation.<sup>(86)</sup> The exact molecular mechanism of TRAF6-mediated Src activation is not clear but could involve direct TRAF6-Src interaction and/or colocalization of TRAF6 with Src in membrane rafts.<sup>(34,86)</sup>

### Parallel paradigms between immunity and bone: TRAF6 and osteoimmunology

There exists an intimate interplay between the bone and the immune system. Skeletal bone is more than a frame on which

to hang flesh and organs, it is also the source of bone marrow-derived hematopoietic cells. Many myeloid lineage hematopoietic cells express receptors such as CD40, RANK and TLRs, which use TRAF6 for signaling and are involved in the generation of adaptive and innate immunity. Recently, it has become apparent that the activity of immune cells affects the balance of bone mineralization and resorption carried out by the opposing actions of osteoblasts and osteoclasts.<sup>(87)</sup> For example, increased bone resorption resulting in lytic bone lesions and osteoporosis is observed in many inflammatory and autoimmune diseases, such as rheumatoid arthritis,<sup>(88)</sup> periodontal disease<sup>(89)</sup> and Paget's disease.<sup>(90)</sup> Bone destruction is also common in many cancers, both those that reside in the bone like leukemias and multiple myeloma, and those that metastasize to the bone such as breast and prostate cancers.<sup>(91)</sup>

Dendritic cells, cells specialized to present antigens, and osteoclasts, cells specialized to resorb bone, exhibit parallel lifecycles (Fig. 3). Dendritic cells arise from multipotent precursors of the monocyte lineage and are essential organizers of immune responses. They are highly specialized cells that capture antigens in peripheral tissues, migrate to lymphoid organs, and organize T cell responses.<sup>(92)</sup> Osteoclasts are derived from the same precursors in response to interactions



with osteoblasts and other bone stromal cells. Upon differentiation into mononuclear osteoclasts and subsequent maturation and fusion into multinucleated cells, osteoclasts actively resorb bone.<sup>(93)</sup> A wealth of genetic and biochemical studies have shown that dendritic cells and osteoclasts undergo parallel differentiation, maturation/activation, and survival/death processes. These processes are dependent on a variety of cytokines, transcription factors, and inflammatory mediators, many of which use TRAF6 for signaling. The parallel lifecycles of these myeloid-derived cells has led to the observation of many molecular and cellular interactions between the bone and the immune system, which has been termed osteoimmunology.<sup>(94)</sup>

TRAF6-deficient mice either completely lack osteoclasts or exhibit defective osteoclast function.<sup>(39,44)</sup> Recent studies have also found that immature dendritic cells derived from TRAF6<sup>-/-</sup> mice have defects in cytokine production and costimulatory molecule upregulation in response to CD40L and microbial products *in vitro* and *in vivo*. These defects result in impaired T cell stimulation (T. Kobayashi and Y. Choi, personal communications). Given that immature dendritic cells have been shown to provoke tolerogenic T cell responses,<sup>(95)</sup> targeting TRAF6 in dendritic cells may ultimately be a useful tool in preventing autoimmunity.

Since TRANCE is expressed on activated T cells, and is crucial for T cell–dendritic cell communication, one might expect massive bone resorption under most inflammatory conditions. Although TRANCE-expressing T cells in chronic inflammatory conditions such as rheumatoid arthritis can stimulate osteoclasts leading to bone destruction,<sup>(96)</sup> the constant activity of T cells fighting the universe of antigens to which we are exposed does not usually cause extensive bone loss. A crucial counter-regulatory mechanism whereby activated T cells can inhibit TRANCE-mediated osteoclast development and activation is through the action of the antiviral cytokine IFN- $\gamma$ . In mice deficient for the IFN- $\gamma$  receptor, bone destruction in an autoimmune arthritis model is greatly exacerbated. While T cells involved in inflammatory responses express TRANCE, they also secrete IFN- $\gamma$ . IFN- $\gamma$  can block TRANCE-mediated osteoclastogenesis, possibly through the activation of the ubiquitin–proteasome pathway leading to TRAF6 degradation.<sup>(94,97)</sup>

### TRAF6 inhibitors

Given the essential roles of TRAF6 in immunity and a diverse array of biological processes, it is desirable to obtain TRAF6 inhibitors to facilitate the development of therapeutics for controlling inflammation and a wide range of diseases, such as osteoporosis and other osteolytic conditions, cystic fibrosis, periodontitis, connective tissue destruction, bladder outlet obstruction and viral infections.<sup>(98–100)</sup> By fusing a TRAF6-binding sequence to a cell permeable tag sequence, we found that the peptide inhibited TRAF6 signaling in the context of

RANK-dependent osteoclast differentiation from RAW264.7 cells or mouse primary monocytes.<sup>(25)</sup> Furthermore, this “decoy” peptide appears to be effective against breast-cancer-induced osteolytic lesions in mice (B. Darnay, personal communications). It will be interesting to test this approach in other TRAF6-dependent disease conditions. In addition to their potential therapeutic value, TRAF6 inhibitors provide powerful tools for dissecting the contribution of TRAF6 to specific biological processes.

### Conclusions

Of the six known TRAF proteins, TRAF6 has several unique features that contribute to its diverse physiological functions. Evolutionarily, TRAF6 is the most ancient of the mammalian TRAF proteins and is the most divergent in its TRAF domain. In parallel to its ancient and modern functionality, it serves as a molecular bridge between innate and adaptive immunity. The vital role of TRAF6 in the life cycles of myeloid-derived cells has revealed many interconnections between the immune system and the bone, and TRAF6 is the central player in osteoimmunology. Its roles in dendritic cell and osteoclast biology have shown it to be a potential therapeutic target for the treatment of autoimmune and inflammatory diseases as well as osteoporosis. TRAF6 appears to mediate kinase activation by non-degradative ubiquitination of both itself and possibly downstream signaling molecules. It may also influence signaling by serving as an adapter molecule, bringing multiple proteins into close proximity, enhancing their interactions and regulating the activation of multiple signals, including NF- $\kappa$ B, MAP kinases, and Src-family kinases. Because TRAF6 is a convergence point for many diverse signals both upstream and downstream, it will remain an important focus of investigation for a wide range of biological interests.

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