CHAPTER 7

Ubiquitination and TRAF Signaling

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Introduction

The Ubiquitin-Proteasome Pathway

Ubiquitin (Ub) is a highly conserved small polypeptide that is ubiquitously expressed in all eukaryotic cells. The best-known function of ubiquitin is to target protein degradation through covalent attachment of this polypeptide on protein substrates.[1-3] This covalent modification, known as ubiquitination, is carried out via a three-step enzymatic cascade. In the first step, Ub is activated by the Ub-activating enzyme (E1) in an ATP-dependent reaction to form an E1-Ub thioester. In the second step, the activated Ub is transferred to a cysteine residue in the active site of a Ub-conjugating enzyme (Ubc or E2) to form an E2-Ub thioester. Finally, in the presence of a Ub-protein ligase (E3), ubiquitin is conjugated to a protein substrate by forming an isopeptide bond between the carboxyl terminus of ubiquitin and the ε-amino group of a lysine residue on the protein target. After Ub is conjugated to a protein substrate, Ub itself can be conjugated by another Ub through one of its seven lysines, typically lysine-48. This process reiterates itself in a highly processive manner to form a polyubiquitin chain, which is then recruited to a large ATP-dependent protease complex called the 26S proteasome. The polyubiquitinated protein substrates are degraded inside the proteasome, whereas the polyubiquitin chains are cleaved to monomeric ubiquitin, which is recycled.

The 26S proteasome is composed of the 20S catalytic core and 19S regulatory particle.[4] The 20S proteasome is a cylinder-like structure formed by four rings, each containing seven subunits. These subunits form an enclosed proteolytic chamber within which the catalytic residues reside. This chamber is impermeable to proteins, except for a narrow channel that connects to the 19S proteasome, which gates the entry of protein substrates. The 19S complex can be further separated into a base and a lid. The base contains multiple ATPase subunits, which presumably function to unfold ubiquitinated protein substrates and propel the unfolded polypeptides through the narrow channel into the catalytic chamber of the proteasome. The lid contains nonATPase subunits, some of which bind to polyubiquitin chains and recruit polyubiquitinated proteins to the proteasome.

The substrate specificity of ubiquitination is dictated by a large family of E2s (more than 40 members in human) and a very large family of E3s (more than 700 members in human). All E2s contain a highly conserved domain called the Ubc domain, which has an invariant cysteine residue in the active site. The vast majority of E3s contain either a RING (Really INteresting Gene) or HECT domain (Homology to E6AP C-Terminus).[5-9] The RING domain E3s function either as a single polypeptide, such as TRAF (TNF Receptor Associated Factor; see below) and IAP (Inhibitor of Apoptosis Protein), or as a subunit of multi-protein complexes. The classical examples of multi-subunit E3s include APC/C (Anaphase Promoting Complex/Cyclosome), which ubiquitinates cell cycle proteins such as cyclins,[10,11] and SCF (Skp1-Cul1-F-box), which ubiquitinates many cellular proteins

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such as the NF-κB inhibitor IκB and the cyclin-dependent kinase inhibitor p27.[12-15] APC/C contains the RING domain protein APC11, whereas SCF contains the RING domain protein Rbx1 (also known as Roc1 or Hrt1) as the catalytic core.[5] The RING domain of E3s interacts with E2s to facilitate polyubiquitination, but the detailed mechanism by which RING E3s facilitate polyubiquitination is not understood. In contrast, the catalytic mechanism of HECT domain E3s is better understood. The HECT domain contains a catalytic cysteine which accepts Ub from an E2 in a thioester relay, and transfers the Ub directly to a lysine residue of the target protein.[16] Examples of the HECT domain E3s include E6AP, which ubiquitinates p53 and targets p53 for degradation in cells expressing the human papillomavirus (HPV) protein E6,[17-19] and NEDD4, which ubiquitinates several cell surface proteins and targets these proteins for endocytosis.[20]

Like other reversible covalent modification such as phosphorylation, ubiquitination can also be reversed by a large family of deubiquitination enzymes (DUBs, also known as isopeptidase).[21] The majority of DUBs are cysteine proteases, which can be classified into four subfamilies based on the following related but distinct domains: UBP (ubiquitin-specific protease), UCH (ubiquitin carboxyl-terminal hydrolase), OTU (ovarian tumor related), and Ataxin-3/Josephin. The fifth subfamily of DUBs are metalloproteases that contain a unique JAMM/MPN+ domain, which was first discovered in a subunit (Rpn11) of the 19S regulatory particle of the proteasome and a subunit (JAB1/CSN5) of the proteasome-like particle termed COP9/Signalosome (CSN).

The NF-κB Pathway

The NF-κB/Rel family of transcription factors controls many physiological processes including inflammation, immunity and apoptosis.[22-24] Members of this family include Rel-A (p65), Rel-B, c-Rel, p50 and p52. These proteins form homo- or hetero-dimers that bind to a consensus DNA sequence known as the κB site, which is present in a large variety of genes. All members of the NF-κB family contain a highly conserved Rel-homology domain (RHD), which is responsible for DNA binding, dimerization, nuclear translocation, and interaction with the NF-κB inhibitor IκB. IκB binds to the nuclear localization sequence of NF-κB, thus sequestering NF-κB in the cytoplasm. IκB is also a multi-member family, which includes IκBα, IκBβ and IκBε. All of these IκB proteins contain 6-7 repeats of ankyrin motifs, which bind to the RHD domain of NF-κB. The ankyrin repeats are also present at the C-termini of the NF-κB precursors p105 and p100, which are processed to the mature subunits p50 and p52, respectively.

The NF-κB activation pathway is broadly classified into the canonical and noncanonical pathways, depending on whether the pathway involves the degradation of IκB or processing of the NF-κB precursors, especially p100.[25] In the canonical pathway, stimulation of cells with an NF-κB agonist, such as tumor necrosis factor-α (TNFα) or interleukin-1β (IL-1β), leads to the activation of a large kinase complex consisting of IKKα, IKKβ and the essential regulatory protein NEMO (also known as IKKγ). This IKK complex, in particular IKKβ, phosphorylates IκB proteins at two N-terminal serine residues, thereby targeting IκB for ubiquitination and subsequent degradation by the proteasome. NF-κB is then liberated to enter the nucleus to carry out its nuclear functions. In the noncanonical pathway, which usually occurs in B cells, stimulation of certain subsets of the TNF receptor superfamily, such as CD40 and BAFF receptor, leads to activation of the protein kinase NIK. NIK in turn phosphorylates and activates IKKα, which then phosphorylates p100 and targets this precursor for polyubiquitination. Unlike IκB, polyubiquitinated p100 is not completely degraded by the proteasome. Rather, the polyubiquitin chain recruits the proteasome to degrade only the C-terminal domain of p100, while leaving the N-terminal RHD domain intact, thus generating the mature p52 subunit. p52 forms a heterodimer with Rel-B, and this dimeric complex translocates to the nucleus to activate target genes involved in B cell maturation. p105 can also be processed to p50 cotranslationally or post-translationally, both requiring the proteasome.[26,27] The cotranslational processing is a constitutive process that may not require phosphorylation or ubiquitination, whereas post-translational processing requires phosphorylation and ubiquitination of p105, which is induced by some agents such as the bacterial lipopolysaccharides (LPS). LPS can also induce the complete degradation of p105, leading to the activation of the p105-associated kinase Tpl2, a MAP3K required for ERK activation.[28]

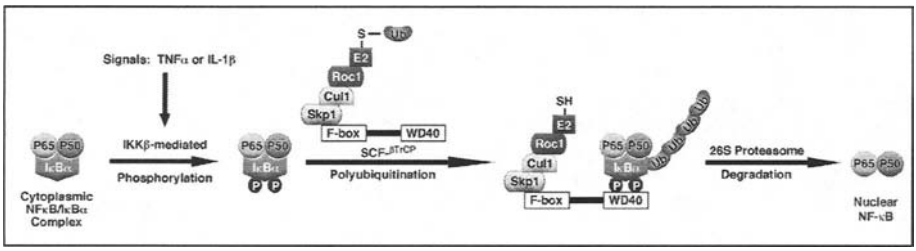

Figure 1. The biochemical pathway of IκBα ubiquitination and degradation. In response to NF-κB stimuli, IκBα is phosphorylated by IKK at two specific N-terminal serine residues. The phosphorylated IκBα is recruited to the $SCF^{\beta TrCP}$ ubiquitin ligase complex, which is composed of Skp1, Cul1, Roc1, and the F-box protein βTrCP. βTrCP contains seven WD40 repeats that bind specifically to the phosphorylated form of IκBα. The RING domain protein Roc1 recruits the E2 Ubc5, and facilitates the transfer of ubiquitin from the E2 to two N-terminal lysine residues of IκBα. After IκBα is polyubiquitinated, it remains bound to NF-κB (shown as the p50/p65 heterodimer), but is selectively degraded by the 26S proteasome. NF-κB then enters the nucleus to regulate the expression of target genes that mediate inflammation, immunity and cell survival.

Both IκB degradation and NF-κB processing require the SCF E3 complex containing Skp1, Cul1, the F-box protein βTrCP, and the RING domain protein Roc1 (Fig. 1).[29] βTrCP contains seven WD40 repeats, which bind specifically to the phosphorylated form of IκB, p100 and p105. The F-box of βTrCP binds to Skp1, which in turn binds to Cul1. Cul1 interacts with Rbx1, which recruits the E2 Ubch5 to ubiquitinate the phosphorylated substrates. This model is verified by the elegant crystal structure of the SCF-βTrCP complex bound to a phosphorylated peptide that contains the destruction motif DpSGΨXpS (where Ψ denotes hydrophobic residue, X any amino acid, and pS phosphoserine).[30,31] This motif is present in several βTrCP targets including IκB, p100 and β-catenin, a transcriptional coactivator in the Wnt pathway.

Roles of Ubiquitination in IKK Activation by TRAF Proteins

Structure and Function of TRAF Proteins

TRAF proteins are crucial signal transducers that mediate the activation of NF-κB and mitogen-activated protein kinases (MAPKs) by TNF receptors (TNFRs), IL-1 receptor (IL-1R) and Toll-like receptors (TLRs).[32,33] The founding members of the TRAF family, TRAF1 and TRAF2, were identified as proteins that associate with the type-2 TNFR (TNF-R2).[34] This family has now expanded to seven members. Except for TRAF7, all TRAF proteins contain a conserved C-terminal TRAF domain, which mediates interaction with cell surface receptors as well as other upstream signaling proteins. The N-terminal segment of the TRAF domain contains a coiled-coil structure that mediates the oligomerization of TRAF proteins. All TRAF proteins except TRAF1 also contain a conserved N-terminal RING domain followed by several zinc finger domains. These N-terminal domains are responsible for downstream signaling to NF-κB and MAPKs such as JNK and p38.

Among TRAF proteins, TRAF2 and TRAF6 have been most extensively studied. TRAF2 mediates the TNFR signaling cascade, whereas TRAF6 is essential for signaling from IL-1R and TLRs. In the TNFR pathway, the binding of the trimeric TNFα ligand leads to the trimerization of the type-I TNF receptor (TNF-R1), which recruits the death domain adaptor protein TRADD.[35] TRADD interacts with TRAF2 as well as the receptor-interacting kinase-1 (RIP1). The formation of these receptor-associated protein complex results in the activation of IKK and JNK, ultimately leading to the activation of NF-κB and AP1, respectively. Genetic ablation of RIP1 abolishes NF-κB activation by TNFα; however, reconstitution experiments show that the kinase activity of RIP1 is not required for NF-κB activation.[36,37] Deletion of TRAF2 in mouse embryonic fibroblasts (MEF) blocks JNK but not NF-κB activation by TNFα.[38] The normal NF-κB activation in TRAF2-deficient cells is likely due to the compensatory function of TRAF5, as the double knockout of TRAF2 and TRAF5 eliminates TNFα-induced NF-κB activation.[39] In the TRAF6 pathways, stimulation of

IL-1R or TLR with a cognate ligand leads to the sequential recruitment of adaptor proteins—MyD88, IRAK4, IRAK1 and TRAF6—to the receptor complex.[40] The kinase IRAK4 phosphorylates IRAK1, resulting in the release of IRAK1 and TRAF6 into the cytoplasm, where they activate the IKK and JNK pathways. Genetic experiments show that TRAF6-deficiency not only prevents NF-κB and JNK activation by IL-1R and the majority of TLRs, but also abolishes signaling by several receptors of the TNFR superfamily, including CD40, lymphotoxin-β receptor, and the latent membrane protein 1 (LMP1) of Epstein-Barr virus.[41-43] Recent studies have also shown that TRAF6 is essential for the development of regulatory T cells that suppress autoimmunity.[44]

TRAF Proteins Are Ubiquitin Ligases

Recent biochemical studies have begun to unravel the signaling mechanism of TRAF proteins. In the course of studying how TRAF6 activates IKK, two intermediary factors that link TRAF6 to IKK activation were identified. The first factor, termed TRIKA1 (TRAF6-regulated IKK activator 1), is a Ub-conjugating enzyme (E2) complex comprised of Ubc13 and a Ubc-like protein Uev1A.[45] The second factor, termed TRIKA2, is a ternary complex consisting of the protein kinase TAK1 and two adaptor proteins TAB1 and TAB2.[46] The identification of Ubc13/Uev1A as an activator of IKK was particularly interesting, and it led to the discovery of TRAF6 as a RING domain ubiquitin ligase (E3) that functions together with Ubc13/Uev1A to synthesize a unique lysine 63 (K63)-linked polyubiquitin chain.[45] Subsequent studies have identified several targets of K63-linked polyubiquitination, including NEMO and TRAF6 itself.[46-56] Through a proteasome-independent mechanism, the K63 polyubiquitination of TRAF6 leads to the activation of TAK1, which subsequently phosphorylates IKKβ at two serine residues in the activation loop, resulting in IKK activation (Fig. 2). TAK1 also phosphorylates an MKK such as MKK6, which activates the JNK and p38 kinase pathways.[46]

Like TRAF6, TRAF2 is also a RING domain protein that catalyzes K63-linked polyubiquitin chain synthesis in conjunction with Ubc13/Uev1A.[45,57] A dominant negative mutant of Ubc13 inhibits NF-κB activation by TRAF2, suggesting that TRAF2 activates NF-κB through a ubiquitination-dependent mechanism.[45] Ubc13 and TRAF2 polyubiquitination have also been shown to mediate the activation of germinal center kinase-related (GCKR) and JNK by TNFα.[54] A recent study confirmed the importance of TRAF2 ubiquitination in JNK activation, but found that TRAF2 ubiquitination is not required for the activation of p38 kinase and NF-κB.[55] This finding is consistent with the phenotypes of TRAF2-deficient MEF cells, which are defective in JNK activation but have normal NF-κB function.[38] Thus, ubiquitination of other proteins such as TRAF5 or RIP may also be important for NF-κB activation in the TNFα pathway.[39,57-59] The ubiquitin ligase activity of TRAF2 may have both positive and negative effects on the NF-κB signaling pathways.[60] For example, while TRAF2 is an activator of the canonical NF-κB pathway, it functions as an inhibitor of the noncanonical pathway, perhaps by targeting certain signaling proteins in this pathway for degradation by the proteasome.[61] Indeed, TRAF2 has been shown to target TRAF3 for ubiquitination and degradation in B cells following CD40 stimulation.[62] TRAF2 itself can also be degraded in certain B cell lines after stimulation with CD40 ligand.[63] Furthermore, stimulation of TNFR2 in T cells by TNFα leads to the polyubiquitination of TRAF2 by another RING domain protein c-IAP1 (cellular inhibitor of apoptosis 1), resulting in TRAF2 degradation by the proteasome.[64] Thus, polyubiquitination of TRAF2 may lead to the activation of downstream kinases or result in proteasomal degradation, perhaps depending on the configuration of the polyubiquitin chains.

The discovery of the role of TRAF ubiquitination in IKK activation provides an explanation for the earlier observations that the RING domains of TRAF2 and TRAF6 are the effector domains in downstream signaling. Removal of the RING domains of TRAF2 and TRAF6 converts these proteins into dominant negative mutants that inhibit the TNFα and IL-1β pathways, respectively.[65,66] Conversely, when the C-terminal TRAF domains of TRAF2 and TRAF6 were replaced with an inducible dimerization domain, it was found that dimerization of the chimeric TRAF proteins was sufficient to activate IKK and JNK.[46,67] Consistent with an essential role of ubiquitination in TAK1 and JNK activation, TRAF6-deficient MEF cells complemented with a TRAF6 mutant lacking the RING domain failed to activate TAK1 or JNK.[68] Surprisingly, these

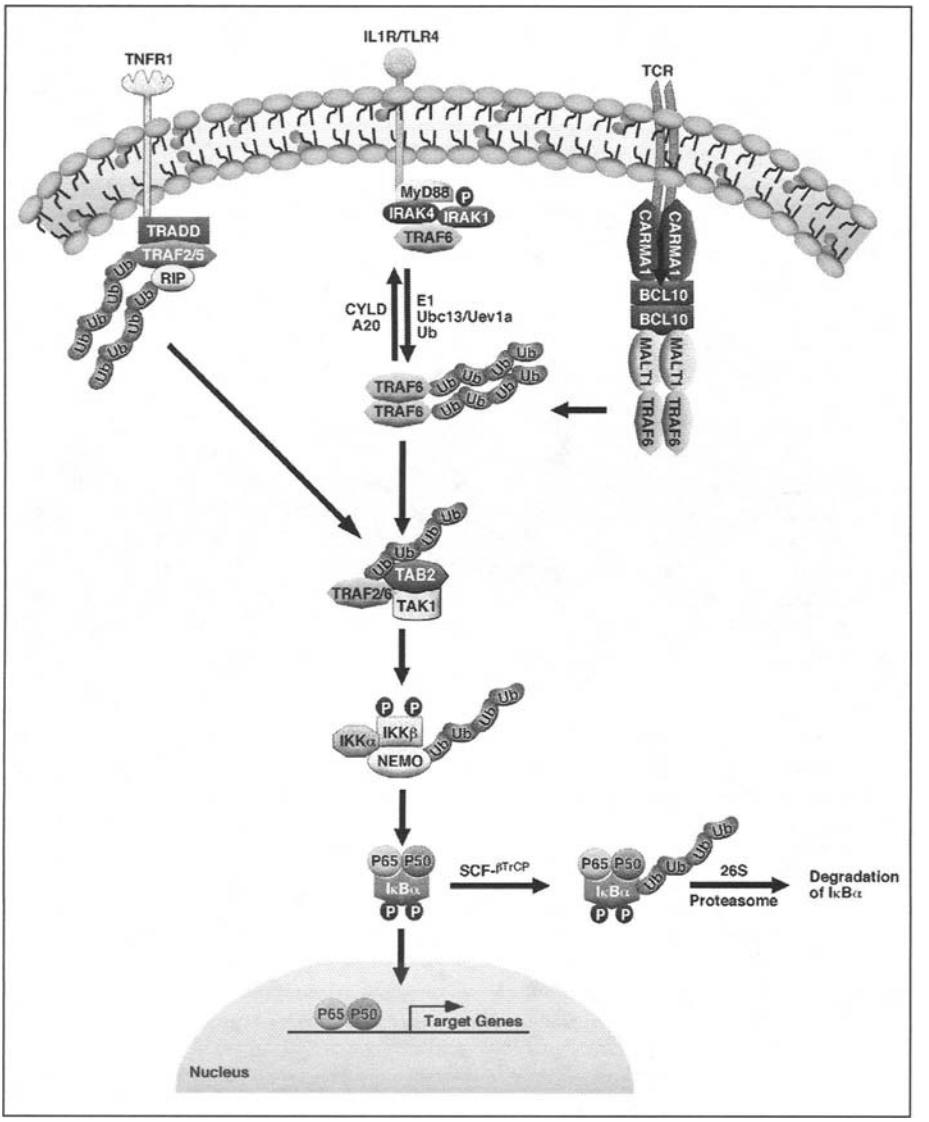

Figure 2. Ubiquitin-mediated activation of TAK1 and IKK by TRAF proteins. In response to proinflammatory cytokines or pathogens, TNF receptors (TNFR), IL-1 receptor (IL-1R), or Toll-like receptors (TLR) bind to their cognate ligands and activate a signaling cascade leading to the activation of TRAF ubiquitin ligases, including TRAF2 and TRAF6. Similarly, in the adaptive immunity pathway, stimulation of the T cell receptors (TCR) with antigenic peptides leads to the membrane recruitment of a protein complex consisting of CARMA1, BCL10 and MALT1. These proteins regulate TRAF2 and TRAF6 by promoting their oligomerization, resulting in the activation of TRAF ubiquitin ligase activity. Activated TRAF proteins catalyze the K63-linked polyubiquitination of target proteins including RIP, NEMO and the TRAF proteins themselves. This polyubiquitination requires E1, Ubc13/Uev1A (E2), and ubiquitin, and can be reversed by deubiquitination enzymes CYLD or A20. The K63-linked polyubiquitin chains facilitate the recruitment of the TAK1/TAB2 complex through interacting with the novel zinc finger (NZF) domain of TAB2. The recruitment of TAK1/TAB2 to ubiquitinated TRAF proteins leads to the activation of TAK1, which in turn activates IKK through direct phosphorylation of IKKβ within the activation loop. IKKβ then phosphorylates IκB and targets this inhibitor for degradation by the ubiquitin-proteasome pathway.

cells were still capable of activating NF-κB in response to IL-1 or LPS, suggesting the existence of a TAK1-independent pathway of NF-κB activation in MEF cells (see below).

Regulation of TRAF Ubiquitin Ligase Activity

As discussed above, chemical-induced dimerization of TRAF6 is sufficient to activate the NF-κB pathway. Interestingly, forced dimerization of TRAF6 also leads to polyubiquitination of TRAF6 itself, suggesting that the ubiquitin ligase activity of TRAF6 is activated by dimerization or oligomerization.[46] Recently, several cellular proteins in the NF-κB pathways have been found to promote the oligomerization of TRAF6. One of these proteins, TIFA [TRAF interacting protein with forkhead associated (FHA) domain], has been identified as a protein that connects IRAK1 to TRAF6 in the IL-1 pathway.[69] Biochemical experiments show that TIFA binds to TRAF6 and induces TRAF6 oligomerization and polyubiquitination, thereby activating IKK.[70]

Another example of TRAF6 regulation by oligomerization is provided from the study of the T cell receptor (TCR) signaling pathway.[48] Stimulation of TCR with an MHC (major histocompatibility complex)-bound antigenic peptide leads to the activation of a tyrosine phosphorylation cascade that in turn activates the serine/threonine kinase PKCθ.[71] PKCθ then facilitates the formation of a complex containing the CARD domain proteins CARMA1 and BCL10, and the paracaspase MALT1.[72,73] This complex is recruited to the lipid rafts where activated TCR and other signaling proteins are localized. The environment within the lipid rafts may promote the oligomerization of BCL10 and MALT1. Two recent studies show that BCL10 and MALT1 activate IKK by inducing K63-linked polyubiquitination of NEMO.[48,49] In one study, it was shown that MALT1 is a ubiquitin ligase that functions together with Ubc13/Uev1A to mediate the polyubiquitination of NEMO at a specific lysine (K399).[49] In the other study, it was found that MALT1 binds to TRAF6 through a C-terminal TRAF6-binding site.[48] Through this binding, the oligomerized forms of MALT1 induce TRAF6 oligomerization and the activation of TRAF6 ubiquitin ligase, which catalyzes the polyubiquitination of NEMO as well as TRAF6 itself. The latter study also showed that the TAK1 kinase complex is involved in IKK activation in T cells, and that the T cell signaling pathway from BCL10 to IκB phosphorylation can be reconstituted in vitro using purified proteins. In any case, these studies show that oligomerization of ubiquitin ligases may be an important mechanism of ligase activation.

Deubiquitination Enzymes Downregulate IKK Activation

The activation of NF-κB by proinflammatory cytokines is a rapid and transient process. For example, in most cells TNFα induces the activation of IKK and nuclear translocation of NF-κB within a few minutes. After NF-κB enters the nucleus, it turns on many genes involved in immune and inflammatory responses, as well as some genes that shut down the NF-κB pathway. One of the immediate early target genes of NF-κB is IκBα, which can enter the nucleus to displace NF-κB from the DNA, and transport it back to the cytoplasm.[74-76] To prevent the newly synthesized IκBα from being degraded, IKK activation must also be turned off. While the mechanisms of IKK down regulation are not fully understood, recent studies suggest that deubiquitination is a key mechanism. Two inhibitors of IKK activation have recently been shown to function as deubiquitination enzymes to disassemble K63-linked polyubiquitin chains from signaling proteins that are required for IKK activation. One of these inhibitors is the cylindromatosis protein CYLD, a tumor suppressor found in human patients with a type of skin tumor called cylindroma.[77] CYLD contains a C-terminal UBP domain that is frequently mutated in cylindroma patients. CYLD binds to TRAF2 and NEMO, and inhibits IKK activation by cleaving K63-linked polyubiquitin chains on TRAF2, TRAF6 and NEMO.[51,52,56] The UBP domain mutations found in the cylindroma patients abrogate the ability of CYLD to inhibit IKK and NF-κB, resulting in hyperactivation of NF-κB, which may contribute to tumorigenesis. However, it is not known why the loss of CYLD function only leads to skin tumor. CYLD is one of the target genes of NF-κB, indicating that the NF-κB pathway has a built-in negative feedback loop to regulate its own activation. CYLD is also regulated by IKK-dependent phosphorylation, which inactivates the ability of CYLD to prevent TRAF2 polyubiquitination.[78] CYLD also inhibits JNK activation by multiple proinflammatory cytokines that signal through

TNFRs, IL-1R and TLRs.[79] As NEMO is not required for JNK activation, the targets of CYLD in the JNK pathway are likely to be TRAF2 and TRAF6, not NEMO.

The other NF-κB inhibitor, A20, is also a well-known target gene of NF-κB and its expression is rapidly induced by TNFα.[80,81] Mice lacking A20 develop severe inflammation in multiple organs, owing to prolonged activation of IKK.[82] A20 contains an N-terminal OTU deubiquitination enzyme domain, and seven zinc finger domains at the carboxyl terminus. Recent studies show that both the N- and C-terminal domains of A20 are utilized to inhibit IKK.[59,83,84] The OTU domain first disassembles K63-linked polyubiquitin chains on RIP1 in the TNFα pathway,[59] and TRAF6 in the LPS pathway,[83] thereby inhibiting IKK. Subsequently, the C-terminal zinc finger domains function as a ubiquitin ligase to synthesize K48-linked polyubiquitin chains on RIP1, thus targeting RIP1 for degradation by the proteasome.[59] Interestingly, it was shown that the K63 polyubiquitin chains on RIP1 must be removed before RIP1 can be conjugated by the K48 chains. This coupling of deubiquitination and ubiquitination by A20 results in the potent suppression of IKK.

Signaling Pathways Downstream of TRAF Proteins

TAK1 and Its Associated Proteins

TAK1 was initially identified as a TGFβ-activated kinase.[85] Subsequent experiments show that TAK1 mediates NF-κB and JNK activation by IL-1β and TNFα.[46,57,86] Biochemical experiments provide the direct evidence that TAK1 is an IKK kinase that phosphorylates IKKβ at key serine residues in the activation loop.[46] Numerous experiments employing different technologies including RNAi and chemical inhibition of TAK1 have now provided strong evidence that TAK1 is required for IKK and JNK activation by IL-1β and TNFα in mammalian cells.[87-89] However, it remains to be seen whether genetic knockout of TAK1 in higher organisms affects NF-κB or JNK activation in vivo. In *Drosophila*, the essential role of TAK1 in IKK and JNK activation in vivo has been demonstrated.[90] *Drosophila* mutants lacking dTAK1 is severely defective in producing antimicrobial peptides in response to bacterial infection, which activates an NF-κB-like (Relish) pathway in *Drosophila*.[22] In addition, RNAi of dTAK1 in *Drosophila Schneider* cells abolishes IKK and JNK activation by bacterial peptidylglycans.[91,92] Thus, the role of TAK1 in NF-κB activation and innate immunity is evolutionarily conserved.

TAK1 forms a complex with TAB1 and TAB2.[93,94] The recently identified TAB2-associated protein, TAB3, can also associate with TAK1 and TAB1.[57,95,96] TAB2 and TAB3 may have redundant functions, as the TAB2-deficient MEF cells have normal activation of NF-κB and JNK in response to TNFα or IL-1β.[97] Indeed, RNAi of both TAB2 and TAB3 markedly reduced IKK and JNK activation by TNFα or IL-1β[57,95,96] TAB2 and TAB3 contain two highly conserved domains, an N-terminal CUE domain, and a C-terminal domain NZF (novel zinc finger) domain. While both domains are Ub-binding domains, the CUE domain appears to be dispensable for NF-κB activation.[57] In contrast, removal or mutation of the NZF domain abolishes the ability of TAB2 and TAB3 to activate TAK1 and IKK. The NZF domain binds preferentially to K63 polyubiquitin chains, and the replacement of the NZF domain with different classes of Ub-binding domains from unrelated proteins restores the signaling function of TAB2 and TAB3.[57] Thus, polyubiquitination may facilitate the interaction between TRAF6 and TAB2 (or TAB3), resulting in the activation of the TAB2-associated kinase TAK1. The mechanism of Ub-mediated activation of TAK1 and IKK by TAB2 and TAB3 is evolutionarily conserved. *Drosophila* has a TAB2-like molecule (dTAB2), which also has the conserved CUE and NZF domains. Remarkably, *Drosophila* harboring mutations in the NZF domain of dTAB2 are defective in antibacterial responses (D. Ferrandon, personal communication). Further supporting the role of ubiquitination in IKK activation in *Drosophila*, RNAi of the *Drosophila* homologues of Ubc13 and Uev1A leads to impaired IKK activation and reduced antibacterial peptide expression.[96a] *Drosophila* also has a TRAF homologue (dTRAF2) that contains the RING domain. The role of dTRAF2 in the immunity pathway is not clear, as RNAi of dTRAF2 in *Schneider* cells has no apparent effect on antibacterial peptide expression.[96a] However, a recent report shows that dTRAF2 mutant larvae are partially defective in the expression of some antimicrobial peptides following *E. coli* challenge.[98]

TAB1 is a potent activator of TAK1, even in the absence of ubiquitination.[93,99,100] However, the endogenous TAK1 complex is inactive, even though it contains TAB1 and TAB2. In vitro reconstitution experiments showed that TRAF6-dependent activation of IKK requires TAK1 and TAB2, but not TAB1.[46] Thus, the role of TAB1 in IKK activation is not clear. In fact, there is no apparent TAB1 homologue in *Drosophila*. Mice devoid of TAB1 are embryonic lethal, and the mutant embryos exhibit abnormal cardiac phenotypes that resemble those of TGF-β2 knockout mice.[101] It is possible that TAB1 is important for TGF-β rather than NF-κB signaling.

TAK1-Independent Signaling Pathways Downstream of TRAF Proteins

Several lines of evidence suggest that TAK1 is not the only mediator of TRAF signaling. First, although the *Drosophila* mutants lacking functional TAK1 or TAB2 are severely defective in antibacterial immunity, these mutants are nevertheless more resistant to bacterial killing than those mutants lacking dIKK or other essential signaling components (e.g, IMD, a RIP1 homologue).[90] Second, in mammalian cells, knockdown of TAK1 expression by RNAi, or chemical inhibition of TAK1 activity, blocks JNK activation, but does not completely inhibit IKK activation by TNFα or IL-1β.[57,87,88] Third, in TAB2-deficient MEF cells,[97] or in MEF cells expressing a TRAF6 mutant lacking the RING domain,[68] IL-1-induced activation of TAK1 is impaired, but NF-κB activation appears to be largely normal. Thus, it is likely that TRAF protein can activate IKK through some pathways that are independent of, or redundant with, the TAK1 pathway. One of these pathways may be mediated through MEKK3, as MEKK3-deficient cells are partially defective in IKK activation in response to TNFα, IL-1β or LPS.[102,103] MEKK3 binds to TRAF2, TRAF6, TRAF7 and RIP, and may link these proteins directly to the IKK complex.[102-104] However, the role of MEKK3 in IKK activation may depend on cell types, as we found that effective silencing of MEKK3 expression in several human cell lines did not inhibit IKK activation by TNFα or IL-1β, whereas silencing of TAK1 expression in the same cell lines markedly reduced IKK activation (C-K.Ea, M. Hong, Z. Chen, unpublished). Furthermore, simultaneous knockdown of both TAK1 and MEKK3 by RNAi did not further inhibit IKK activation beyond what was achieved with TAK1 RNAi alone.

Several other kinases may also be the downstream targets of TRAF proteins. One of these kinases is GCKR, a MAP3K that can be activated by TNFα or TRAF2. It has been shown that TRAF2 and Ubc13/Uev1A promote GCKR polyubiquitination and activation, resulting in the activation of JNK.[54] Another TRAF-interacting MAP3K, apoptosis signal-regulating kinase 1 (ASK1), is required for sustained activations of JNK, p38 and apoptosis.[105] ASK1 interacts with and is activated by several TRAF proteins, including TRAF2 and TRAF6.[106] Interestingly, a TRAF2 mutant lacking the RING domain inhibits the TNFα-dependent activation of ASK1. A recent study shows that the binding of LPS to TLR4 induces the production of intracellular reactive oxygen species, which leads to the formation of a complex containing TRAF6 and ASK1.[107] Through an unknown mechanism, TRAF6 activates ASK1, which in turn activates the p38 kinase required for innate immune responses against bacteria. Another example of TRAF6 activating a downstream kinase in innate immunity is provided from the study of interferon-α induction by TLRs that bind to viral RNA (TLR7-8) and bacterial DNA (TLR9).[108-110] The induction of interferon-α requires MyD88, TRAF6 and the transcription factor IRF7. Following the activation of TLRs by viral RNA or bacterial DNA, IRF7 forms a complex with MyD88, IRAK1, IRAK4 and TRAF6. TRAF6 then activates a putative IRF7 kinase that phosphorylates IRF7, allowing IRF7 to dimerize and translocate to the nucleus to turn on interferon-α. Interestingly, Ubc13 and the RING domain of TRAF6 are required for IRF7 activation, suggesting that K63-linked polyubiquitination may play a role in the activation of an IRF7 kinase.

Conclusions and Perspectives

Research in the past few years has firmly established the central role of TRAF proteins in inflammation and immunity. The discovery of TRAF proteins as ubiquitin ligases and the in vitro reconstitution of TRAF6 signaling pathways have set the stage for a detailed study of the TRAF signaling mechanism. This mechanism involves, at least in part, the lysine-63 polyubiquitination of several proteins in the NF-κB pathway, including RIP, NEMO, and TRAF proteins themselves. However,

the roles of polyubiquitination of these proteins in the NF-κB pathway have not been fully investigated. In addition, the mechanism by which polyubiquitination activates TAK1 and IKK requires further studies. In this regard, the identification of TAB2 and TAB3 as polyubiquitin chain binding proteins provides some clues to the mechanism of TAK1 activation, but more work employing modern biophysical techniques is clearly needed in order to understand how the binding of a polyubiquitin ligand to the receptors (TAB2 and TAB3) activates the receptor-associated kinase (TAK1). Future research should also address the in vivo functions of TAK1 in higher organisms, and to investigate other mechanisms of IKK activation that may be independent of TAK1. Finally, it will be of enormous interest to explore the possibility that the ubiquitin signaling mechanism learnt from TRAF proteins may be applicable to other signaling pathways.

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