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News and Commentary

Ubiquitin, TAK1 and IKK: is there a connection?

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The basic framework of NF-κB signaling was established within the first decade of the groundbreaking discovery of NF-κB.[1] According to this framework, NF-κB is a transcription factor sequestered in the cytoplasm of unstimulated cells through its association with an inhibitory protein of the IκB family. Stimulation of cells with a variety of agents, including proinflammatory cytokines such as TNFα and IL-1β, leads to the activation of a protein kinase that phosphorylates IκB at specific sites. This signal-induced site-specific phosphorylation targets IκB for polyubiquitination and subsequent degradation by the proteasome, thereby allowing NF-κB to enter the nucleus to turn on target genes. Building on this framework, subsequent research in the second decade was centered on understanding the regulation and function of the IκB kinase, which apparently integrates signals from diverse pathways. In 1996, a large ($\sim$700 kDa) IκB kinase complex was partially purified; surprisingly, this kinase could be activated by polyubiquitination through a mechanism independent of proteasomal degradation.[2] This was a rather provocative finding as there was no precedence of proteasome-independent function of ubiquitin and the results were obtained entirely from *in vitro* biochemical experiments. The idea that ubiquitin can activate IκB kinase, which was later called IKK when the subunits of the kinase complex were molecularly cloned, was revived when some components of the NF-κB pathway were found to be connected to ubiquitination.[3,4] In particular, TRAF6, an essential signaling protein of the NF-κB pathway, was found to be a ubiquitin ligase (E3) that catalyzes the synthesis of a unique polyubiquitin chain required for IKK activation.[5] Subsequent studies showed that TRAF6-catalyzed polyubiquitination leads to activation of the TAK1 kinase complex, which in turn phosphorylates and activates IKK.[6] However, these studies were also carried out largely *in vitro*. Although the role of TAK1 in the NF-κB pathway was later supported by multiple lines of evidence, including those derived from RNA interference and Drosophila genetic experiments, the final *in vivo* proof in higher organisms was still lacking – until now. Through genetic ablation of TAK1 in mice, Sato *et al*.[7] have now provided convincing *in vivo* evidence that TAK1 is essential for IKK activation in multiple signaling pathways. In this commentary, we will review the current knowledge of the connection between ubiquitin, TAK1 and IKK, and discuss some important questions that remain to be resolved.

Ubiquitin-mediated activation of TAK1 and IKK by IL-1β and TNFα

The role of ubiquitin and TAK1 in IKK activation was discovered in the course of studying the interleukin-1 (IL-1) signaling pathway. In this pathway, the binding of IL-1β to its receptor (IL-1R) leads to the recruitment of several proteins including MyD88, IRAK1, IRAK4 and TRAF6 to the receptor complex. IRAK4 is a protein kinase that phosphorylates IRAK1, which is also a kinase but its catalytic activity is not required for signaling. Following phosphorylation, IRAK1 and TRAF6 are released to the cytoplasm to activate downstream kinases including IKK, JNK and p38. The IKK complex consists of two catalytic subunits IKKα and IKKβ, and an essential regulatory subunit NEMO/IKKγ.[8] Recent genetic and biochemical studies have shown that IKKβ and NEMO regulate the phosphorylation and subsequent degradation of IκB proteins in response to stimulation by a large variety of NF-κB stimuli, including TNFα and IL-1β. IKKα, on the other hand, is responsible for the phosphorylation of the NF-κB precursor p100 in response to stimulation of a subset of TNF receptor (TNFR) superfamily in certain cells such as B cells. Phosphorylation of p100 leads to its ubiquitination and subsequent processing to the mature subunit p52 by the proteasome.

Genetic experiments had demonstrated the essential role of TRAF6 in the activation of IKK and other downstream kinases by IL-1β, but the biochemical mechanism by which TRAF6 signals to IKK was unknown. To investigate this mechanism, biochemical fractionation experiments were carried out to identify intermediary factors that link TRAF6 to IKK activation.[5] Two such factors were identified. The first factor, termed TRIKA1 (TRAF6-regulated IKK activator 1), was found to be a dimeric ubiquitin-conjugating enzyme (Ez) complex consisting of Ubc13 and a Ubc-like protein Uev1A. This finding led to the realization that TRAF6 may serve as a ubiquitin ligase (E3). Indeed, several members of the TRAF family, including TRAF6, contain a highly conserved N-terminal RING domain that is found in a large family of ubiquitin ligases. Biochemical experiments provided the direct evidence that TRAF6 is a ubiquitin ligase that functions together with Ubc13/Uev1A to catalyze the synthesis of a unique polyubiquitin chain linked through lysine-63 (K63) of ubiquitin. K63 polyubiquitination was previously implicated in DNA repair and stress response in yeast, but the mechanism was unknown.[9,10] Through the use of a panel of ubiquitin mutants and proteasome inhibitors, it was found that K63 polyubiquitination by TRAF6 led to the activation of IKK through a proteasome-independent mechanism.[5]

The second factor, termed TRIKA2, turned out to be a protein kinase complex composed of TAK1, TAB1 and TAB2.[6]

TAK1 was first identified as a TGF-β activated kinase, but later shown to be involved in the IL-1β pathway as well.[11,12] It was initially proposed that TAK1 activates IKK through a kinase called NIK. However, NIK activates IKKα, but not IKKβ.[8] Biochemical reconstitution experiments showed that TAK1 directly phosphorylates IKKβ within the activation loop, leading to activation of the IKK complex.[6] Furthermore, it was found that TAK1 is activated by auto-polyubiquitination of TRAF6. The ubiquitin-activated TAK1 can also phosphorylate another kinase of the MKK family such as MKK6, leading to the activation of JNK and p38 kinase. These results place TAK1 in a pivotal position that links TRAF6 to the activation of IKK and other stress kinase pathways (Figure 1).

TAK1 can be activated by TAB1 *in vitro* and in over-expression experiments, but the endogenous TAK1 complex, which contains both TAB1 and TAB2, is inactive in unstimulated cells.[13] TAB2 is neither an activator of TAK1 nor an inhibitor of TAK1/TAB1 complex in reconstitution experiments.[6,14] However, TAB2, but not TAB1, is required for ubiquitin-dependent activation of TAK1 by TRAF6.[6] A mechanism by which TAB2 and its homologous protein TAB3 activate TAK1 and IKK was recently uncovered.[15] Both TAB2 and TAB3 contain a highly conserved C-terminal zinc-finger domain that binds preferentially to K63 polyubiquitin chain. Point mutations within this domain that impair polyubiquitin binding also abolish the ability of TAB2 and TAB3 to activate TAK1 and IKK. Conversely, replacement of the zinc-finger domain with a heterologous ubiquitin-binding domain restores the activation of TAK1 and IKK by TAB2 and TAB3. Thus, TAB2 and TAB3 appear to activate TAK1 and IKK by binding to K63 polyubiquitin chains of target proteins such as TRAF6 itself. It is still not clear how binding to polyubiquitinated TRAF6 leads to the activation of TAK1. One possibility is that the binding of polyubiquitinated TRAF6 to TAB2 or TAB3 facilitates the dimerization or oligomerization of TAK1 complex, thereby promoting the *trans*-autophosphorylation and activation of TAK1. Consistent with this possibility, TAK1 is phosphorylated at Thr-187 within the activation loop, and a mutation of Thr-187 abolishes its kinase activity.[16]

The TAK1 complex was initially thought to be involved in the IL-1β pathway, but not the TNFα pathway.[12,14] Subsequent studies, however, provided evidence that TAK1 and TAB2 are also critical for TNFα signaling.[15,17,18] In the TNFα pathway, binding of the trimeric TNFα ligand to TNFR leads to the trimerization of TNFR and subsequent recruitment of signaling proteins including TRADD, TRAF2 and the protein kinase RIP1, which then activates IKK and JNK. Genetic experiments have demonstrated that RIP1 is essential for the activation of IKK and NF-κB by TNFα, but the kinase activity of RIP1 is dispensable.[19,20] The role of RIP1 in JNK activation is controversial, as one study showed that RIP1-deficient MEF cells could still activate JNK in response to TNFα,[20] whereas another study showed that JNK activation was defective in the same cell line.[21] TRAF2-deficent MEF cells failed to activate JNK but NF-κB activation was normal in response to TNFα.[22,23] The normal NF-κB activation was likely due to the functional redundancy between TRAF2 and TRAF5, as cells lacking both TRAF proteins were completely defective in NF-κB signaling.[24] Both TRAF2 and TRAF5 contain an N-terminal RING domain, suggesting that they may activate

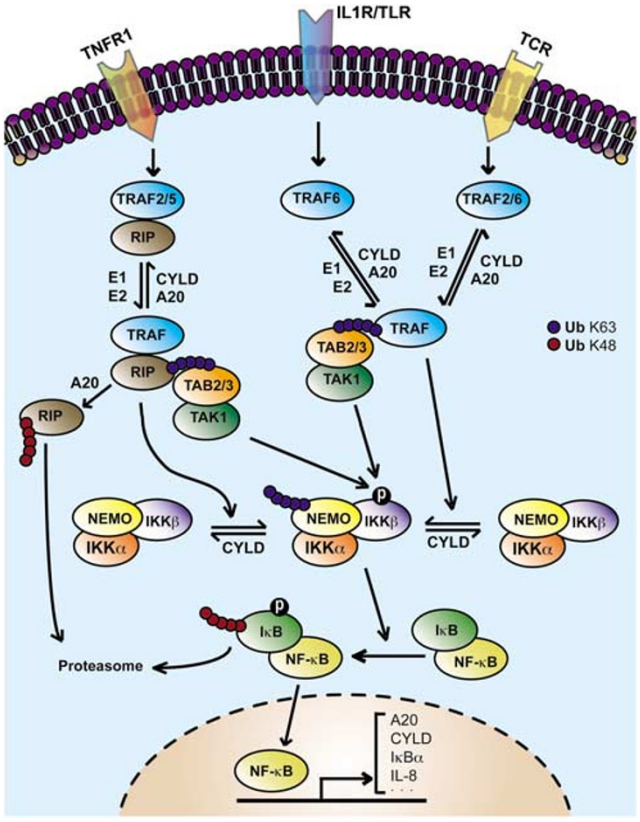

Figure 1 The central role of ubiquitin and TAK1 in multiple NF-κB signaling pathways. NF-κB is activated by multiple signaling pathways emanated from the TNF receptors (TNFR), IL-1 receptor (IL-1R), Toll-like receptors (TLR) and T-cell receptors (TCR). The binding of TNFα to TNFR leads to the recruitment of proteins including TRADD (not shown), TRAF2, TRAF5 and RIP1 to the receptor complex. The TRAF proteins promote the K63-linked polyubiquitination of RIP1, as well as autopolyubiquitination of TRAF proteins themselves (not shown). Polyubiquitinated RIP1 recruits the TAK1 kinase complex through the binding between polyubiquitinated RIP1 and TAB2 (or TAB3). The TAK1 kinase is then activated to phosphorylate IKKβ in the activation loop, resulting in IKK activation. The phosphorylation of IKK by the TAK1 complex may be assisted by the polyubiquitination of NEMO, which may also be carried out by the TRAF proteins. Following activation, IKK phosphorylates IκB proteins and targets these inhibitors for degradation by the proteasome, thereby allowing NF-κB to enter the nucleus to activate a large array of downstream target genes including proinflammatory cytokines such as interleukin-8 (IL-8), and negative regulators of the NF-κB pathway such as A20, CYLD, and IκBα. A20 and CYLD inhibit IKK activation by functioning as deubiquitination enzymes to cleave K63-linked polyubiquitin chains from the target proteins, such as TRAFs, RIP1, and NEMO. A20 can also function as an E3 to catalyze K48-linked polyubiquitination of RIP1, which is subsequently degraded by the proteasome. The activation of IKK by IL-1R, TLR and TCR also requires K63-linked polyubiquitination and TAK1, except that TRAF6 is the ubiquitin ligase and a major ubiquitination target in these pathways. TRAF6 and TRAF2 may function redundantly in the TCR pathway. The role of TRAF proteins and TAK1 in the TCR pathway remains to be investigated using an *in vivo* model

IKK through a ubiquitin-dependent mechanism. Indeed, a dominant-negative mutant of Ubc13 was found to inhibit NF-κB activation by TNFα.[5] Furthermore, TRAF2 was found to be polyubiquitinated in TNFα-stimulated cells.[25,26] In addition, *in vitro* experiments showed that TRAF2 has ubiquitin ligase activity.[15] However, unlike the IL-1β pathway in which TRAF6 functions downstream of IRAK1, in the TNFα pathway RIP1 appears to function downstream or at the level of TRAF2. Correspondingly, RIP1 is polyubiquitinated and

recruited to the receptor complex following TNFα stimulation[15,27–29] It has been shown that polyubiquitinated RIP1 preferentially recruits the TAK1 complex through the binding between the polyubiquitin chains and TAB2.[15] However, it is still not clear at present whether the ubiquitination of RIP1 or TRAF2, or both, is required for TNFα signaling to IKK.

Gene silencing experiments using RNA interference provided the initial evidence that TAK1 is required for the activation of IKK and JNK by IL-1β and TNFα.[18] This conclusion was further supported by the use of a chemical inhibitor of TAK1, 5Z-7-oxozeaenol, which blocks IKK and JNK activation in both pathways.[17] The roles of TAB1, TAB2 and TAB3 in the NF-κB pathway seem to be more complicated. Knockout of TAB1 in mice led to early embryonic lethality and the mutant embryos exhibited abnormal cardiac phenotypes that resembled those of TGF-β2 knockout mice.[30] TAB1-deficient cells had normal NF-κB signaling in response to TNFα or IL-1β. Thus, TAB1 may be involved in TGF-β, but not NF-κB signaling. TAB2 knockout mice died of liver apoptosis before embryonic day E12.5, and the embryonic fibroblasts (MEFs) derived from these mice had no defect in IKK and JNK activation in response to IL-1β or TNFα stimulation.[31] The normal signaling observed in the TAB2-deficient cells, however, appears to be due to the redundancy between TAB2 and TAB3, as RNAi of both TAB2 and TAB3 effectively blocks IKK and JNK activation by IL-1β or TNFα.[15,32]

Sato *et al.*[7] now provide strong genetic evidence that TAK1 is indeed essential for signaling by TNFα and IL-1β. As TAK1 knockout mice died before embryonic day 10.5, the Cre-LoxP strategy was used to obtain MEF cell lines lacking functional TAK1. These cells were severely defective in activating IKK, JNK and p38 kinase following stimulation with TNFα or IL-1β. As TNFα can trigger apoptosis if NF-κB induction is abrogated, TAK1-deficient MEF cells underwent apoptosis following TNFα treatment. The apoptotic phenotype was rescued when TAK1 was introduced back to the knockout cell line, indicating that the observed defects in the mutant cells were indeed due to the loss of TAK1.

The conserved role of ubiquitination and TAK1 in innate immunity

Sato *et al.*[7] also used the Cre-LoxP strategy to delete TAK1 specifically in mouse B cells and found that TAK1-deficiency in B cells impaired innate immune response mediated by several Toll-like receptors (TLRs). The TLR family of receptors is known to recognize the pathogen-associated molecular patterns (PAMP) and elicit the first line of defense against microbial pathogens by activating several signaling pathways including that of NF-κB.[33] Most TLRs activate NF-κB through the MyD88-IRAK-TRAF6 signaling module, while some other TLRs, such as TLR3, which recognizes extracellular viral double stranded RNA, utilizes another adaptor TRIF to signal to NF-κB and other transcription factors such as IRF3. TLR4, the receptor for bacterial lipopolysaccharides (LPS), recruits MyD88 and TRIF as well as other adaptors for signaling. Previous studies have suggested that TAK1 is activated in LPS-stimulated macrophages, suggesting the involvement of TAK1 in TLR signaling.[34] The new study showed that TAK1-deficient B cells failed to proliferate and were defective in activating IKK, JNK, p38 and ERK in response to LPS, CpG DNA (a TLR9 ligand) or poly (I-C) (a TLR3 ligand).[7] Thus, like TRAF6, TAK1 is essential for signaling in multiple TLR pathways.

The role of TAK1 in innate immunity is evolutionarily conserved.[35] Homologs of TAK1 and TAB2, but not TAB1, have been found in Drosophila, which possesses NF-κB-like pathways that are essential for innate immune responses against microbial pathogens, including fungi and bacteria. Infection with fungi and Gram-positive bacteria leads to activation of the Toll pathway that activates the NF-κB-like proteins Dorsal and Dif, which turn on the expression of antimicrobial genes. Infection with Gram-negative bacteria activates a distinct pathway known as the IMD pathway through the binding of bacterial peptidylglycans to specific peptidylglycan recognition proteins (PGRPs). These receptors activate the death domain protein IMD, which in turn activates the Drosophila homolog of the IKK complex (dIKK). dIKK phosphorylates the NF-κB-like precursor Relish, leading to the cleavage of Relish by a caspase. The N-terminal fragment of Relish then enters the nucleus to activate the expression of a battery of antibacterial peptides. Genetic screens for components of the IMD pathway led to the identification of dTAK1[36] and dTAB2/galere (D. Ferrandon, personal communication). Mutations of dTAK1 and dTAB2 loci cause severe defects in the production of antimicrobial peptides in response to bacterial, but not fungal, infection. In addition, RNAi and biochemical experiments in a Drosophila culture cell line have shown that the loss of dTAK1 prevents the activation of dIKK and JNK by bacterial peptidylglycans.[37]

The mutations in the dTAB2/galere mutants isolated from the genetic screen are particularly interesting, as these mutations have been mapped to the conserved C-terminal zinc-finger domain of dTAB2, which is likely to bind K63 polyubiquitin chains based on data obtained from mammalian TAB2 and TAB3. That K63 polyubiquitination is important for Drosophila innate immunity receives further support from recent studies demonstrating that the Drosophila homologs of Ubc13 and Uev1A are required for the activation of dIKK and induction of antibacterial genes.[38] It is still not clear whether there is an E3 required for dTAK1 and dIKK activation in the IMD pathway. Although Drosophila has several TRAF homologs, including dTRAF2 that contains a RING domain, none of the dTRAF proteins appear to be involved in the IMD pathway based on RNAi experiments.[38] Recently, RNAi screens led to the identification of Drosophila Inhibitor of Apoptosis 2 (dIAP2) as an essential component of the IMD pathway.[39,40] Epistasis experiments suggest that dIAP2 functions upstream or at the level of dTAK1. As dIAP2 contains a C-terminal RING domain, it may function as an E3 to activate dTAK1.

The role of ubiquitination and TAK1 in adaptive immunity

Recent studies have shown that K63 polyubiquitination plays an important role in the activation of T cells, which are central

mediators of adaptive immunity. T cells are activated upon binding of the T-cell receptors (TCRs) to a cognate MHC–peptide complex.[41] This binding triggers a cascade of tyrosine phosphorylation that in turn leads to the activation of the serine/threonine kinase PKCθ. PKCθ then promotes the sequential recruitment of several signaling proteins to the lipid rafts. These proteins include the CARD domain proteins CARMA1 and BCL10, and the paracaspase MALT1. Genetic experiments have demonstrated the essential role of CARMA1, BCL10 and MALT1 in the activation of IKK by TCR stimulation. Two independent studies have shown that K63 polyubiquitination by Ubc13/Uev1A is required for the activation of IKK by BCL10 and MALT1.[42,43] Both studies showed that NEMO is a ubiquitination target, but they differed in the identity of the E3 that catalyzes NEMO polyubiquitination. One study showed that MALT1 is a ubiquitin E3 although it lacks any known E3 domain such as the RING domain.[42] The other study showed that MALT1 binds to TRAF6, which then serves as a ubiquitin E3 to polyubiquitinate NEMO.[43] The latter study also employed *in vitro* reconstitution and RNAi experiments in Jurkat T cells to show that TAK1 is required for IKK activation in the TCR pathway. RNAi of both TRAF2 and TRAF6 led to more potent inhibition of IKK than RNAi of TRAF2 or TRAF6 alone, suggesting that these proteins may function redundantly in T cells. This may explain why there is no reported defect in T cell development or activation in TRAF2 or TRAF6 knockout mice, whereas transgenic mice expressing a dominant-negative mutant of TRAF2 in T cells are severely defective in mounting immune responses to antigenic stimulation.[44] Future experiments employing conditional deletion of both TRAF2 and TRAF6 in T cells should help determine the role of these proteins in T cell activation *in vivo*.

The studies on the biochemical mechanism of IKK activation by BCL10 and MALT1 also uncover a mechanism by which the TRAF6 ubiquitin ligase activity is regulated.[43] It was found that a fraction of BCL10 and MALT1 proteins forms high molecular weigh oligomers *in vitro*. Interestingly, only these large oligomers can induce TRAF6 oligomerization and lead to the activation of IKK. On the basis of these studies, it was proposed that TCR stimulation leads to the oligomerization of BCL10 and MALT1 in the lipid rafts, which in turn promote the oligomerization and activation of TRAF6 (and possibly TRAF2). This oligomerization-induced activation of ubiquitin ligase activity may underlie not only the physiological activation of NF-κB by TCR, but also the pathologic activation of NF-κB in certain cancers, such as MALT lymphoma. Aberrant activation of BCL10 and MALT1 has been proposed as the major culprit of MALT lymphoma. In particular, the most frequent chromosomal translocation associated with MALT lymphoma is t(11;18)(q21;q21), which generates a fusion protein consisting of the N-terminal BIR domains of IAP2 and the C-terminal paracaspase domain of MALT1. This fusion protein is a potent activator of NF-κB. Recent studies show that this fusion protein forms oligomers and its overexpression greatly induces K63-linked polyubiquitination of cellular proteins, including NEMO.[45] Importantly, it was found that NEMO polyubiquitination was enhanced in MALT lymphoma patient samples expressing the IAP2-MALT1 fusion protein. This study also showed that mutations of the TRAF6 binding sites at the C-terminus of the fusion protein did not impair NF-κB activation. However, the same group has previously shown that the IAP2 portion of the fusion protein can bind to TRAF2.[46] Thus, the normal signaling function of the IAP2-MALT1 mutant lacking the TRAF6 binding sites may be due to the recruitment of TRAF2. The role of TRAF2 and TRAF6 in signaling by the oncogenic as well as wild-type MALT1 requires further study.

The CARMA1-BCL10-MALT1 complex is also important for signaling by B-cell receptors (BCR).[41] Therefore, Sato *et al*.[7] investigated the role of TAK1 in B-cell activation. As expected, B-cell proliferation following stimulation of BCR and CD40 (a member of TNFR superfamily) was impaired in TAK1-deficient B cells. Similarly, the activation of JNK in response to BCR stimulation was also diminished in the absence of TAK1. Unexpectedly, however, BCR-induced activation of NF-κB was normal in B cells lacking TAK1. These results suggest that either TAK1 is not involved in NF-κB activation by BCR, or there is another protein that functions redundantly with TAK1 in this pathway. Recently, Shinohara *et al*.[47] knocked out TAK1 in the chicken B-cell line DT40 by homologous recombination, and found that the activation of IKK and JNK, but not ERK, by BCR was completely abolished in the absence of TAK1. Moreover, BCR-mediated activation of IKK and JNK in TAK1-deficient cells was rescued by the wild type, but not by the kinase-dead mutant, of TAK1. Thus, at least in DT40 cells, TAK1 is essential for IKK and JNK activation by BCR. It is not clear why there is a discrepancy between the two studies. One possibility is the difference in cell types (chicken *versus* mouse). Another possibility is the difference in knockout strategies. Shinohara *et al*.[47] used a conventional targeting strategy to delete the chicken TAK1 locus, whereas Sato *et al*.[7] used a conditional knockout strategy in which a Cre recombinase driven by the CD19 promoter was used to remove TAK1 in B cells. Thus, it is possible that the Cre-mediated removal of TAK1 was incomplete and some B cells containing TAK1 might preferentially accumulate.

Negative control of TAK1 and IKK by deubiquitination

Like phosphorylation, ubiquitination is a reversible covalent modification. Deubiquitination is carried out by members of a large family of enzymes called isopeptidases or Dubs (deubiquitination enzymes).[48] Recently, two Dubs have been found to inhibit IKK by cleaving K63 polyubiquitin chains from the target proteins. One of these Dubs is CYLD, a tumor suppressor protein linked to familial cylindromatosis, a disease characterized by numerous skin adrenal tumors often referred to as 'turban tumors'.[49] CYLD contains a C-terminal domain called UBP, which is the catalytic domain of a large subfamily of Dubs. The cancer-causing mutations of CYLD are often found within the UBP domain. Overexpression of CYLD inhibits IKK and NF-κB activation, whereas RNAi of CYLD further enhances IKK and NF-κB activation[50–52] CYLD interacts with TRAF2 and NEMO, and causes the deubiquitination of these proteins. CYLD is also a potent-negative regulator of JNK activation,[53] suggesting that it may inhibit a

common upstream regulator of IKK and JNK, such as TRAF2 and TAK1. It has been proposed that mutations in the UBP domain of CYLD obliterate its ability to suppress NF-κB activation, thereby promoting the survival of certain tumor cells. It is not clear why the defect in CYLD leads to cylindromas specifically. One possibility is that CYLD is the major inhibitor of IKK in the skin appendage, whereas in other tissues, additional inhibitors of IKK may compensate for the loss of CYLD.

Another Dub that inhibits IKK is the zinc-finger protein A20, a well-known target gene of NF-κB, which also functions as an NF-κB inhibitor to provide negative feedback control.[54] A20 contains a novel type of deubiquitination enzyme domain known as the OTU domain at the N-terminus and seven zinc-finger domains at the C-terminus. Mice lacking A20 develop inflammation in multiple organs due to prolonged activation of IKK. Recent studies have shown that A20 inhibits IKK in the TNFα pathway through two distinct but concerted mechanisms.[55] The N-terminal OTU domain of A20 first cleaves K63 polyubiquitin chains from RIP1, and then one of the C-terminal zinc-finger domains catalyzes K48 polyubiquitination of RIP1 and targets it for proteasomal degradation. A20 has also been shown to disassemble K63 polyubiquitin chains from TRAF6, thereby dampening the inflammatory responses elicited by LPS stimulation of TLR4.[56] However, the mechanism by which A20 inhibits NF-κB may be more complicated, as it has been shown that A20 mutants lacking the N-terminal OTU domain or carrying point mutations in the catalytic cysteine can still potently inhibit NF-κB.[57]

Conclusions and perspectives

The past decade has witnessed significant progress towards elucidating the role of ubiquitination in the NF-κB pathway. Research in this area has not only uncovered the traditional role of ubiquitin in targeting the degradation of IκB inhibitors and processing of NF-κB precursors by the proteasome, but also the nontraditional role of ubiquitin in regulating the activation of IKK through a proteasome-independent mechanism. While the traditional role of ubiquitin in the NF-κB pathway is well established, the nontraditional role of ubiquitin in the IKK pathway has just begun to receive support from the recent discoveries of several key signaling proteins in the IKK pathway that are intimately linked to the process of ubiquitination, deubiquitination and ubiquitin-binding. The biochemical studies on the mechanism of IKK activation by TRAF proteins also led to the discovery of TAK1 as a direct IKK kinase. The work by Sato *et al.*[7] has now closed the loop from *in vitro* discovery to *in vivo* validation of the role of TAK1 in IKK and stress kinase activation by multiple pathways. A map that connects ubiquitin, TAK1 and IKK is now emerging. However, important questions remain. First, although TAK1-deficient cells are severely defective in IKK activation in multiple pathways, there is still a residual amount of IKK activity remaining in these cells. Is this residual activity due to another kinase functioning upstream of IKK, or could it be due to direct activation of IKK by upstream proteins such as TRAF6 or RIP1? Second, the roles of TAK1 and TRAF proteins in TCR signaling remain to be resolved, and conditional deletion of these proteins in T cells will be required to address this question. Third, although several proteins in the IKK pathway are known to be polyubiquitinated in response to signals, the significance of ubiquitination has not been validated for most of these targets. Fourth, the biochemical mechanism by which K63 polyubiquitination activates TAK1 and IKK remains to be fully elucidated. Finally, the existence of large families of ubiquitination and deubiquitination enzymes as well as ubiquitin-binding proteins, coupled with the complexity of polyubiquitin chains with different configurations, suggests that the regulatory mechanism involving ubiquitination may not be confined to TAK1 and IKK. Perhaps there are more dots to be connected through ubiquitin on the NF-κB map.

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Note added in proof

Shim *et al*. have also shown that TAK1 is essential for IKK and JNK activation in the TNFR and IL-1R/TLR pathways (Shim *et al*. (2005) *Genes Dev*. **19**: 2668–2681).

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