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JB Commentary

Role of K63-linked polyubiquitination in NF-κB signalling: which ligase catalyzes and what molecule is targeted?

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Nuclear factor-κB (NF-κB) is a master regulator of immunity and also involved in malignant transformation. It has been widely accepted that Lys-48 (K48)-linked polyubiquitination plays a critical role in NF-κB signalling by targeting inhibitor of NF-κB (IκB), thereby leading to its degradation by the proteasome. Alternatively, studies on IL-1 and TNF signalling have revealed that proteins modified with K63-linked polyubiquitin chains do not undergo the proteasomal degradation, instead, function as the signalling platforms required for the activation of the IκB kinase (IKK) complex. From the studies on lymphoid malignancies, human T cell leukaemia virus 1-derived protein, Tax, has been shown to activate the IKK complex, although the mechanism is largely unknown. Recently, Shibata *et al.* (Activation of the IκB kinase complex by HTLV-1 Tax requires cytosolic factors involved in Tax-induced polyubiquitination. *J. Biochem.* 150: 679–686, 2011) has established a cell free IKK assay system and demonstrated that recombinant Tax protein can activate the IKK complex in a K63-linked-polyubiquitination-dependent manner. This cell free assay system will be useful for the identification of various key players responsible for Tax-induced IKK activation.

Keywords: IKK/K63/NF-κB/Tax/ubiquitin.

Abbreviations: IAP, Inhibitor of apoptosis; IL-1, interleukin-1; NEMO, NF-κB essential modulator; RIP1, receptor-interacting protein 1; TNF, tumor necrosis factor; TRAF, tumor necrosis factor receptor-associated factor.

The nuclear factor kappaB (NF-κB) family of transcription factors plays a key role in the immune system by regulating the expression of wide variety of genes responsible for various immune responses and is also involved in malignant transformation (*1*). NF-κB consists of five members, NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), RelA (also called p65), RelB and c-Rel, respectively, all of which conserve an N-terminal Rel homology domain responsible for DNA binding and homo- and heterodimerization (*1*).

In unstimulated cells, the NF-κBs are retained in the cytoplasm in an inactive state by interacting with inhibitors called IκBs or the precursor forms of NF-κB1 and NF-κB2. Among NF-κB dimers, RelA:p50 dimer is most intensively studied and the activating process of this dimer is described as the 'canonical pathway' that is initiated by the activation of the IκB kinase (IKK) complex containing two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ (also known as NEMO) (*1*). This pathway is triggered in response to bacterial and viral infections, or exposure to proinflammatory cytokines, such as TNF-α and IL-1β (*1*), leading to phosphorylation-induced IκB degradation. The other pathway, 'non-canonical pathway', leads to selective activation of RelB:p52 dimer by inducing processing of the NF-κB2/p100 precursor protein, which mainly forms a heterodimer with RelB in the cytoplasm. This pathway is activated by certain members of the TNF cytokine family such as lymphotoxin β (LTβ) and B cell-activating factor belonging to the TNF family (BAFF) through the selective activation of IKKα homodimer by NIK (*1*). Two pathways switch on different gene sets and therefore mediate different immune functions (*1*).

Following phosphorylation by the IKK complex or IKKα homodimer, phosphorylated forms of IκBs or NF-κB2 precursors are modified with polyubiquitin chains, thereby recognized by the proteasome resulting in their degradation or processing. Ubiquitin has seven lysines (K6, K11, K27, K29, K33, K48 and K63) and polyubiquitin chains can be formed by conjugating any one of these lysine residues to another ubiquitin. Polyubiquitin chains synthesized through different lysine linkages are known to regulate distinct functions in the cell (*2*, *3*). For example, K48- and K11-linked polyubiquitin chains usually target proteins for proteasomal degradation, whereas K63-linked polyubiquitin chains participate in the cell signalling through proteasome-independent mechanisms (*2*, *3*). In addition to the isopepide-linkage-mediated polyubiquitination, linear polyubiquitination, which generates head-to-tail linked linear ubiquitin chains by conjugating the C-terminal of glycine of ubiquitin to the N-terminal methionine of another ubiquitin, has recently emerged as the novel signalling process involved in the NF-κB activation (*3*).

Conjugation of ubiquitin to the substrate proceeds via a three-step mechanism. Initially, the ubiquitin-activating enzyme (E1) activates ubiquitin by forming a thiol ester bond. Then activated ubiquitin is transferred to one of several ubiquitin-conjugating enzymes (E2s). The E2 and ubiquitin complex interacts with a member of the ubiquitin ligase family (E3s) that binds

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to the subustrate protein and facilitates ubiquitin transfer (*4*). Thus, E3s are thought to determine the specificity in polyubiquitination. E3s are mainly classified into two groups: the HECT (homologous to E6-AP carboxy terminus) domain-containing E3s and the RING finger-containing E3s (*4*, *5*). Furthermore, RING-finger-containing E3s come into two varieties: the single subunit RING E3s and the multisubunit RING E3s (*4*). In the case of IKK complex-phosphorylated IκBs and IKKα dimer-phospholyrated NF-κB2 precursors, they are both targeted by the ubiquitin ligase $SCF^{\beta\text{-TrCP}}$ that are assembles of four subunits, Skp1, Cul1, F-box protein β-TrCP and RING-finger-containing Roc1 (also known as Rbx1 or Hrt1) (*5*). Thus K48-linked polyubiquitin chains are conjugated to IκBs or NF-κB2 precursors leading to the degradation or processing by the proteasome.

As an alternative ubiquitin modification on the signalling molecules involved in NF-κB activation, K63-linked polyubiquitination has been characterized (*6*, *7*). This area of research began with the biochemical characterization of TRAF6 as an E3 that functions together with dimeric E2 complex (Ubc13:Uev1A) to catalyze K63-linked polyubiquitination, inducing the activation of IKK complex *in vitro* (*8*). Subsequently, several signalling components have been shown to be modified by K63-linked ubiquitin moiety following the stimulation that induces canonical NF-κB activation. As shown in Fig. 1, IL-1 receptor (IL-1R) recruits the adaptor protein MyD88 upon ligand binding. MyD88 in turn recruits the IL-1 receptor associated kinases, IRAK4 and IRAK1. IRAK4 phosphorylates IRAK1, releasing IRAK1 into the cytosol, where it forms a complex with TRAF6. TRAF6 then catalyzes the formation of K63-linked polyubiquitin chains, which serve as the signalling platform that recruits TAB2 and TAB3, adaptor proteins for TGF-β-activated kinase (TAK1), through their ubiquitin-binding domains (UBDs) that binds preferentially to K63-linked polyubiquitin chains. Subsequently, activation of TAK1:TAB1 complex occurs on the platform. On the other hand, the IKK complex is also recruited to the platform by binding to K63-linked polyubiquitin chains through the UBD conserved in IKKγ/NEMO. Thus proximal recruitment of TAK1:TAB1 complex and the IKK complex enables TAK1 to phosphorylate the activation loop of IKKβ, leading to its activation thereby phosphorylation of IκBs (*7*). Among proteins known to be involved in IL-1 signalling, the K63-linked polyubiquitination sites that are critical for NF-κB activation have been mapped on TRAF6 itself (*9*) and IRAK1 (*10*). However, physiological significance of K63-linked polyubiquitination of these two molecules is still controversial. For example, complementation of TRAF6-deficient cells with a lysine-free TRAF6 mutant restores normal IKK activation by IL-1β, suggesting that ubiquitination of TRAF6 is dispensable (*11*). Regarding the E3 for K63-linked polyubiquitination of IRAK1, separate groups have proposed TRAF6 (*10*) and Pellino proteins (*12*) as candidates. It should be noted that TRAF6 deficiency causes complete defects in IL-1-triggered signalling including NF-κB activation (*13*, *14*) whereas IκB degradation in IRAK1-deficient cells is impaired at low concentrations of IL-1 but fully observed at higher concentration (*15*). Thus, it is likely that TRAF6 plays a critical role in IL-1 signalling as an E3 responsible for the K63-linked polyubiquitination, however, its target acting as the signalling platform may be dispensable. Indeed, unanchored K63-linked polyubiquitin chains that are not conjugated to any cellular protein can directly activate the TAK1 complex *in vitro* (*16*).

In contrast, RIP1 seems to represent the critical molecule as the signalling platform by accepting K63-linked polyubiquitin chains thereby activating the IKK complex upon TNF stimulation (*7*). After TNF binding to TNF receptor 1 (TNFR1), TRADD provides a scaffold for the assembly of RIP1 and TRAF2 and IAPs (IAP1 and IAP2). In this complex (called complex I) formed at the plasma membrane, cIAPs direct the formation of polyubiquitin chains through K63 of ubiquitin on RIP1. Although TRAF2 possesses the RING-finger domain, structural analyses demonstrated that the TRAF2 RING structure is very different from the known TRAF6 RING structure, *i.e.*, the TRAF2 RING lacks the structural interface necessary for interacting with E2 (*17*). Therefore, it is likely that IAPs associated with TRAF2 act as E3 for K63-linked ubiquitination in TNF-induced NF-κB activation. Indeed, knockdown of both cIAP1 and cIAP2 severely impairs TNF-induced NF-κB activation, indicating their essential and redundant roles in this signalling (*18*). Thus K63-linked polyubiquitin chains formed on RIP1 in complex I facilitate the recruitment and activation of TAK1 and the IKK complex by binding to TAB2/3 and NEMO, respectively (Fig. 1). Recently, HOIL-1 and HOIP, which form a linear ubiquitin chain assembly complex (LUBAC) that acts as the E3 complex responsible for linear polyubiquitination, have been identified as the additional signalling modules recruited to the complex I in response to TNF stimulation (*19*). Although LUBAC is reported to conjugate linear chains of ubiquitin onto IKKγ/NEMO (*20*), the exact role of this modification in NF-κB activation needs to be further investigated. Subsequent to the formation of the receptor-associated complex I and the activation of TAK1 and the IKK complex, TRADD, RIP1 and TRAF2 dissociate from the receptor to form complex II in the cytoplasm together with FADD and procaspase-8. Within complex II, procaspase-8 is processed into the mature caspase-8, which then cleaves and activates caspase-3, an executer of apoptosis (*7*). Initial activation of NF-κB by complex I leads to the induction of anti-apoptotic proteins including cIAPs and c-FLIP that binds to procaspase-8 and prevents its activation.

Among various inducers of NF-κB activation, human T-lymphotropic virus type I (HTLV-1)-derived Tax protein shows unique characteristics. Tax binds directly to IKKγ/ NEMO and activates the IKK complex (*1*, *7*). However, the precise mechanism how Tax binding leads to the activation of the IKK complex is largely unknown. For example, the involvement of TAK1 in Tax-induced NF-κB activation is the subject

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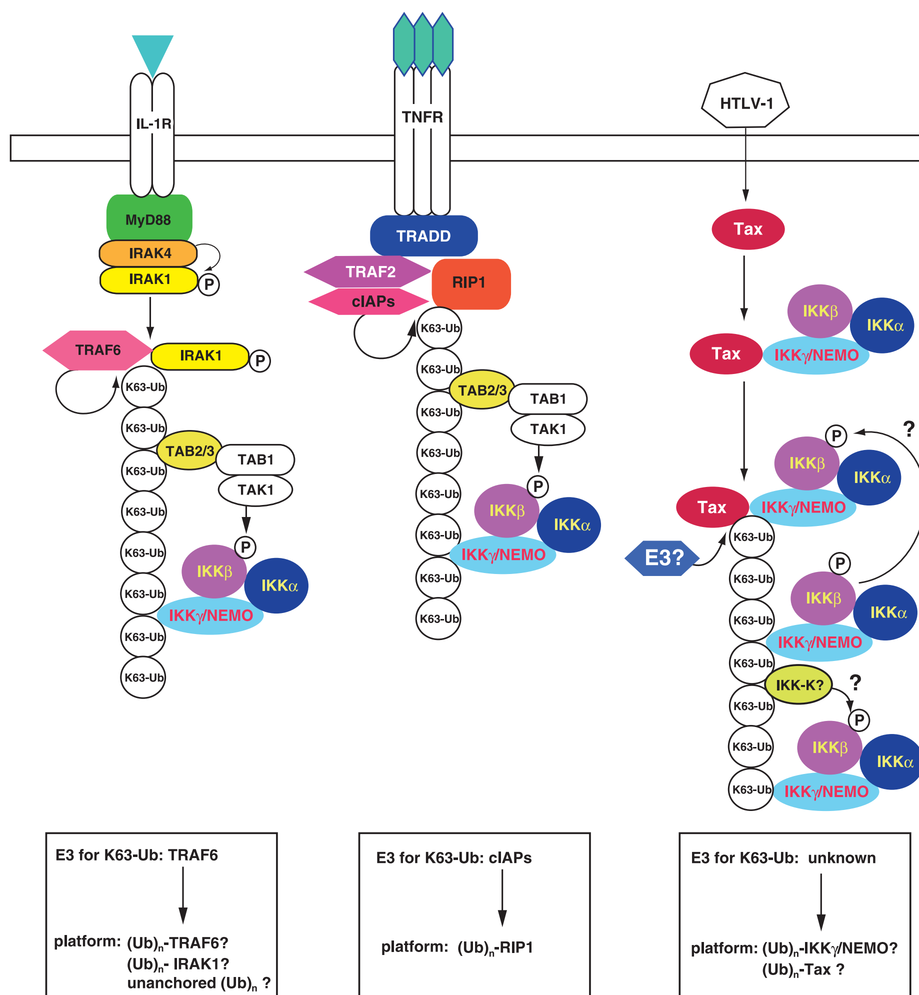

Fig. 1 Mysterious black box in HTLV-1 Tax-triggered canonical NF-κB activating pathway. IL-1-, TNF- and HTLV-1 Tax-triggered signalling pathways leading to IKK activation are illustrated. In the case of Tax-triggered signalling, it is unclear which E3 is responsible for the formation of K63-linked polyubiquitin-based platform and how the IKK complex is activated there.

of controversy. Wu *et al.* (*21*) reported that Tax failed to activate NF-κB when expressed in TAK1-deficient mouse embryonic fibroblasts (MEFs) but not in wild-type MEFs whereas, Gohda *et al.* (*22*) demonstrated that Tax-dependent IKK activation was observed not only in wild-type MEFs but also in TAK1-deficient MEFs. In most studies including those referred above, experiments are carried out using intact cells expressing Tax protein, therefore, it is difficult to rule out the possibility that the primary NF-κB activation induced by Tax results in the production of proinflammatory cytokines, leading to the secondary NF-κB activation that may undergo independently of Tax-mediated pathways. In a recent study by Shibata *et al.* (*23*), they established a cell-free IKK assay system, in which highly purified recombinant Tax protein was reconstituted with cytosolic fractions from unstimulated cells or with various recombinant proteins as the candidates of signalling modules in the presence of the IKK complex isolated from unstimulated cells, enabling the secondary NF-κB activation to be negligible. Using this system, they showed the following observations. (i) The IKK complex contained in the crude cytosolic fractions was successfully activated *in vitro* during the incubation with recombinant Tax. However, recombinant Tax failed to activate the purified IKK complex in the absence of the components contained in the cytosolic fractions. (ii) Tax-induced IKK activation was almost completely blocked in the presence of recombinant ubiquitin with K63R substitution, indicating that K63-linked polyubiquitintion but not linear polyubiquitination is responsible for this process. (iii) Both recombinant Tax and IKKγ/NEMO derived from the crude cytosolic fractions are ubiquitinated during the cell free assay, although dominant negative K63R-ubiquitins do not prevent the interaction between Tax and IKKγ/NEMO and (iv) Tax-dependent IKK activation was significantly observed using cytosolic fractions from TRAF6-deficient cells. Thus we can assume that Tax-induced IKK activation occurs as follows (Fig. 1): after the infection with HTLV-1, Tax proteins bind directly to IKKγ/NEMO in the IKK complex without requiring K63-linked polyubiquitination. By unknown mechanism, certain types of E3 (except TRAF6) target Tax and IKKγ/NEMO, leading to their modification with K63-linked polyubiquitin chains. Polyubiquitinated Tax and IKKγ/NEMO

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may act as the signalling platforms that recruit the IKK kinases responsible for the phosphorylation of IKKβ activation loop. Alternatively, proximal recruitment may cause the trans-autophosphorylation of IKK complexes.

The cell free IKK assay system shown above will support the identification of the E3 responsible for Tax-induced NF-κB activation. In addition, requirement of IKK kinases such as TAK1 and MEKK3 and identification of the acceptor sites for K63-linked ubiquitination in Tax and IKKγ/NEMO should be examined using this system.

Conflict of interest

None declared.

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