

## News and Commentary

# Finding NEMO by K63-linked polyubiquitin chain

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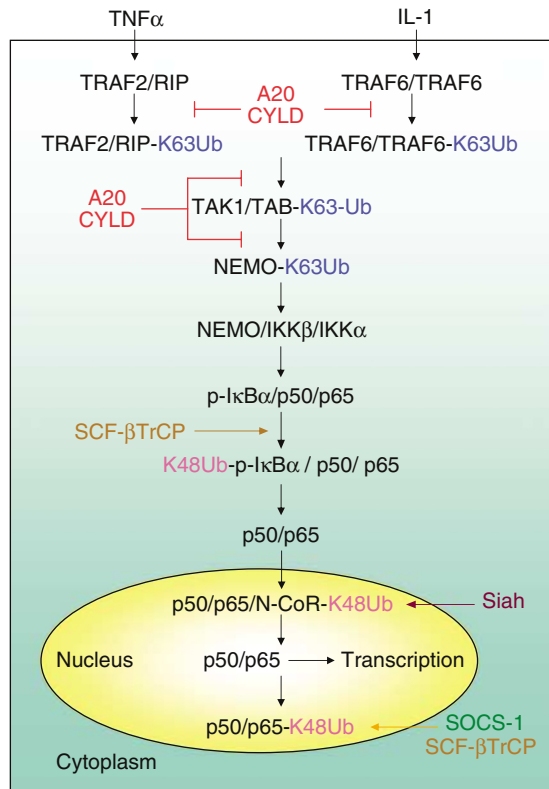
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Few would argue that protein ubiquitination is the most important process in the signaling pathway to activate nuclear factor- $\kappa$ B (NF- $\kappa$ B), a critical transcription factor governing the expression of genes involved in inflammation, carcinogenesis and cell apoptosis. Ubiquitination of I $\kappa$ B $\alpha$ , an intrinsic inhibitor of NF- $\kappa$ B, was found more than one decade ago.<sup>1</sup> In response to extracellular inducers, I $\kappa$ B $\alpha$  is first phosphorylated on the N-terminal two serine (S) residues, S32 and S36, by IKK kinase complex composed of two catalytic subunits, IKK $\alpha$  and IKK $\beta$ , and one regulatory subunit, NEMO (IKK $\gamma$ ). This phosphorylation leads to the conjugation of lysine (K)48-linked polyubiquitin chains on two lysine (K) residues, K21 and K22, of I $\kappa$ B $\alpha$  protein (Figure 1). The conjugated polyubiquitin chains will ferry I $\kappa$ B $\alpha$  to the protein disposal machinery, proteasome, for degradation, leading to the activation and nuclear translocation of NF- $\kappa$ B. The most abundant form of NF- $\kappa$ B is the p50/p65(RelA) heterodimer, although other forms of NF- $\kappa$ B dimers, such as p50/p50, p52/p52, p52/p65, p50/c-Rel, c-Rel/c-Rel and p50/RelB, have also been found. Later studies suggested that SCF- $\beta$ TrCP, a F-box ubiquitin ligase complex that uses phosphorylated S32 and S36 as a docking site, is responsible for the ubiquitination of I $\kappa$ B $\alpha$  protein.<sup>2</sup>

SCF- $\beta$ TrCP-dependent K48-linked polyubiquitination has also been implicated in the generation of NF- $\kappa$ B p50 and p52 subunits from their respective precursors, p105 and p100. Both p105 and p100 proteins contain C-terminal ankyrin repeats similar to those in I $\kappa$ B proteins. Partial degradation of the C-terminal ankyrin repeats by proteasome results in the generation of mature forms of p50 and p52. The processing of p105 is dependent on IKK $\beta$ -mediated phosphorylation of S927 and S932, whereas the partial degradation of p100 requires phosphorylation of S866 and S870 by IKK $\alpha$  and its upstream kinase, NIK. Similarly, the processes of ubiquitination-dependent proteasomal degradation may be important in the elevation of NF- $\kappa$ B transcriptional activity in the nuclei.<sup>3</sup> In this case, the protein targeted for

ubiquitination and degradation is N-CoR, a ubiquitous nuclear receptor co-repressor, rather than I $\kappa$ B or NF- $\kappa$ B family proteins. N-CoR can associate with a number of transcription factors including NF- $\kappa$ B to repress their transcriptional activity. The biological consequences of N-CoR ubiquitination include the dismissal and subsequent degradation of N-CoR, which will lead to recruitment of the co-activators and transcriptional activation of target genes regulated by NF- $\kappa$ B or other transcription factors. The ubiquitin ligase responsible for N-CoR ubiquitination appears to be Siah, a RING-type family E3 ligase.<sup>4</sup>

Although the NF- $\kappa$ B field was abuzz with the activating role of protein ubiquitination in the earlier years, the opposite effect of ubiquitination on NF- $\kappa$ B was outlandish until the finding that p65 or c-Rel undergoes proteasomal degradation under certain circumstances. The first evidence indicating ubiquitination of NF- $\kappa$ B subunits is from a study of HTLV-1-infected T cells where ubiquitinated c-Rel proteins were noted.<sup>5</sup> The C-terminal region of c-Rel was believed to be responsible for the ubiquitination and subsequent proteasome-mediated degradation of c-Rel. It remained obscure how such ubiquitination and degradation of c-Rel affects the activity of NF- $\kappa$ B. Following the observation of c-Rel ubiquitination, ubiquitin-dependent degradation of p65 was discovered in cells with deficiency in Pin1, a peptidyl-prolyl isomerase that isomerizes phosphorylated serine (S) or threonine (T) residues in S/T-P (proline) motif.<sup>6</sup> In wild-type cells where the Pin1 is normally expressed, Pin1 is able to bind to the T254-phosphorylated p65 and stabilize p65 by preventing its ubiquitination and proteasomal degradation. Protein pull-down assay indicates an interaction between p65 and the suppressor of cytokine signaling 1 (SOCS-1).<sup>6</sup> Based on the capability of SOCS-1 to interact with elongin B/C protein, SOCS-1 has been previously considered to be a receptor subunits (analogous to VHL) of VCB-like RING ubiquitin ligase complex. The association of SOCS-1 with COMMD1/Murr1, a protein involved in copper metabolism and NF- $\kappa$ B inhibition, has been implicated in the ubiquitination and degradation of p65, RelB, p50 and p52 in nuclei (Maine GN *et al.*, personal communication). However, whether SOCS-1 is truly involved in the ubiquitination of p65 is under question, as this protein is mainly cytoplasmic.<sup>7</sup> Given the fact that p65 contains a less conserved SCF- $\beta$ TrCP docking site (536S-I-A-D-M540) and that S536 can be phosphorylated by either IKK $\beta$  or IKK $\alpha$ , the SCF- $\beta$ TrCP may not be exonerated completely in the ubiquitination of p65. In addition, the STAT-interacting LIM protein (SLIM), a potential RING-type ubiquitin E3 ligase, may also play a role on the ubiquitination of p65 (T Tanaka and T Kaisho, personal communication). Ubiquitination of p65 is perhaps one of several mechanisms involved in the termination of activated NF- $\kappa$ B signals in inflammation resolution.<sup>8</sup> It may also contribute to the observed oscillation of nuclear NF- $\kappa$ B activity or the exchange of NF- $\kappa$ B dimers following cytokine stimulation.<sup>9</sup>



**Figure 1** Ubiquitination in NF- $\kappa$ B signaling. In response to cytokine signals, RIP or TRAF6 is modified by the K63-linked polyubiquitin chain (K63Ub) catalyzed by the E2 enzyme UBC13/Uev1 along with the TRAF2 or TRAF6 ubiquitin ligase. This ubiquitination will recruit TAK/TAB complex and IKK complex to RIP or TRAF6 through the ubiquitin binding domains of TAB and NEMO, respectively. Then TRAF2 or TRAF6 induces K63-linked polyubiquitination of NEMO, which activates IKK possibly through bringing NEMO into TAK and stabilizing the complex of RIP, TRAF2 or TRAF6, TAK1/TAB, and IKK oligomer. The activated IKK phosphorylates S32 and S36 of I $\kappa$ B $\alpha$  protein (p-I $\kappa$ B $\alpha$ ) associated with NF- $\kappa$ B p50/p65 heterodimer. SCF- $\beta$ TrCP, an ubiquitin ligase, binds to the phosphorylated S32 and S36 motif and conjugates K48-linked polyubiquitin chain (K48Ub) to K21 and K22 of the I $\kappa$ B $\alpha$  protein. The ubiquitinated I $\kappa$ B $\alpha$  is recognized and degraded by the proteasome, which liberates NF- $\kappa$ B dimer for nuclear translocation. In the nucleus, N-CoR, a repressor of NF- $\kappa$ B and other transcription factor, will be subjected to K48-linked ubiquitination and proteasomal degradation, leading to full activation of NF- $\kappa$ B for the transcription of the target genes. The nuclear NF- $\kappa$ B may be ubiquitinated and degraded by the proteasome, which is possibly responsible for the inflammation resolution, the oscillation of the NF- $\kappa$ B activity and the exchange of NF- $\kappa$ B dimers induced by the extracellular signals

The pursuit of protein ubiquitination in NF- $\kappa$ B activation or activity never appears to languish. There has been explosive interest in the role of ubiquitination in the regulation of signaling pathways upstream, rather than downstream, of IKK activation since 2000. Nevertheless, the requirement of protein ubiquitination for IKK activation was first observed even before the formal identification of IKK in an *in vitro* I $\kappa$ B $\alpha$  phosphorylation assay using a cell-free system.<sup>10</sup> In contrast to I $\kappa$ B $\alpha$  degradation, proteasomal degradation for ubiquitinated proteins is not involved in IKK activation based on the fact that proteolytic activity is not required for I $\kappa$ B $\alpha$  phosphorylation. This conclusion was further supported by the use of proteasome inhibitors and the K48-mutated ubiquitin that interrupts the formation of K48-linked polyubiquitin chains.<sup>10</sup> It

was a vexing mystery for many researchers at that time as to how ubiquitination activates IKK and which molecules were ubiquitinated. By a series of biochemical fractionations that have never been obsolete in identifying intermediate molecules, later studies elucidated an essential role of K63-linked polyubiquitin chain in this process.<sup>11</sup> Unlike the K48-linked polyubiquitin chain, the K63-linked polyubiquitin chain does not promote proteasomal degradation of the target proteins. Both E2 enzyme UBC13/Uev1A and E3 enzyme TRAF2 or TRAF6 are required for the synthesis of K63-linked polyubiquitin chain on several target proteins, including receptor interaction protein (RIP), IKK subunit NEMO (also named IKK $\gamma$ ), TAB2 and TRAF6 itself. It was originally proposed that the K63-linked ubiquitination on RIP or TRAF6 allows binding of TAB1, TAB2 and/or TAB3 regulatory subunits in the TAK1 kinase complex. The recruitment of TAK1 through the TAB proteins bound to these ubiquitinated proteins activates TAK1 that then phosphorylates and activates IKK $\beta$ .<sup>11</sup> A20 and CYLD, two de-ubiquitinating enzymes, are capable of removing the K63-linked polyubiquitin chains conjugated on RIP or TRAF6 protein, which diminishes TAK1 activation and the subsequent IKK $\beta$  activation.<sup>12</sup>

Evidence suggests that in addition to RIP and TRAF proteins, NEMO, a regulatory subunit of IKK complex, is conjugated with K63-linked polyubiquitin chain. The conjugation of the polyubiquitin chain of NEMO was first observed in TNF $\alpha$ -stimulated HEK293 T and HeLa cells engineered with overexpression of NEMO and cIAP1 constructs.<sup>13</sup> It was demonstrated that cIAP1, another RING-type E3 ligase, along with E2 enzyme UbcH5C synthesizes K6-linked, but not K48- or K63-linked, polyubiquitin chain on NEMO, which does not cause subsequent proteasomal degradation of the NEMO. An intriguing observation is that genotoxic stress induces mono- or di-ubiquitination, rather than polyubiquitination, of NEMO following SUMO modification on K277 and K309 of NEMO.<sup>14</sup> A hallmark of genotoxic stress or DNA damage is the activation of protein kinase ATM that phosphorylates several key components of DNA repair and checkpoint machineries. The latest studies indicate that ATM is also capable of phosphorylating SUMO-conjugated NEMO, which leads to mono- or di-ubiquitination and nuclear export of NEMO.<sup>15</sup> Using an elegant biochemical purification strategy, direct evidence of K63-linked polyubiquitination of NEMO was obtained in T cells in a manner that was Bcl10 and MALT1 dependent.<sup>16</sup> An association of Bcl10 with UBC13 and Uev1b, which form E2 complex for the assembly of K63-linked polyubiquitin chain, was noted in GST-Bcl10 column elution using cytosolic S100 extracts prepared from either untreated or TPA-treated Jurkat T cells. Such an association causes K63-linked polyubiquitination of NEMO in the activated T cells. K399 substitution with arginine (R) in NEMO abrogated its ubiquitination and NF- $\kappa$ B activation. The MALT1 might be acting as an E3 ubiquitin ligase in this process, despite the fact that this protein does not possess any domain conserved among the known ubiquitin ligases. The K63-linked NEMO ubiquitination was further confirmed in a cell-free system by reconstituting key factors including Bcl10, MALT1, TRAF6, Ubc13/Uev1A and TAK1/TAB1/TAB2.<sup>17</sup> Intriguingly, NOD2, a CARD domain-containing protein functioning as an

intracellular receptor for bacteria, has been associated with the K63-linked polyubiquitination of NEMO through the CARD domain-mediated interaction with RIP.<sup>18</sup> However, NOD2-RIP-triggered K63-linked ubiquitination occurred at K285 of NEMO, rather than K399 as mentioned above. It is unclear whether this property of NOD2 is related to the pathological characteristics of Crohn's diseases that usually result from polymorphisms of the NOD2 gene.

Although RIP, TRAF6 and NEMO have been found to be modified by K63-linked polyubiquitin chain(s), there are considerable gaps in our understanding of how IKK is activated through these ubiquitination events. These gaps are partially filled by an amalgamated effort of two well-established research groups in this field recently.<sup>19,20</sup> By asking whether NEMO has other interacting partners in addition to IKK $\alpha$  and IKK $\beta$  for the essential IKK activation, Ashwell *et al.*<sup>19</sup> demonstrated interaction between NEMO and polyubiquitin molecules. Using a strategy of GST pull down, they were able to identify the binding of the NEMO to the K63-linked polyubiquitin chain conjugated with RIP in TNF $\alpha$ -stimulated cells. A NEMO fragment containing the coiled-coil2 (CC2) and the leucine zipper (LZ) motifs is sufficient for such binding. A leucine (L) to proline (P) substitution in the LZ motif of NEMO (L329P) abrogated the ability of NEMO to bind the polyubiquitin chain on RIP and the subsequent IKK activation by TNF $\alpha$ . Similarly, Chen *et al.*<sup>20</sup> observed that NEMO was capable of binding to RIP that has K63-linked polyubiquitin chain, but not the K399R-mutated RIP that was unable to be ubiquitinated in response to TNF $\alpha$ . The ubiquitin-binding domain of NEMO was further narrowed down to a 66 residue fragment covering the C-terminus of CC2 motif and the N-terminus of LZ motif in an experiment using a series deletion constructs of NEMO. Site-directed mutation of the hydrophobic residue tyrosine 308 (Y308S) at the N-terminus of LZ motif disrupted the binding activity of NEMO to the K63-linked polyubiquitin chain on RIP and abolished the restoration of IKK activation in NEMO-deficient cells. Taken together, despite some subtle discrepancy in the point mutation results, both groups identified that the region encompassing the CC2 and LZ motifs of NEMO is the ubiquitin-binding domain critical for signal-induced IKK activation.

The finding that NEMO can bind to K63-linked, but not K48-linked, polyubiquitin chain is surprising, as the CC2 and LZ motifs of NEMO do not contain any known ubiquitin-binding domain. As is the case with many novel discoveries, more exciting or even confusing questions were raised with regard to the biological significance and consequence of NEMO binding to K63-linked polyubiquitin chain. The majority of the key proteins, including RIP, TRAF6, TAB2 and NEMO, in the IKK activation modules appear to be modified by the K63-linked polyubiquitin chain. There is little evidence suggesting that the ubiquitination of these proteins is in an ordered or a simultaneous fashion. Based on the limited information from the present literature, we know that the RIP ubiquitination in lipid rafts can be seen within 1–2 min following cytokine receptor activation.<sup>19,20</sup> The IL-1-induced TRAF6 ubiquitination can be detected at 5 min and decreases thereafter,<sup>11</sup> whereas ubiquitination of NEMO appears to be gradually enhanced following TPA/ionomycin stimulation for 10 min to

60 min in T cells.<sup>16</sup> Can we conclude that the NEMO ubiquitination is the last event induced by TRAF2 or TRAF6? It is difficult to provide a clear-cut answer to this question, as the activation of IKK usually occurs within the same time range of RIP ubiquitination and the fact that NEMO ubiquitination is essential for IKK activation.<sup>16,17</sup> We also have no clear picture of whether NEMO can bind to K63-linked polyubiquitin chain conjugated to any type of proteins, including proteins involved in DNA repair, or the proteins only associated with the activation of IKK. In response to IL-1 and Toll-like receptor (TLR) signals in which RIP is not involved, the auto-ubiquitinated TRAF6 might be acting as a surrogate of the ubiquitinated RIP for NEMO binding. Although there is no evidence suggesting that NEMO binds to the K63-linked polyubiquitin chain conjugated to TRAF6, it is highly possible that IKK activation in response to IL-1 or TLR requires NEMO binding to the ubiquitin chain of TRAF6. NEMO itself is modified by the K63-linked polyubiquitin chains. It remains open whether NEMO can bind to itself through these conjugated K63-linked polyubiquitin chains to form oligomers of IKK complex. Previous studies indicated that NEMO could form a dimer, trimer or tetramer *in vivo* and *in vitro* through its CC2-LZ domain that was currently known as the ubiquitin-binding domain of NEMO. An interesting question is whether the binding of NEMO to the ubiquitin chains changes the overall stoichiometry and conformation of NEMO oligomers.

It is imaginable that NEMO binding to the K63-linked polyubiquitin chain may bring the IKK complex into close proximity of upstream signal molecules, such as TAK1 and TRAF6, for full activation of IKK. However, there might be additional biological consequences of such binding. Indeed, studies by Wu *et al.*<sup>19</sup> suggest that NEMO binding to the K63-linked polyubiquitin chain of RIP can stabilize RIP protein, possibly through competing with A20. A20 is a de-ubiquitinating enzyme for removing K63-linked polyubiquitin chain and an ubiquitin ligase conjugating K48-linked polyubiquitin chain to target proteins for proteasomal degradation. Thus, binding of NEMO to RIP may reduce the chance of A20 that converts K63-linked to K48-linked polyubiquitination on RIP, leading to the stabilization of RIP for an extended propagation of IKK activation signal. Furthermore, it is tempting to speculate that such binding might result in a conformational change of NEMO itself to exposure the sheltered IKK $\alpha$  and IKK $\beta$  subunits bound to the N-terminal CC1 region of NEMO for phosphorylation by TAK1 or other kinases.

Regardless of many unanswered questions, the discovery that NEMO binds to the K63-linked polyubiquitin chain suggests an additional layer of regulation of the signaling pathway leading to the activation of IKK. Along with the K63-linked ubiquitination of RIP, TRAF6, TAB2 and NEMO, the binding of NEMO to these ubiquitin chains may be an important step of the signaling cascade dedicated to controlling propagation of IKK/NF- $\kappa$ B activation. Understanding how IKK activation is regulated by the upstream signal pathways and how this event may be coupled to the initiation of certain diseases will offer valuable insight into the control of chronic inflammation and carcinogenesis. Further studies into the structure and function of NEMO, such as the X-ray crystallography of monomer and oligomer NEMO with or without ubiquitin modification, should shed

new light on how cells are able to tightly regulate the functions of IKK.

## Disclaimer

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