

NF- κ B signaling: Flipping the Switch with Polyubiquitin Chains

Dispatch

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Protein modification by ubiquitin has emerged as an important cellular regulatory mechanism. Recent studies illustrate the surprising ways in which polyubiquitin chains are manipulated in the regulation of NF- κ B signaling.

Cells respond rapidly to changes in their internal and external environments, often by using post-translational protein modifications to transmit signals from the cell surface or internal sites to the nucleus. These signaling pathways allow cells to respond to growth signals, tolerate stresses or trigger programmed cell death. In general, signaling pathway activation is transient, with feedback mechanisms that reset the regulatory network. Protein phosphorylation, for example, is a well-characterized post-translational mechanism for transmitting transient cellular signals [1]. Phosphate groups, covalently attached to target proteins by substrate-specific protein kinases, can be removed by specific phosphatases, thereby terminating the signaling cascade. Recent studies on the NF- κ B signaling network indicate that, in addition to transient protein phosphorylation, polyubiquitin chain attachment and removal work as on/off switches at several points along the signal transduction pathways that lead to activation of NF- κ B family transcription factors.

The carboxyl terminus of the ubiquitin polypeptide is enzymatically appended to lysines on target proteins as well as on other ubiquitin molecules to form polyubiquitin chains. Protein ubiquitination involves the sequential action of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-protein ligase (E3) [2]. E3s bind both E2 and substrate, and facilitate the transfer of ubiquitin molecules from the E2 to the substrate. Ubiquitin is best known as a signal that targets proteins for degradation by the proteasome. In the ubiquitin–proteasome pathway, proteins destined for degradation are usually attached to a polyubiquitin chain in which ubiquitin molecules are linked to one another by amide bonds involving a specific ubiquitin lysine, Lys48 [2]. Proteasomes recognize the Lys48-linked chain, leading to degradation of the substrate and recycling of the ubiquitin molecules. Ubiquitin removal from proteins is catalyzed by a diverse group of enzymes collectively termed deubiquitinating enzymes.

Interplay of Ubiquitination and Phosphorylation in NF- κ B Regulation

As noted, protein phosphorylation and ubiquitination are now both known to participate in the NF- κ B signaling pathway (Figure 1) [3]. NF- κ B molecules can concentrate in the nucleus and activate specific genes only when inhibitory controls on NF- κ B are relieved. This occurs in response to DNA damage, during stimulation by proinflammatory cytokines such as tumor necrosis factor α (TNF α) or with an innate immunity response initiated when a ligand interacts with a Toll-like receptor (TLR) [4]. Mature NF- κ B is normally kept inactive by tight binding to an inhibitory protein called I κ B. Activation of NF- κ B is triggered by site-specific phosphorylation of I κ B, which allows I κ B to be recognized by a ubiquitin-ligation complex. Polyubiquitinated I κ B is degraded by the proteasome, releasing active NF- κ B [3]. Regulation of NF- κ B is thus directed toward controlling cellular levels of I κ B.

Remarkably, multiple studies now indicate that polyubiquitin chain formation promotes protein–protein associations that activate several steps in the signaling cascades leading to activation of the I κ B kinase (IKK) complex [4]. These ubiquitin polymers are Lys63-linked chains, rather than the Lys48-linked chains that target proteins for proteasomal degradation. In the case of TNF α stimulation (Figure 1), activated TNF receptor recruits several adaptor proteins, including receptor-interacting protein (RIP) and TNF receptor-associated factor 2 (TRAF2). TNF α also induces polyubiquitination of RIP (and TRAF2), allowing binding of TAB2, a regulatory subunit in the TAK1 kinase complex, and this activates the TAK1 catalytic subunit [4] [5]. Active TAK1 then phosphorylates, and thereby activates, IKK (Figure 1).

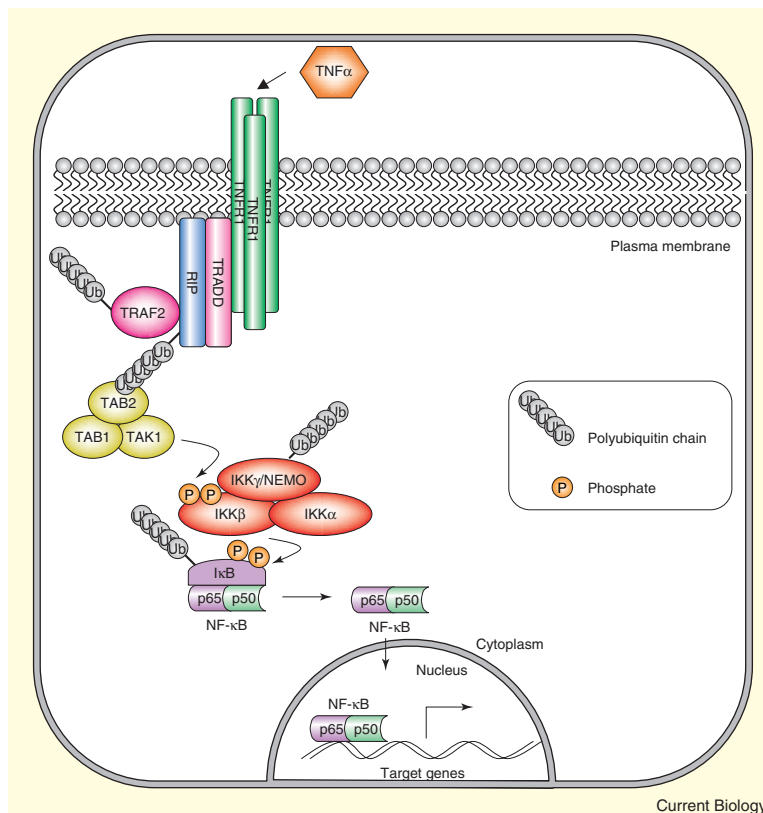
A20 protein: A Dual-Function Ubiquitin Chain Exchanger

The activation of IKK is transient, and recent findings indicate that negative regulation of IKK depends on specific deubiquitinating enzymes. This was first demonstrated for the tumor suppressor CYLD [4,6]. Loss of CYLD leads to a disfiguring cancer syndrome of the skin appendages called cylindromatosis. The CYLD protein disassembles Lys63 polyubiquitin chains on TRAF2 and the related TRAF6 protein. Reduction of CYLD expression, or mutations that abrogate its deubiquitinating activity, increase NF- κ B signaling, whereas CYLD overexpression has the opposite effect.

Failure to attenuate the NF- κ B signaling pathway may lead to chronic inflammation and immune system dysfunction. A human gene called A20, originally identified by an mRNA that is strongly induced by TNF α [7], encodes a key negative regulator of NF- κ B signaling. Studies in mice have shown that loss of A20 results in severe inflammation, cachexia and premature death, and that fibroblasts derived from these mice are

Figure 1. Model for the roles of ubiquitination and phosphorylation in NF- κ B activation.

The binding of a ligand to a plasma membrane receptor, in this case TNF α to TNFR1, causes trimerization of the receptor and recruitment of the receptor-associated proteins TRADD, RIP and the ubiquitin ligase TRAF2, which is activated as a result. Polyubiquitination of RIP (and TRAF2) by Lys63-linked chains appears to stimulate binding of the kinase complex TAK1-TAB1-TAB2 to the membrane-bound complex, thereby activating the TAK1 kinase. TAK1 phosphorylates the activation loop of the IKK β subunit of IKK, which in turn phosphorylates I κ B. This step is controlled by the NEMO subunit of IKK which is also modified by polyubiquitination. However, the mechanistic effects of the NEMO modification are not yet clear. Phosphorylated I κ B is modified with Lys48-linked polyubiquitin chains by a specific ubiquitin-ligation complex and is degraded by the proteasome, allowing NF- κ B to enter the nucleus to turn on target genes.



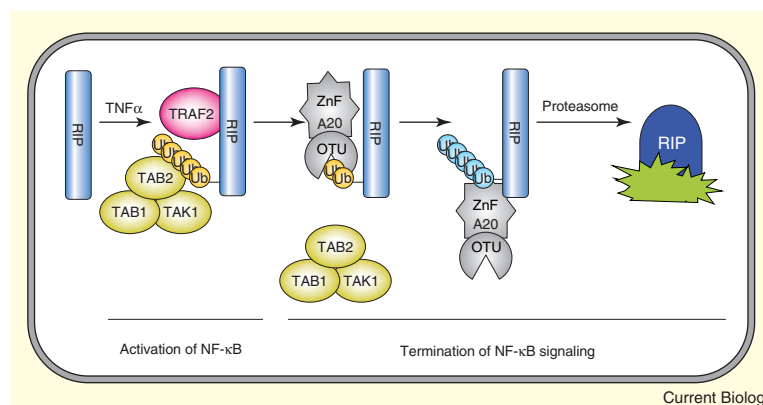
hypersensitive to TNF α and fail to downregulate NF- κ B [8]. The first hint that A20 function might be linked to ubiquitin was the finding that it has an amino-terminal ‘ovarian tumor’ (OTU) domain. A20 and several other OTU-related proteins were shown to have deubiquitinating activity [4]. Therefore, a reasonable hypothesis to explain how A20 downregulates NF- κ B signaling was that A20 removes the Lys63-polyubiquitin signal from proteins such as TRAF6 or RIP.

Wertz *et al.* [9] have recently reported data that support this idea, but also provide some unexpected twists. They identified RIP as a target for A20 deubiquitinating activity. This activity was traced to the OTU

domain, which preferentially removes Lys63-linked ubiquitin chains from RIP. A20 with an active site mutation in the OTU domain failed to turn down NF- κ B signaling in transfected cells from A20^{-/-} mice. Surprisingly, purified A20 was also shown to stimulate ubiquitin chain formation in the presence of E1, E2 and ubiquitin. Not only was this E3 activity unanticipated, but A20 lacks any motifs that characterize the majority of the known E3s [2]: the carboxy-terminal half of A20 has seven zinc fingers, and the E3-like activity was traced to the fourth of these (ZnF4). Intriguingly, polyubiquitin chains synthesized by A20 are of the Lys48 variety. This suggested that A20

Figure 2. A sequential two-step mechanism for downregulating TNF α signaling by A20.

In this model, TNF α induces TRAF2-mediated polyubiquitination of RIP. The Lys63 ubiquitin (Ub) chain on RIP is recognized by TAB2, an adaptor subunit of the TAK1 kinase complex. Polyubiquitin binding to TAB2-TAK1 activates TAK1 kinase activity by an uncharacterized mechanism. TNF α also increases A20 expression and the association of A20 with RIP. The OTU domain of A20 removes the Lys63-linked ubiquitin chains from RIP, possibly dissociating TAB2-TAK1, although this could occur later. Deubiquitination of RIP is followed by A20-ZnF-mediated modification of RIP by Lys48-linked ubiquitin chains, targeting RIP for proteasomal degradation. Gold Ub ovals, Lys63-linked ubiquitin; cyan Ub ovals, Lys48-linked ubiquitin.



might stimulate the degradation of a positively acting factor(s) in the NF- κ B pathway.

RIP also turns out to be a key target of A20 E3 ligase activity. Wertz *et al.* [9] found that receptor-associated RIP is ubiquitinated in an A20-dependent manner *in vivo* and is degraded by the proteasome. Direct ubiquitination of RIP by A20 was suggested by *in vitro* assays, and ubiquitin ligase activity depended on ZnF4 both *in vivo* and *in vitro*. Although it would at first appear that the A20 polypeptide has opposing enzyme activities, the distinct ubiquitin chains synthesized or disassembled by the respective A20 domains allow a way out of this paradox. In fact, the deubiquitinating and E3 activities of A20 appear to function in an ordered fashion: Lys63-linked ubiquitin chains on RIP (attached by another ubiquitin ligase, possibly TRAF2) are first removed by the OTU domain of A20 and this enables the ZnF to assemble a Lys48-linked chain in its place (potentially on the same lysines) (Figure 2). Kanayama *et al.* [5] recently demonstrated that TAB2, the adaptor subunit of the TAK1 complex, binds specifically to Lys63-linked ubiquitin chains on RIP. This binding activates the TAK1 kinase. Thus, the net effect of A20 on RIP is to exchange a Lys63 chain necessary for signal transmission for a Lys48 chain that targets RIP for destruction. In other words, both A20 activities negatively regulate NF- κ B signaling.

The function of A20 in NF- κ B regulation goes beyond the response to TNF α [10]. In mice lacking both A20 and TNF α , TLR-induced proinflammatory gene expression in macrophages persists abnormally, and the mutant mice are highly susceptible to endotoxin shock. This pathway is apparently independent of RIP.

Ubiquitin and Signal Transduction

The data of Wertz *et al.* [9] strongly imply that modification of a protein with ubiquitin polymers of different linkage has very different consequences [9]. To date, there is little evidence that chains of different topology have different binding affinities to particular ubiquitin binding domains, although intact TAB2 does prefer Lys63 over Lys48 chains [5]. This preference is lost if the major ubiquitin binding domain in TAB2 is expressed by itself. Perhaps multivalent interactions involving several ubiquitin binding domains in a protein can confer greater chain discrimination. Because proteasomes carry tightly associated E3 ubiquitin-ligase as well as deubiquitinating enzyme subunits [11], another possibility is that the proteasomal E3(s) preferentially extends Lys48 chains but not Lys63 ones or that the deubiquitinating enzyme(s) preferentially cleaves Lys63 chains. The net effect would be to enhance degradation of Lys48-linked proteins while sparing Lys63-linked ones.

Removal of Lys63-ubiquitin chains from RIP by A20 is insufficient to downregulate TNF α signaling; signal attenuation also seems to require that A20 synthesize a Lys48-ubiquitin chain on RIP [9]. It is known that proteasomes have a sophisticated 'unfoldase' activity that can pluck individual subunits from a multisubunit complex and degrade them selectively [12]. Lys48-polyubiquitin-modified RIP

might be subject to this activity. In this view, Lys63-linked chains facilitate activated complex assembly but Lys48-linked chains trigger proteasome-mediated disassembly (and usually degradation). These ubiquitin chain-dependent mechanisms might be crucial for controlling the duration and strength of signaling in the NF- κ B pathway and perhaps other signaling pathways.

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