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Ubiquitin Signaling in the NF- κ B Pathway

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Summary

The transcription factor NF- κ B (nuclear factor kappa enhancer binding protein) controls many processes including immunity, inflammation and apoptosis. Studies of the NF- κ B pathway have revealed new functions for ubiquitination in addition to its well-known role in targeting protein degradation. Ubiquitin is involved in at least three steps in this pathway: degradation of the NF- κ B inhibitor I κ B, processing of NF- κ B precursors, and activation of the I κ B kinase (IKK) through a degradation-independent mechanism.

Introduction

In this post-genomic era filled with cutting-edge technologies, it is refreshing to look back at the history of ubiquitin and NF- κ B as both were discovered through an ingenious combination of simple “bucket” biochemistry and a clear mind^{1–5}. Since these classic experiments, both ubiquitin and NF- κ B have taken center stage in biology, as each controls a vast array of cellular and organismal processes ranging from the birth and death of a cell to the neuronal behavior of an organism. Again, it was through biochemistry that the essential role of ubiquitination in NF- κ B activation was revealed^{6–8}. Ubiquitin targets not only I κ B for degradation, but also the NF- κ B precursors, p105 and p100, for processing into mature forms by the proteasome. More surprisingly, a critical role for ubiquitination in activating protein kinases in the NF- κ B pathway through a degradation-independent mechanism has also been discovered^{9–11}. Thus, the convergence of ubiquitin and NF- κ B research has not only helped elucidate the biochemical mechanism underlying NF- κ B activation, but also broadened our understanding of ubiquitin as a versatile signaling tag. As several extensive reviews of ubiquitination in the NF- κ B pathway are available^{12,13}, here I will summarize the current view of the traditional role of ubiquitin in the NF- κ B pathway, and then focus on recent advances in understanding the non-traditional role of ubiquitin in this pathway.

The traditional role of ubiquitin in the NF- κ B pathway

NF- κ B is a dimeric transcription factor consisting of Rel family members, which include Rel-A (also known as p65), c-Rel, Rel-B, p50 and p52¹⁴. p50 and p52 are derived from the larger precursors p105 and p100, respectively, through proteolytic processing by the proteasome. All NF- κ B proteins contain a highly conserved Rel-homology domain (RHD) that is responsible for DNA binding, dimerization, nuclear translocation and interaction with the I κ B proteins. The I κ B proteins, including I κ B α , β and ϵ , bind to NF- κ B via ankyrin repeats and block its nuclear import and thereby, its transcriptional activity. The C-termini of p105 and p100 also contain the I κ B-like ankyrin repeats that must be degraded in order to generate the mature Rel subunits.

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The NF- κ B activation pathways are broadly classified as the canonical and non-canonical pathways, depending on whether activation involves I κ B degradation or p100 processing (Figure 1)¹⁵. In the canonical pathway, which is the predominant NF- κ B signaling pathway, stimulating cells with an agonist such as tumor necrosis factor α (TNF α) or interleukin-1 β (IL-1 β) activates the IKK complex that is composed of two catalytic subunits IKK α and IKK β and a regulatory subunit NEMO (also known as IKK γ). Genetic experiments have demonstrated that IKK β , but not IKK α , phosphorylates I κ B proteins at two N-terminal serine residues. This signal-induced phosphorylation targets I κ B for polyubiquitination and subsequent degradation by the proteasome⁷, thus releasing NF- κ B. The non-canonical pathway of NF- κ B activation operates mainly in B cells in response to stimulation of a subset of the TNF receptor superfamily, including receptors for BAFF, lymphotoxin- β (LT β) and CD40 ligand. Stimulation of these receptors activates the protein kinase NIK, which in turn activates IKK α . IKK α then phosphorylates p100 at two C-terminal serine residues leading to the selective degradation of its I κ B-like domain by the proteasome^{16,17}. The mature p52 subunit and its binding partner Rel-B translocate into the nucleus to regulate gene expression.

The ubiquitin-proteasome pathway plays a crucial role in both the canonical and non-canonical pathways of NF- κ B activation. Ubiquitination is a reversible covalent modification catalyzed by three enzymatic steps¹⁸. In the first step, ubiquitin is activated by a ubiquitin-activating enzyme (E1) in an ATP-dependent reaction. In the second step, the activated ubiquitin is transferred to a ubiquitin-conjugating enzyme (E2 or Ubc), forming an E2-Ub thioester. Finally, in the presence of a ubiquitin-protein ligase (E3), ubiquitin is attached to a target protein through an isopeptide bond between the carboxyl terminus of ubiquitin and the ϵ -amino group of a lysine residue in the target protein. Ubiquitin contains seven lysines, which can be attached to another ubiquitin in a highly processive reaction to form a polyubiquitin chain. Typically, a polyubiquitin chain that targets a protein for degradation by the proteasome is linked through lysine-48 (K48) of ubiquitin¹⁹. However, ubiquitin chains linked through other lysines of ubiquitin have also been found in cells²⁰. In particular, K63-linked polyubiquitin chains have recently been found to regulate DNA repair and protein kinase activation through a degradation-independent mechanism (see below).

Ubiquitination of I κ B is carried out by an E2 of the Ubc4/5 family^{7,9,21} and the SCF- β TrCP E3 ligase (Skp1-Cul1-F-box ligase containing the F-box protein β TrCP)²²⁻²⁶. Two β TrCP proteins, β TrCP1 and β TrCP2, have been found in mammalian cells. Genetic deletion of β TrCP1 in mice leads to only modest retardation of I κ B α degradation, suggesting that β TrCP1 and β TrCP2 may have redundant functions²⁷. Indeed, silencing of both β TrCP1 and β TrCP2 expression by RNAi block I κ B α degradation. β TrCP1 and β TrCP2 bind specifically to the phosphorylated form of I κ B through their C-terminal WD40 repeats, and to the rest of the SCF complex through the F-box. The SCF complex contains the RING domain protein Roc1/Rbx1, which binds to Ubc4/5, allowing this E2 to ubiquitinate I κ B at two conserved N-terminal lysine residues. The polyubiquitinated I κ B remains associated with NF- κ B, but is selectively degraded by the 26S proteasome, while NF- κ B itself is spared⁷.

The ubiquitin-proteasome pathway is also responsible for the processing of p105 and p100 to p50 and p52, respectively. p105 can be processed either co-translationally or post-translationally, both requiring the proteasome^{6,28}. Co-translational processing of p105 appears to be a constitutive process that does not require phosphorylation or ubiquitination and is likely the major source of p50, which is constitutively present in unstimulated cells. Some agents such as phorbol ester (PMA) and lipopolysaccharides (LPS) can stimulate post-translational processing of p105 by activating IKK, which phosphorylates p105 at a C-terminal domain²⁹. On the other hand, the processing of p100 is tightly regulated by the non-canonical pathway of NF- κ B activation and depends on both phosphorylation and ubiquitination. As discussed above, stimulation of certain receptors of the TNFR family on B cells activates

IKK α , which phosphorylates p100 at two C-terminal serine residues (Figure 1). This phosphorylation relieves the inhibition of p100 processing by a C-terminal death domain, and recruits the SCF- β TrCP ligase to polyubiquitinate p100 at a specific lysine^{16,17,30,31}. Subsequently, the C-terminal I κ B-like domain is selectively degraded by the proteasome, generating the mature p52 subunit.

How does the proteasome partially degrade a protein? More specifically, what defines where degradation starts and stops? A plausible model for regulated ubiquitin/proteasome-dependent processing has recently been proposed³². According to this model (Figure 2), the ubiquitin tag on a substrate recruits the proteasome to an internal unstructured sequence such as the glycine rich region (GRR) of p105 and p100³³. It is postulated that this region forms a hairpin-like loop that inserts into the proteolytic chamber of the 20S proteasome. Proteolysis begins at the loop and then proceeds in both the N- and C-terminal directions of the target protein. In most cases, the N- and C-terminal domains can be completely unfolded by the 19S subcomplex of the proteasome and threaded into the proteolytic chamber so that the target is completely degraded. In the cases of substrates such as p105 and p100, the RHD domain is a tightly folded structure that may be difficult to unfold. Thus, after initiating proteolysis from the putative GRR loop of p105 or p100, the proteasome chews along the C-terminal polypeptide until it reaches the end, but comes to a halt at the N-terminus when it encounters the tightly folded RHD structure.

The non-traditional role of ubiquitin in activating protein kinases

Serendipity played a major role in the discovery of a ubiquitination-dependent I κ B kinase complex⁹. In 1995, a cell-free system was established to demonstrate the signal-induced ubiquitination of I κ B α ⁷. This system was later used to identify the enzymes involved in I κ B ubiquitination by separating HeLa cytosolic extracts into two fractions, dubbed fraction I and II^{1,9}. Surprisingly, each fraction alone no longer supported I κ B α phosphorylation but when both were combined, or when fraction I was replaced with ubiquitin and Ubc4 or Ubc5, I κ B α phosphorylation was restored. Further purification of fraction II led to the identification of a 700 kDa kinase complex whose activity can be regulated by polyubiquitination in a degradation-independent manner⁹.

A major issue concerning the early work on IKK activation by ubiquitin was the lack of a connection between ubiquitination and signaling pathways known to activate IKK. This issue was largely resolved by the discovery that TRAF (TNF receptor associated factor) proteins are ubiquitin E3 ligases¹⁰. TRAF proteins play a pivotal role in signaling pathways for NF- κ B activation from many cell surface receptors including the TNF receptor superfamily (TNFR), IL-1 receptors (IL-1R) and Toll-like receptors (TLRs)³⁴. Seven members of this family have been identified in the human genome. With the exception of TRAF1, all TRAF proteins contain an N-terminal RING domain followed by several zinc finger domains. Among all TRAF proteins, TRAF2 and 6 have been most intensively studied. TRAF2 is recruited to the TNF receptor complex through binding to the adaptor protein TRADD³⁵. Both TRADD and TRAF2 also interact with the receptor interacting protein RIP, a protein kinase that signals in the TNF pathway through a kinase-independent mechanism. TRAF6 is essential for IKK and JNK activation in the IL-1 and TLR pathways^{36,37}. In these pathways, stimulation of the receptors leads to the recruitment of MyD88, an adaptor protein that further recruits the protein kinase IRAK4 and IRAK1. IRAK1 then binds to TRAF6, which subsequently activates downstream signaling cascades including those of IKK and JNK.

IKK activation by the TRAF6 and TRAF2 ubiquitin ligases

In a series of biochemical experiments aiming at elucidating the mechanism of IKK activation by TRAF6, two TRAF6-regulated IKK activators (TRIKAs) were identified^{10,11}. TRIKA1

is an E2 enzyme complex consisting of Ubc13 and a Ubc-like protein Uev1A^{10,38}. This E2 functions with TRAF6, a RING domain E3, to synthesize a K63-linked polyubiquitin chain on target proteins including NEMO and TRAF6 itself. TRIKA2 is a trimeric complex consisting of the protein kinase TAK1 and two adaptor proteins TAB1 and TAB2^{11,39}. TAK1 phosphorylates IKK β at two serine residues in the activation loop, thereby activating IKK. TAB2 and its related protein TAB3 contain a highly conserved novel zinc finger (NZF) domain that binds preferentially to K63-linked polyubiquitin chains⁴⁰. Mutation of the NZF domain abolishes the ability of TAB2 and TAB3 to activate TAK1 and IKK, whereas the replacement of the NZF domain with a heterologous ubiquitin-binding domain restores the function of TAB2 and TAB3, suggesting that ubiquitin-binding by the NZF domain is necessary for activating TAK1 and IKK. These studies have led to the following model of IKK activation by TRAF6-mediated polyubiquitination (Figure 3). Upon ligand binding to IL-1R or TLRs, TRAF6 is recruited to the receptor complexes and forms oligomers. TRAF6 oligomerization activates its ligase activity, leading to K63-linked polyubiquitination of targets including TRAF6 itself. Ubiquitinated TRAF6 recruits TAB2 and activates the TAB2-associated TAK1 kinase, which then phosphorylates and activates IKK. Ubiquitin-activated TAK1 also phosphorylates and activates MKK kinases such as MKK6, which in turn activates the JNK and p38 kinase pathway¹¹. Thus, TRAF6-mediated polyubiquitination initiates protein kinase cascades through a degradation-independent mechanism. Recent structural studies have shown that the K48- and K63-linked ubiquitin chains adopt very different configurations, potentially providing the molecular basis for the distinct signaling functions of these polyubiquitin chains⁴¹.

Like TRAF6, TRAF2 is also a RING domain ubiquitin ligase (Figure 4)^{40,42–44}. A dominant negative mutant of Ubc13 has been shown to block NF- κ B activation by TRAF2 or TNF α ¹⁰. Overexpression of TRAF2 leads to its polyubiquitination by Ubc13/Uev1A, and TRAF2 ubiquitination appears to be important for the activation of JNK⁴². A recent study showed that TRAF2 ubiquitination is essential for JNK, but not p38 or NF- κ B, activation by TNF α ⁴⁴. This is likely due to the redundant role of TRAF2 and TRAF5 in NF- κ B activation by TNF α , as TRAF2-deficient cells are defective in JNK but not NF- κ B activation⁴⁵, whereas TRAF2/TRAF5 double knockout cells are defective in both JNK and NF- κ B activation⁴⁶. TRAF2 and/or TRAF5 may also ubiquitinate RIP, which is essential for NF- κ B activation^{40,47}. It has been shown that TNF α induces RIP polyubiquitination, and that ubiquitinated RIP associates with the TNF receptor as well as TAB2^{40,48,49}. Thus, it is possible that polyubiquitination of RIP recruits the TAK1/TAB2 complex, which subsequently activates IKK. However, definitive proof that RIP polyubiquitination is required for TNF α signaling is still lacking.

Roles of the TAK1 kinase complex in IKK and JNK activation

Since TAK1 knockout mice are early embryonic lethal, it has not been possible to determine whether this kinase is essential for NF- κ B activation in mice⁵⁰. However, multiple lines of evidence support a crucial role for the TAK1 complex in IKK and JNK activation. First, TAK1 phosphorylates IKK β and MKK6 at key serine residues within the activation loop, leading to their activation¹¹. Second, silencing of TAK1 expression by RNAi or inhibition with a specific kinase inhibitor in mammalian cells drastically reduced IKK activation and completely blocked JNK activation by IL-1 β and TNF α ^{40,51,52}. Third, mutations of TAK1 in *Drosophila melanogaster*, or silencing of TAK1 in a *D. melanogaster* cell line, blocked IKK and JNK in response to bacterial challenge^{53–55}. Thus, independent genetic and biochemical studies have indicated that TAK1 is important for IKK and JNK activation in both invertebrates and vertebrates. With regard to the role of TAB2 in the NF- κ B pathway, a recent study showed that a TAB2-deficient cell line has normal NF- κ B activation in response to IL-1 β ⁵⁶. However, this is likely due to the redundant functions of TAB2 and its closely related homologue TAB3, as RNAi against TAB2 and TAB3 in mammalian cells abrogated IKK activation by IL-1 β and

TNF α . Moreover, mutations in the *D. melanogaster* homologue of TAB2 led to severe defects in antibacterial responses (D. Ferrandon, personal communication). Interestingly, most of these mutations have been mapped to the ubiquitin-binding domain of dTAB2, suggesting that ubiquitin-mediated activation of TAK1 and IKK is an evolutionarily conserved mechanism. The TAK1 kinase complex also contains TAB1; however, the role of TAB1 in the NF- κ B pathway is not clear, as reconstitution experiments showed that TAB1 is dispensable for IKK activation *in vitro*¹¹. In addition, there is no evidence that RNAi or genetic ablation of TAB1 impairs IKK activation⁵⁷. Furthermore, there is no apparent homologue of TAB1 in the *Drosophila* genome. Thus, TAB1 may not be involved in NF- κ B activation, but in the regulation of other pathways such as the Wnt or TGF β pathway^{58,59}. In fact, TAK1 was first isolated as a TGF β -activated kinase, but its mechanism of activation in this pathway is poorly understood⁶⁰.

While TAK1 and TAB2 clearly play a major role in innate immune responses in *D. melanogaster*, the phenotypes of dTAK1 and dTAB2 mutants are not as severe as those of dIKK mutants⁵³. Similarly, although RNAi against TAK1 or TAB2 and TAB3 severely impaired IKK activation, there is still residual IKK activity (approximately 20–30%). While some of this residual activity may be because depletion by RNAi is incomplete, it is also likely that there is another minor pathway contributing to IKK activation. One possible activator of IKK is p62, a UBA domain containing protein that binds to TRAF6 and RIP^{61–63}. Mice lacking p62 are partially defective in prolonged NF- κ B activation by RANK, a TNFR that activates IKK through TRAF6 in osteoclasts⁶⁴. Interestingly, p62 forms a complex with an atypical PKC (ζ PKC), representing potentially another example of a ubiquitin receptor-containing kinase complex similar to the TAB2/TAK1 complex. Another potential activator of IKK is MEKK3, as murine cells lacking MEKK3 are partially defective in NF- κ B activation in response to certain stimuli^{65,66}. It is also possible that the residual IKK activation does not require an upstream kinase, but is induced by the oligomerization of IKK itself. In this case, TRAF6 or TRAF2 and RIP may somehow interact with IKK/NEMO to induce its oligomerization, causing IKK to autophosphorylate itself, resulting in its activation.

Ubiquitin signaling in adaptive immunity

Recent studies have revealed that ubiquitin signaling plays an important role in adaptive immunity, particularly T cell activation (Figure 4). Stimulation of T cell receptors (TCR) activates a tyrosine kinase cascade that subsequently activates the serine/threonine kinase PKC θ . PKC θ then induces the formation of a protein complex composed of CARMA1, BCL10 and MALT1, which are recruited to the lipid rafts where TCR resides^{67,68}. Two studies have shown that BCL10 and MALT1 induce the K63-linked polyubiquitination of NEMO by Ubc13/Uev1A, leading to IKK activation^{69,70}. However, there is a discrepancy concerning the E3 ubiquitin ligase for NEMO between these studies. In one study, MALT1 directly ubiquitinates NEMO at K399, despite the fact that MALT1 does not contain any known E3 domain⁶⁹. In the other study, MALT1 was found to recruit TRAF6 via its TRAF6-binding sites, and TRAF6, in turn ubiquitinates NEMO⁷⁰. The latter study showed that MALT1 also induces auto-ubiquitination of TRAF6, which then recruits the TAK1/TAB2 complex to activate IKK. This entire process from MALT1-mediated polyubiquitination to I κ B phosphorylation was reconstituted *in vitro* using purified recombinant proteins⁷⁰. TRAF6 and TRAF2 are redundant in the TCR pathway, as RNAi of both TRAF2 and TRAF6 effectively blocked IKK activation and IL-2 production by TCR stimulation, whereas RNAi of either TRAF alone was less effective⁷⁰. However, it remains to be seen whether TRAF2 and TRAF6 are required for T cell activation *in vivo*.

Emerging evidence for the regulatory role of ubiquitin in diverse signaling pathways

NF- κ B is regulated by a large variety of stimuli, including microbial pathogens and DNA damaging agents, which have recently been shown to activate IKK through a ubiquitin-dependent but degradation-independent mechanism. An elegant example was provided from the study of DNA damage-induced IKK activation⁷¹. DNA damaging agents, especially those that generate double strand breaks, such as topoisomerase inhibitors and gamma irradiation, can activate NF- κ B in certain cancer cells. This DNA damage response, which requires the cytoplasmic IKK complex, may be responsible for the resistance of some cancer cells to chemotherapy. A mechanism by which the nuclear DNA damage signal is transduced to the cytoplasmic IKK complex has recently been uncovered (Figure 4)⁷¹. Upon DNA damage, a small fraction of nuclear NEMO is modified by the ubiquitin-like protein SUMO, which further retains NEMO in the nucleus. DNA damage also activates the kinase ATM, which phosphorylates NEMO. This phosphorylation leads to the replacement of SUMO by ubiquitin at two lysine residues (K277/K309) within NEMO. Ubiquitinated NEMO subsequently enters the cytoplasm, where it associates with the rest of the IKK complex, resulting in IKK activation.

Another recent study has provided evidence that K63 polyubiquitination may be involved in IKK activation by NOD1 and NOD2, two cytosolic receptors for intracellular bacteria⁷². Mutations in NOD2 have been closely linked to Crohn's disease, an inflammatory bowel disease. NOD1 and NOD2 activate IKK through RIP2, a RIP1-like kinase. Through a process that is still not well understood, NOD2 and RIP2 induce the K63-linked polyubiquitination of NEMO at K285, thereby activating IKK (Figure 4)⁷³. Interestingly, the NOD2 mutations found in Crohn's disease patients impair NOD2's ability to activate NF- κ B and to induce NEMO polyubiquitination in cell culture experiments⁷³. However, mouse knock-in experiments show that the NOD2 mutations associated with the disease lead to NF- κ B activation rather than inhibition⁷⁴. Thus, the signaling mechanisms of NOD proteins and the potential role of ubiquitination in NOD signaling require further investigation.

The regulatory role of ubiquitin may not be limited to the NF- κ B pathway. Indeed, TRAF6 ubiquitination has recently been found to be important for the activation of the transcription factor IRF7⁷⁵. IRF7 regulates the production of type-I interferons in response to stimulation of certain Toll-like receptors (TLR7, 8 & 9) by viral RNA or bacterial DNA. IRF7 is activated through its phosphorylation by an unknown kinase, which is in turn activated by the MyD88-IRAK4-TRAF6 complex (Figure 4). Recent studies have shown that MyD88 and TRAF6 bind to IRF7, inducing its phosphorylation and nuclear translocation. Interestingly, Ubc13 and TRAF6 ubiquitination are required for IRF7 activation⁷⁵, suggesting that K63 polyubiquitination of as yet unidentified targets may be involved in the activation of an IRF7 kinase.

Regulation of IKK activation by deubiquitination

Like phosphorylation, ubiquitination is a reversible covalent modification. Two deubiquitinating enzymes (DUBs) have recently been shown to play an important role in suppressing NF- κ B activation at a step upstream of IKK. One of these DUBs is the cylindromatosis tumor suppressor protein CYLD, which inhibits IKK activation by cleaving K63-linked polyubiquitin chains on several proteins including TRAF2, TRAF6 and NEMO⁷⁶⁻⁷⁸. Mutations within the DUB domains of CYLD, which are found in patients with cylindromas, lead to the enhanced activation of NF- κ B, thereby contributing to the pathogenesis of the tumors. A recent study found that CYLD also inhibits JNK activation⁷⁹, consistent with the idea that CYLD interferes with TRAF ubiquitination or TAK1 activation, which functions upstream of both IKK and JNK. However, this study found that CYLD inhibits IKK activation by IL-1 β , LPS and CD40, but not TNF α , whereas previous studies showed that CYLD inhibits IKK activation by TNF α ⁷⁶⁻⁷⁸. The reason for this discrepancy is not clear.

Another DUB that acts in this pathway is A20, an NF- κ B induced protein that inhibit NF- κ B in a negative feedback loop^{80–82}. A20 deficient mice develop severe inflammatory diseases in multiple organs partly due to the enhanced and prolonged activation of NF- κ B by proinflammatory stimuli including TNF α and LPS⁸³. A20 contains a novel OTU type deubiquitination enzyme domain at the N-terminus, and several zinc finger domains in the remainder of the protein. The OTU domain disassembles K63-linked polyubiquitin chains from RIP (in the TNF α pathway)⁸¹ and TRAF6 (in the LPS pathway)⁸⁰, thereby suppressing IKK activation. Moreover, A20 also functions as a ubiquitin ligase through its zinc finger domains to assemble K48-linked polyubiquitin chains on RIP after the K63-linked chains are removed by the OTU domain⁸¹. K48-linked polyubiquitination targets RIP for degradation by the proteasome, further diminishing IKK activation. Thus, A20 provides a dramatic example that a switch in polyubiquitin chains can profoundly influence the signaling and degradation of targets. A20 also inhibits the activation of the transcription factor IRF3 by RNA viruses⁸⁴. In this case, the role of ubiquitin is less clear, as overexpression of an A20 mutant lacking the OTU domain still inhibits IRF3. It appears that A20 binds directly to and inhibits TBK1, an IKK-like kinase that phosphorylates IRF3.

Conclusions and Perspectives

The past decade has witnessed tremendous progress in elucidating the intertwining between ubiquitination and phosphorylation in the NF- κ B pathway. It is now well established that phosphorylation is a prerequisite for ubiquitination of I κ B and the NF- κ B precursors p100 and p105. The identification of the ubiquitination machinery, especially the ubiquitin ligase complex containing β TrCP, which binds specifically to phosphorylated substrates, provides the molecular basis for the signal-dependent degradation of I κ B and processing of p100 and p105. Recent studies have also identified several ubiquitin pathway components that function upstream of IKK, including the TRAF proteins as E3s, Ubc13/Uev1A as E2s, TAB2 and TAB3 as ubiquitin receptors, and CYLD and A20 as DUBs (Table 1). These results lend strong support to the thesis that ubiquitination can also regulate phosphorylation by activating IKK through a proteasome-independent mechanism.

While the traditional role of ubiquitin in degrading and processing NF- κ B inhibitors is widely accepted, there are several important issues that remain to be addressed. For example, what is the mechanism underlying the highly processive polymerization of ubiquitin to form polyubiquitin chains? How are different E2 selected to a common SCF ubiquitin ligase scaffold to ubiquitinate distinct substrates? Is the assembly of a specific SCF complex regulated by the availability of substrates? How is ubiquitinated I κ B, but not NF- κ B present within the same complex, selectively degraded by the proteasome? How do p100 and p105 escape complete degradation by the proteasome? Are there additional proteins that escort the ubiquitinated I κ B or p105/p100 to the proteasome?

Much also remains to be learnt about the non-traditional role of ubiquitin in the NF- κ B pathway. Specifically, how does binding of a ubiquitinated protein (*e.g.*, TRAF6 or RIP) to a ubiquitin receptor (*e.g.*, TAB2 or TAB3) activate the receptor-associated kinase (*e.g.*, TAK1)? What is the function of Nemo ubiquitination? Does NIK activation in the non-canonical pathway of NF- κ B activation involve TRAF proteins and/or ubiquitin? Are there other NF- κ B activation pathways that utilize ubiquitin as a signaling molecule for IKK activation? Are there other E2s (*e.g.*, Ubc4/5) and E3s that may regulate IKK? In more general terms, is ubiquitin signaling involved in activating other enzymes including other kinases and ATPases? In this regard, the 19S proteasome contains multiple ATPases and ubiquitin binding proteins. Does the binding of polyubiquitinated protein substrates to the 19S proteasome activate its associated ATPase activity? If so, ubiquitin regulation of enzymes may be a unified mechanism underlying both the traditional and non-traditional functions of this protein tag.

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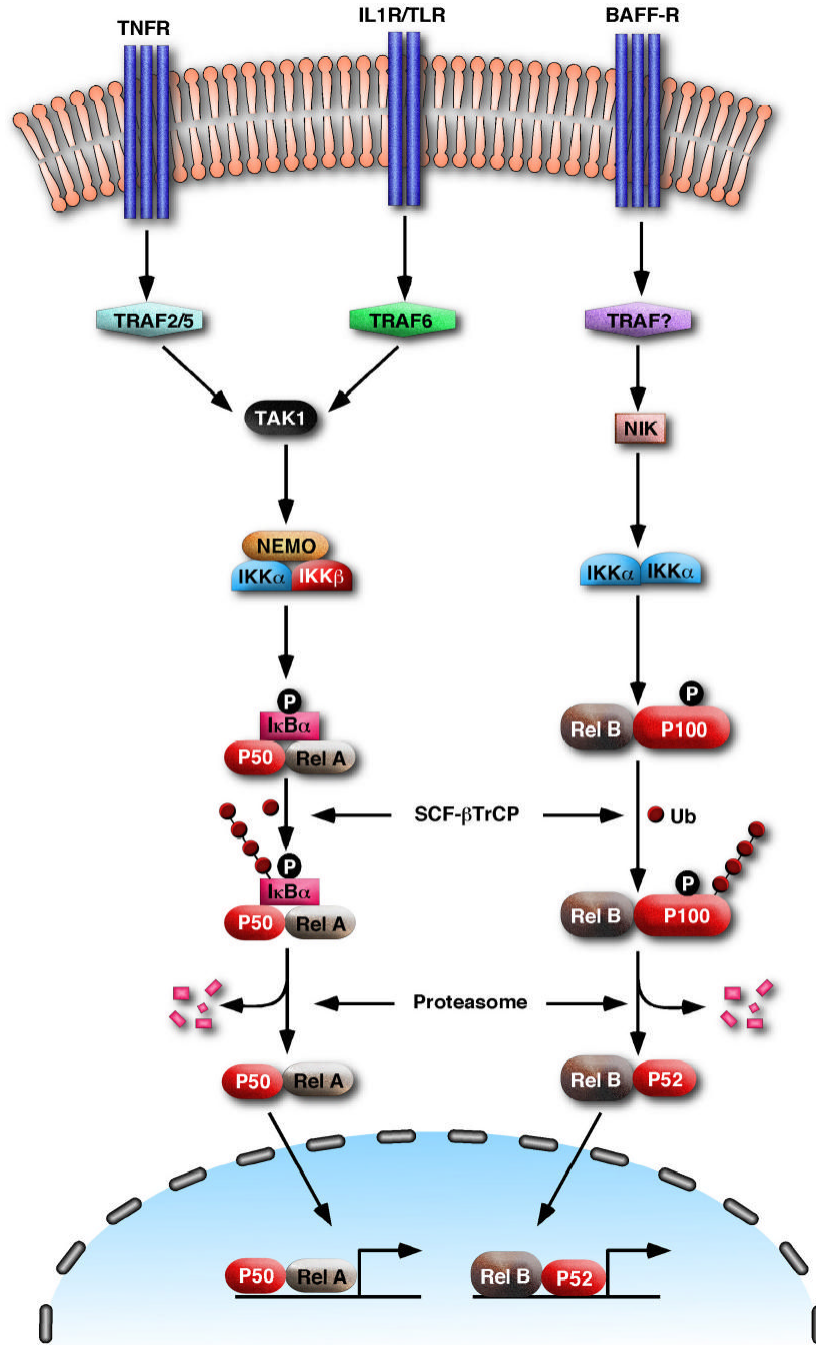


Figure 1. The NF-κB signaling pathways

In the canonical pathway (left), stimulation of TNF receptors (TNFR), IL-1 receptors (IL-1R) or Toll-like receptors (TLR) with their cognate ligands activates TRAF proteins and subsequently the kinase TAK1, which phosphorylates and activates IKK β . IKK β then phosphorylates I κ B resulting in its ubiquitination by the SCF- β TrCP ubiquitin ligase complex and subsequent degradation by the proteasome. The NF- κ B dimer consisting of p50 and Rel A can then enter the nucleus to regulate the expression of targets genes involved in inflammation, immunity and cell survival. In the non-canonical pathway, a subset of receptors belonging to the TNFR superfamily, such as the B cell receptor for BAFF (BAFF-R), activates the kinase NIK through an unknown mechanism. NIK then phosphorylates IKK α , which in

turn phosphorylates the NF- κ B precursor p100. p100 is subsequently polyubiquitinated and then processed to the mature subunit p52 by the proteasome. p52 and its binding partner Rel-B translocate to the nucleus to turn on genes that are important for the maturation of B cells.

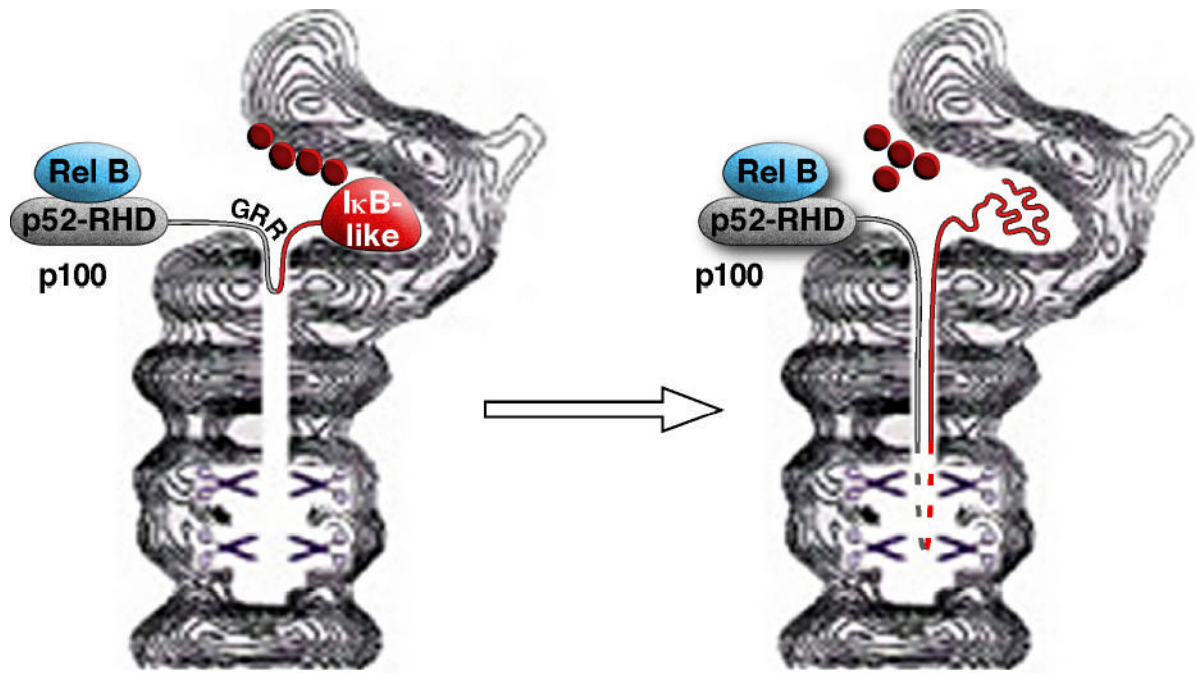


Figure 2. A model for the processing of p100 by the proteasome

Polyubiquitinated p100 is recruited to the proteasome, allowing the flexible glycine rich region (GRR) to insert into the proteasome as a loop. Degradation of the polypeptide proceeds in both the N- and C-terminal directions. The degradation of the C-terminus proceeds to the end, whereas the degradation along the N-terminus stops when the tightly folded Rel-homology domain (RHD) cannot be unfolded to allow insertion into the proteasome. The resulting p52 subunit and its dimeric partner Rel-B is then released from the proteasome.

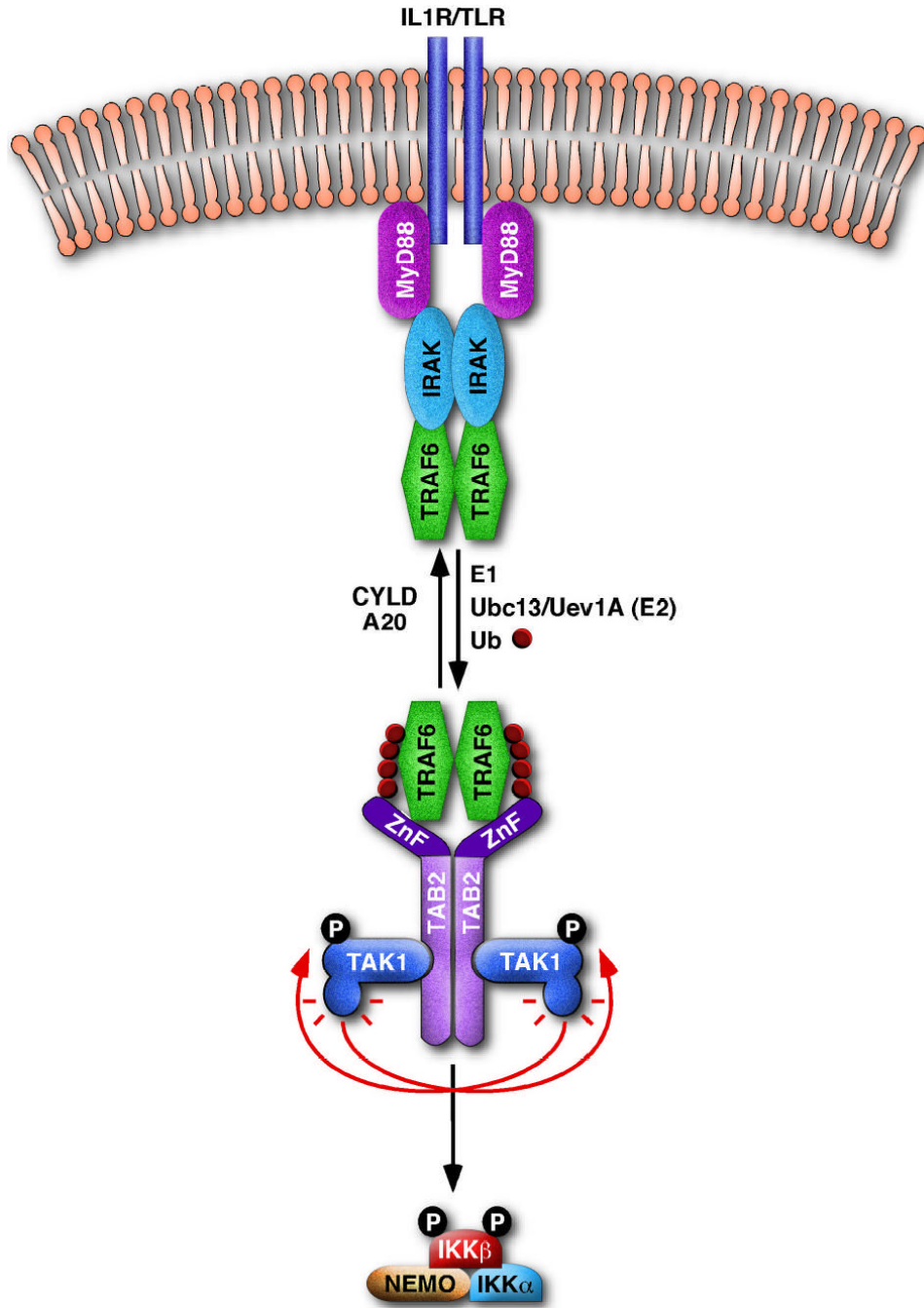


Figure 3. A model for IKK activation by TRAF6 ubiquitination

Stimulation of IL-1R or TLR leads to the recruitment of MyD88, IRAK and TRAF6 to the receptor complex. This may facilitate TRAF6 oligomerization and activate its ubiquitin ligase activity, leading to K63-linked polyubiquitination of targets including Nemo (not shown) and TRAF6 itself. This polyubiquitination reaction requires E1 and Ubc13/Uev1A, and can be reversed by deubiquitination enzymes such as CYLD and A20. Ubiquitinated TRAF6 is recruited to the TAK1/TAB2 complex through binding of K63-linked polyubiquitin chains to the NZF domain of TAB2 as well as by the direct interaction between TRAF6 and TAB2. This binding may facilitate the dimerization or oligomerization of the TAK1/TAB2 complex,

promoting its autophosphorylation and TAK1 activation. TAK1 then phosphorylates IKK β , resulting in its activation.

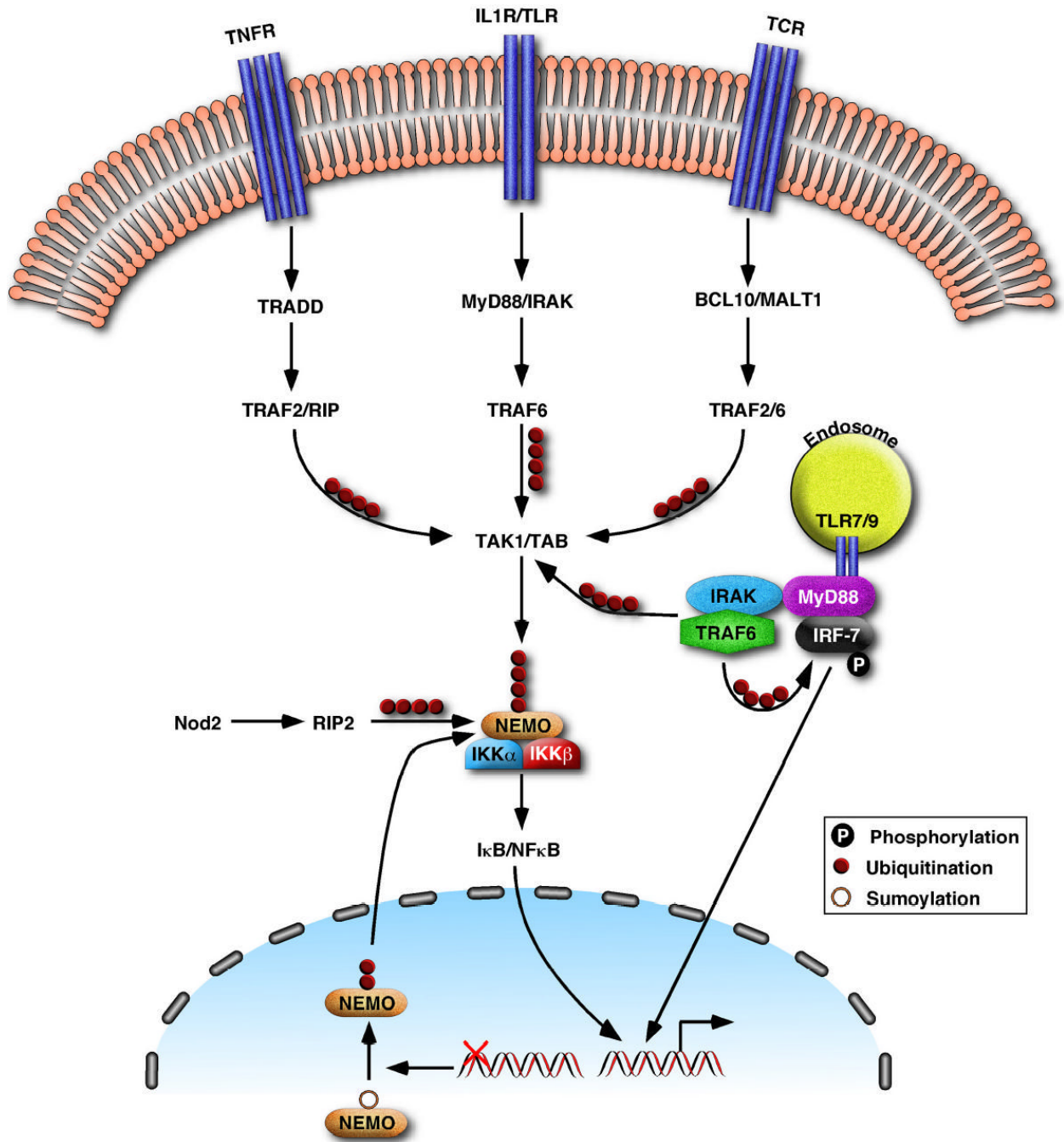


Figure 4. A central role for ubiquitin in multiple signaling pathways

Polyubiquitination by TRAF2 and TRAF6 not only mediates signaling by TNFR, IL1R and TLR (see Fig. 3) in the innate immunity pathways, but also plays an important role in adaptive immune responses by activating IKK in response to stimulation of TCR. In T cells, BCL10 and MALT1 activate the ligase activity of TRAF6 (and possibly TRAF2), to catalyze the K63-linked polyubiquitination of NEMO and TRAF6 itself. These ubiquitination events mediate the activation of TAK1 and IKK. NOD2 and RIP2, which trigger innate immune responses against intracellular bacteria, also facilitate the K63-linked polyubiquitination of NEMO. DNA damage leads to the sumoylation and subsequent ubiquitination of NEMO in the nucleus. The ubiquitinated NEMO then exits the nucleus and associates with the other subunits of the

cytosolic IKK complex, resulting in IKK activation. Some receptors of the TLR family, including TLR7, 8 and 9, are localized in the endosomal membrane. Stimulation of TLR7/8 and TLR9 by viral RNA and CpG DNA, respectively, leads to the activation of both NF- κ B and IRF7, which coordinately regulate interferon production. IRF7 binds to MyD88, which is associated with the endosomal TLRs as well as IRAK and TRAF6. TRAF6 polyubiquitination is required for the activation of IRF7, presumably by promoting the phosphorylation and subsequent nuclear translocation of IRF7.

Ubiquitin Mediated Activation of Protein Kinases	Protein/Enzyme Category	Protein/Enzyme Name	Functional Domains
	E2	Ubc13/Uev1A	Ubc/Ubc-like
		Ubc4; Ubc5	Ubc
	E3	TRAF6	RING; Zn finger; Coiled-coil; TRAF/MATH
		TRAF2	
		c-IAP1	RING; CARD; BIR
	Ub Linkage	K63-linked	
	Ub Receptor	TAB2; TAB3	CUE; NZF (Zn finger)
		p62 (?)	UBA; Zn finger
	Ub Target	TRAF6	(See above)
		TRAF2	
		RIP	Kinase; DD
		NEMO	Coiled-coil; leucine zipper; Zn finger
	Kinase Target	TAK1	Kinase
		IKK	Kinase; HLH; leucine zipper; Ub-like
	E3 Regulator	BCL10	CARD
		MALT1	DD; Ig-like; Caspase-like; TRAF6-binding
		TIFA	TRAF6-binding; FHA
	Deubiquitination Enzyme (Dub)	CYLD	UCH; CAP-Gly; SH3
		A20	OTU; Zn Finger

Table 1. Protein and enzymes involved in ubiquitin-mediated activation of IKK

Protein name abbreviations: c-IAP1: cellular inhibitor of apoptosis 1; RIP: receptor interacting protein; NEMO: NF- κ B essential modulator; MALT1: mucosal associated lymphoid tissue 1; TIFA: TRAF-interacting protein with a forkhead-associated domain. Protein domain abbreviations: Ubc: ubiquitin conjugating enzyme; RING: really interesting new gene; CUE: cue1 homologous; NZF: novel zinc finger; UBA: Ub association; DD: death domain; HLH: helix-loop-helix; CARD: caspase activation and recruitment domain; Ig-like: immunoglobulin like; FHA: forkhead-associated; UCH: ubiquitin C-terminal hydrolase; CAP-Gly: cytoskeleton-associated protein; SH3: Src homology-3; OTU: ovarian tumor type cysteine protease.