No one can whistle a symphony alone – how different ubiquitin linkages cooperate to orchestrate NF-κB activity

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Summary

Although it has been known for a long time that ubiquitylation has a major role in the activation and regulation of the nuclear factor kappa B (NF-κB) pathway, recent studies have revealed that the picture is a lot more complex than originally thought. NF-κB and ubiquitylation initially became linked when it was recognised that lysine (K)48-linked ubiquitin chains are involved in the processing of NF-κB precursors and the degradation of inhibitor of kappa B (IκB) proteins. Soon thereafter, it was reported that K63-linked chains were involved in the assembly of IκB kinase (IKK)-activating complexes and required for activation of the NF-κB signalling pathway. Recently, the discovery that atypical ubiquitin linkages, including linear and K11 linkages, are also involved in the activation of NF-κB has led to the need to re-evaluate existing models of how activation of this transcription factor is initiated and regulated. It is now becoming apparent that not only the canonical types of ubiquitin chains but possibly all linkage types have to be investigated in order to fully comprehend NF-κB activation. This can be considered a turning point in our view of the regulation of one of the most important pathways of gene induction. Hence, in this Commentary, we summarise the information that is currently available and incorporate it into a new model of NF-κB activation, thereby highlighting the emerging new challenges in understanding the role of ubiquitylation in NF-κB activation.

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Key words: NF-kappaB, Atypical linkage types, Ubiquitin

Introduction

Activation of the transcription factor nuclear factor kappa B (NF-κB) is a crucial step in cellular responses to a variety of internal and external stimuli, such as bacterial or viral infections, physical or oxidative stress and hyperosmotic shock (Hoffmann and Baltimore, 2006; Oeckinghaus and Ghosh, 2009). Consistent with its role downstream of a multitude of stimuli, the target genes induced by NF-κB are numerous and comprise functionally diverse proteins, including immunoregulatory factors, cytokines, cyclins and regulators of apoptosis as well as of the NF-κB pathway itself (Hoffmann and Baltimore, 2006; Oeckinghaus and Ghosh, 2009).

Owing to its role as a central mediator of processes that are essential to homeostasis and the integrity of the organism, aberrant activation of NF-κB can have deleterious consequences (Ben-Neriah and Karin, 2011; Courtois and Gilmore, 2006; Hayden and Ghosh, 2008; Wullaert et al., 2011). To avoid pathological outcomes, multiple regulatory mechanisms are in place to prevent untimely or excessive NF-κB activation. Many aspects of this regulation are based on post-translational modifications (Liu and Chen, 2011; Perkins, 2006; Wertz and Dixit, 2010). The two main regulatory modifications are phosphorylation (reviewed by Karin and Ben-Neriah, 2000; Schmitz et al., 2001; Sebban et al., 2006; Viatour et al., 2005) and ubiquitylation.

The term ubiquitylation refers to the covalent attachment of the small protein ubiquitin to target proteins. This reaction involves the concerted action of a ubiquitin-activating enzyme (E1) and a ubiquitin-conjugating enzyme (E2), which, in the presence of a ubiquitin ligase (E3), mediates the formation of an isopeptide bond between the C-terminus of ubiquitin and the ε-amino group of a lysine residue within a target protein. Because all seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) present in ubiquitin, as well as the N-terminal methionine (M1) can be used to create inter-ubiquitin linkages, eight different ubiquitin chain types exist in total. Depending on the linkage type, ubiquitin chains differ both structurally and functionally. The mechanism of ubiquitylation, the structural differences between ubiquitin chains, their recognition by specific ubiquitin-binding domains (UBDs) and the resulting functional differences have been reviewed elsewhere (Behrends and Harper, 2011; Dikic and Dotsch, 2009; Fushman and Walker, 2010; Hochstrasser, 2006; Komander, 2009; Passmore and Barford, 2004), and these aspects will therefore not be covered in detail here. Instead we will focus on ubiquitylation as a regulatory mechanism that is employed at multiple stages of NF-κB activation: depending on the linkage type, ubiquitylation mediates the processing of NF-κB precursors, the degradation of the inhibitor of kappa B (IκB) proteins (Kanarek et al., 2010) and favours the assembly of protein complexes that are required for NF-κB activation. Recent studies have revealed that, in achieving regulation of NF-κB through these mechanisms, the classical linkage types (i.e. K48- and K63-linked chains) are assisted by

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atypical ubiquitin chains, such as linear and K11-linked chains (Dynek et al., 2010; Gerlach et al., 2011; Haas et al., 2009; Ikeda et al., 2011; Tokunaga et al., 2011; Tokunaga et al., 2009; Emmerich et al., 2011). In this Commentary, we discuss the effects of both the well-established, as well as these more recently discovered, ubiquitin chains on NF-κB activation.

The NF-κB family of transcription factors

The term NF-κB refers not to a single protein but to a family of dimeric transcription factors. The dimers are formed by combinations of members of the Rel protein family that are characterised by the presence of a Rel homology domain (RHD), which enables the formation of homo- and hetero-dimers (Hayden and Ghosh, 2008) and is important for DNA-binding. The Rel protein family comprises RelA (also known as p65), RelB, Rel, p52 and p50 (Fig. 1A); p50 and p52 are generated by the processing of their precursors, p100 (encoded by *NFKB1*) and p105 (encoded by *NFKB2*), respectively.

The combinatorial association of the five NF-κB monomers results in the formation of 15 different dimers, of which nine are potential transcription factors. The others either cannot bind DNA or, owing to the lack of transactivation domains, do not exhibit transcriptional activity (Hoffmann and Baltimore, 2006; O'Dea and Hoffmann, 2009). A common feature of all transcription factors of the NF-κB family is that their activity is inducible. In the absence of a stimulus, all NF-κB subunits are expressed and pre-formed dimers exist within the cell (Baltimore, 2011; Hoffmann and Baltimore, 2006; Oeckinghaus and Ghosh, 2009). In this latent state, DNA binding of the NF-κB dimers is prevented through their association with members of the IκB family. This family is characterised by the presence of between five and seven ankyrin repeat motifs (Oeckinghaus and Ghosh, 2009; Zheng et al., 2011) and comprises the typical members IκBα, IκBβ and IκBε, the atypical IκBs B-cell CLL/lymphoma 3 (BCL3) and IκBζ, and the NF-κB precursors p105 and p100, which can also act as inhibitors of NF-κB activity (Fig. 1B) (Kanarek et al., 2010).

When bound to a p65–p50 dimer, IκBα, which is the best-studied member of the IκB family, masks the p65 nuclear localisation sequence (NLS), thereby leading to a predominantly cytoplasmic localisation of the IκB-bound NF-κB dimer. Freeing NF-κB dimers from IκBs drastically shifts the balance towards localisation in the nucleus, where NF-κB dimers can bind κB sequences in regulatory elements of NF-κB target genes and initiate transcription (Baltimore, 2011; Kanarek et al., 2010; Oeckinghaus and Ghosh, 2009). The removal of IκBs involves both phosphorylation and ubiquitylation events and can be achieved through two distinct and evolutionarily conserved pathways that are referred to as the canonical (or classical) and the non-canonical pathways (Hoffmann and Baltimore, 2006; Sun, 2011).

The canonical pathway can be activated by a variety of stimuli and consists of a fast-acting cascade of events that relies on IκB kinase β (IKKβ) and NF-κB essential modulator (NEMO, also referred to as IKKγ) as essential mediators in freeing NF-κB dimers from canonical IκBs (Box 1, Fig. 2). The canonical pathway is independent of de novo protein synthesis and is regulated by multiple feedback mechanisms (Hoffmann and Baltimore, 2006; Shih et al., 2011; Sun, 2011). By contrast, the non-canonical pathway acts more slowly and uses the kinase activities of the NF-κB-inducing kinase (NIK, also known as MAP3K14) and an IKKα homodimer to remove p100 inhibition from NF-κB dimers and to provide long-lasting NF-κB activity (Box 2) (Hoffmann and Baltimore, 2006; Shih et al., 2011; Sun, 2011). Consistent with these differences, the canonical pathway is predominantly involved in regulating proliferation and death of lymphoid cells during the immune response, whereas the non-canonical pathway is essential for the development of lymphoid organs (Sun, 2011).

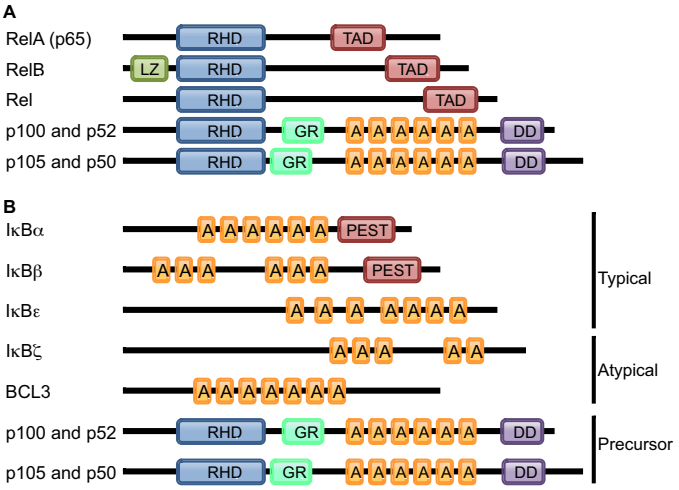


Fig. 1. Members of the NF-κB and IκB protein families. (**A**) Schematic representation of the five NF-κB (Rel) family members. The proteins p50 and p52 are generated from the precursor proteins p105 and p100, respectively, and lack a transactivation domain. The characteristic Rel homology domains, as well as other typical features, are indicated schematically. (**B**) Members of the IκB protein family are characterised by the presence of ankyrin repeats and their ability to bind and sequester NF-κB dimers. The NF-κB precursors p100 and p105 fulfil both of these criteria and can therefore be assigned to the IκB family of proteins. According to mechanistic and structural differences, members of this family can be subdivided into typical, atypical and precursor IκBs. RHD, Rel homology domain; TAD, transactivation domain; LZ, leucine zipper; GR, glycine-rich region; A, ankyrin repeats; DD, death domain; PEST, proline-, glutamic acid-, serine- and threonine-rich sequence.

The role of K48-linked ubiquitin in NF-κB signalling

K48-linked ubiquitin in the removal of non-functional proteins

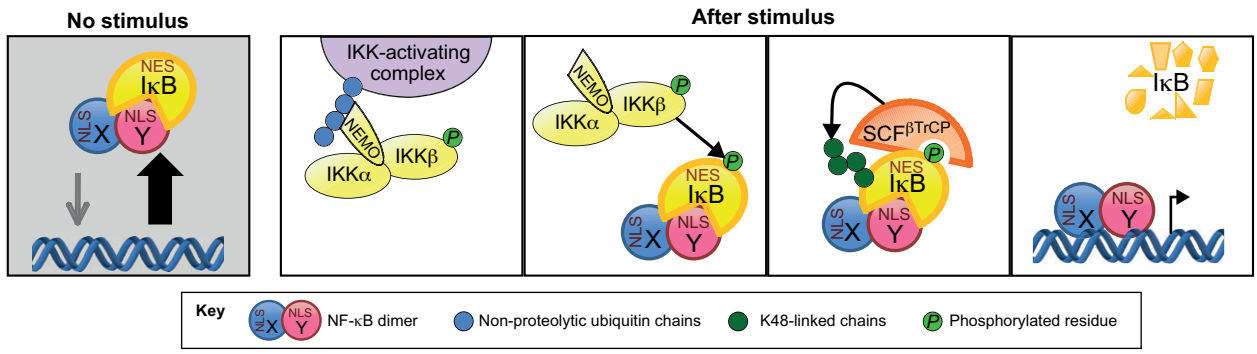
K48-linked ubiquitin chains were the first linkage type to be functionally characterised (Chau et al., 1989) and the attachment of ubiquitin molecules linked in this fashion is an important step in targeting proteins for proteasomal degradation (Pickart, 1997; van Nocker and Vierstra, 1993). However, K48-linked chains are not only components of a cellular waste-removal system but also have an important role in signal transduction pathways by mediating signal-induced degradation of both agonists and antagonists of these signalling cascades.

Positive regulation of the canonical pathway through the removal of IκBs

The stimulation-induced phosphorylation of the canonical IκBs by the IKK complex generates a degron motif that is recognised by the $SCF^{\beta TrCP}$ E3 complex (βTrCP is also known as FBW1A) (Kanarek et al., 2010), which initiates the attachment of K48-linked ubiquitin chains to and proteasomal degradation of IκB proteins (Kanarek et al., 2010). Phosphorylation of p105 by IKKβ generates a similar degron, which results in ubiquitylation of p105 at multiple lysine residues and complete degradation of the protein

Box 1. The canonical NF-κB pathway

The canonical NF-κB pathway can be activated by a variety of stimuli, including inflammatory cytokines, bacterial or viral products and oxidative or genotoxic stress conditions (Hadian and Krappmann, 2011; Miyamoto, 2011; Oeckinghaus and Ghosh, 2009). By activating their respective receptors, these stimuli induce the formation of large protein complexes that serve as platforms for the recruitment and activation of a preassembled protein complex that contains the kinases IKKα, IKKβ and the regulatory subunit NEMO. Activation of this IKK complex is essential for the induction of IκB degradation. Depending on the stimulus, these IKK-activating complexes can either be receptor-associated or secondary complexes that are dissociated from the originally stimulated receptor. Furthermore, they can differ greatly in their localisation and composition (see Fig. 2) (Harhaj and Dixit, 2011; Liu and Chen, 2011; Ruland, 2011; Wertz and Dixit, 2010).



Despite their distinct compositions, IKK-activating complexes share certain common features: in all of these protein assemblies, kinase recruitment and activation is achieved in a ubiquitin-dependent manner (Chen and Sun, 2009; Harhaj and Dixit, 2011; Liu and Chen, 2011; Wertz and Dixit, 2010). Polyubiquitin modifications on complex components are specifically recognised by NEMO and the TAK1-binding proteins 2 and 3 (TAB2 and TAB3) – the regulatory subunits of the IKK and TAB–TAK complexes, respectively (Cheung et al., 2004; Rothwarf et al., 1998; Shibuya et al., 1996) – and hence serve as recruitment platforms for these kinase complexes (Ea et al., 2006; Kanayama et al., 2004; Wu and Ashwell, 2008; Wu et al., 2006). It is not clear whether the phosphorylation events that are required for IKK activation occur by trans-autophosphorylation or are carried out by an upstream kinase, possibly TAK1 (Hayden and Ghosh, 2008; Oeckinghaus and Ghosh, 2009; Wang et al., 2001; Yang et al., 2001). In addition, binding of NEMO to ubiquitin chains results in conformational changes in the protein itself and possibly also in the associated kinases IKKα and IKKβ. These changes are likely to favour IKK activation. Furthermore, the recruitment of IKK to clusters of signalling proteins induces sufficient proximity between kinases for phosphorylation to occur through either of the two mechanisms (Hayden and Ghosh, 2008). In the context of the canonical pathway, activation of IKKβ is both necessary and sufficient to phosphorylate IκBs. Phosphorylated, canonical IκBs are recognised by the E3 complex $SCF^{\beta TrCP}$ (Skp, cullin, F-box containing complex together with β-TrCP) (Shirane et al., 1999; Wu and Ghosh, 1999; Yaron et al., 1998). This leads to IκB ubiquitylation and proteasomal degradation, thereby exposing the nuclear localization sequence (NLS) of NF-κB subunits and allowing NF-κB dimers to translocate to the nucleus and to initiate transcription (Kanarek et al., 2010). The resulting response is limited by different mechanisms, including the induction of the expression of IκBs (Hayden and Ghosh, 2008; Hoffmann and Baltimore, 2006). NES, nuclear export sequence.

(Perkins, 2006). This process is favoured when p105 is bound to other NF-κB subunits and differs from both constitutive and stimulation-induced partial processing of p105 to p50, which occur independently of K48-linked ubiquitylation (Oeckinghaus and Ghosh, 2009).

K48-chain-mediated regulation of the non-canonical pathway

Processing of p100, when induced by the non-canonical pathway, depends on K48-linked ubiquitylation. The E3 involved in attaching these chains to p100 is $SCF^{\beta TrCP}$, which recognises its substrate once p100 has been phosphorylated by the upstream kinases NIK and IKKα (Kanarek et al., 2010; Perkins, 2006). Processing of p100 at the proteasome is terminated at the glycine-rich domain that is present N-terminally of the ankyrin repeats and results in the generation of p52 (Hoffmann and Baltimore, 2006; Oeckinghaus and Ghosh, 2009). K48-linked ubiquitylation also positively regulates activation of the non-canonical pathway by causing degradation of components of the NIK-destruction complex. For example it has been shown that in CD40 signalling, TNF-receptor-associated factor (TRAF) 2 and 3 are degraded following their ubiquitylation by cIAP1 and/or cIAP2 (cellular inhibitor of apoptosis, also known as BIRC2 and BIRC3) (Sun, 2011).

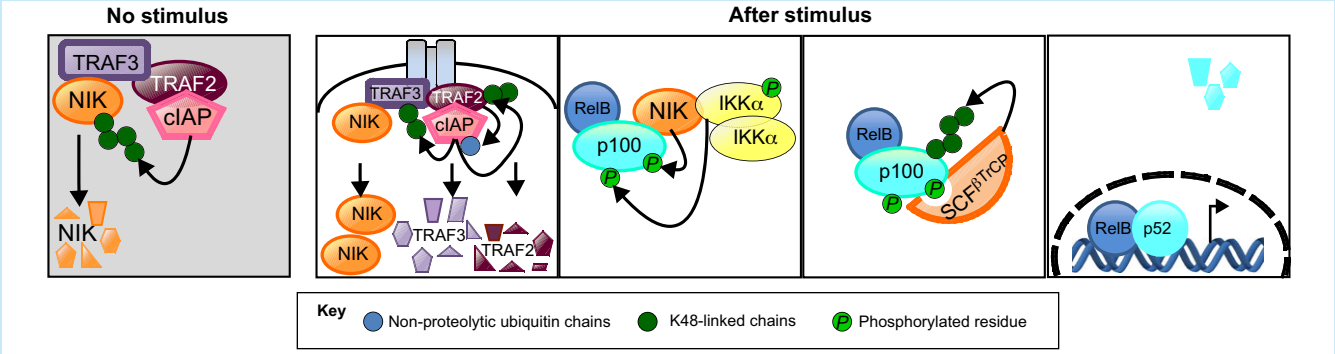
In contrast with the above examples, K48-linked ubiquitylation can also inhibit NF-κB activation: in the absence of a stimulus, the same destructive activity of cIAP1 and/or cIAP2 that is required for TRAF degradation in CD40 signalling is directed towards NIK, thereby restricting constitutive activation of the non-canonical pathway (Matsuzawa et al., 2008; Vallabhapurapu et al., 2008; Zarnegar et al., 2008).

Destabilisation of agonists of the canonical pathway

Other inhibitory effects of K48-linked ubiquitin on NF-κB activation are conveyed by the degradation of individual NF-κB subunits, such as Rel and RelA, following ubiquitylation (Perkins, 2006). Apart from the NF-κB subunits that are induced by different stimuli, stimulus-specific agonists of NF-κB activation can also be degraded in a ubiquitin-dependent manner. One example is the destabilisation of receptor-interacting protein 1 (RIP1, also known as RIPK1) in the context of the TNF receptor 1 (TNFR1)-associated signalling complex. Here, the non-proteolytic K63-linked chains attached to this protein are replaced with degradative K48-linked ubiquitylation by the A20 ubiquitin-editing complex [for more detail, refer to Harhaj and Dixit (Harhaj and Dixit, 2011)]. Other E3s suggested to be involved in RIP1 ubiquitylation and degradation are RING finger protein 216 (RNF216, also known as

Box 2. The non-canonical NF-κB pathway

The non-canonical NF-κB pathway differs from the classical signalling cascade in terms of its slower and more persistent kinetics, its physiological functions and its components (Sun, 2011). One of the first steps required for activation of the non-canonical pathway is the stabilisation of the kinase NIK. In the resting state a 'destruction complex' comprising TRAF2, cIAP1 and cIAP2 as well as TRAF3, which links NIK to this complex, mediates the proteasomal degradation of NIK, thereby keeping its levels very low (Liao et al., 2004; Vallabhapurapu et al., 2008; Zarnegar et al., 2008). Following recruitment to a receptor, TRAF2 modifies cIAP1 and cIAP2 with non-proteolytic ubiquitin chains, thereby changing their substrate specificity from NIK to TRAF3. This results in degradation of TRAF3, dissociation of the destruction complex from NIK and, consequently, in NIK accumulation (Sun, 2011).



Consistent with this mechanism, receptors that can induce the non-canonical NF-κB pathway commonly contain a TRAF-binding motif in their intracellular domain (Sun, 2011). This group of receptors comprises a subset of TNFR superfamily members, including BAFFR, CD40, LTBR, RANK, TNFR2 and FN14 (also known as TNFRSF12A) (Sun, 2011). FN14 employs a mechanistic variant of NIK stabilisation, whereby removal of TRAF2 and cIAPs involves lysosomal rather than proteasomal degradation (Vince et al., 2008).

Once sufficient levels of NIK have accumulated, the kinase mediates the phosphorylation of p100 and activates IKKα homodimers, which allows activated IKKα to phosphorylate the p100 subunit at several additional sites (Shih et al., 2011; Xiao et al., 2004; Xiao et al., 2001). Phosphorylated p100 is then recognised by the $SCF^{\beta TrCP}$ complex and the subsequent ubiquitylation of p100 results in partial processing by the 26S proteasome and the formation of the p52 subunit. This favours the activation of certain, mainly RelB-containing, NF-κB dimers. Considering the selectivity of the non-canonical pathway in activating only certain NF-κB dimers, it is not surprising that the targets induced and the functional roles served by this signalling cascade differ from those of the canonical pathway. In fact, this specificity also contributes to the different kinetic properties because the main mediators of the non-canonical pathway, the RelB-containing dimers, exhibit low affinities for binding to canonical IκBs and are therefore less responsive to the highly dynamic feedback regulation exerted by these inhibitors (Derudder et al., 2003; Shih et al., 2011). Instead, other mechanisms, such as the NF-κB-inducible expression of TRAF3 and the inhibitory phosphorylation of NIK by the downstream kinase IKKα (Razani et al., 2010; Shih et al., 2011) are in place to limit activation of the non-canonical pathway.

TRIAD3 and ZIN) (Fearns et al., 2006) and CARP2 (for caspases 8 and 10-associated RING finger protein 2) (Liao et al., 2008). Their importance for TNF-induced NF-κB activation is, however, controversial (Ahmed et al., 2009).

Destabilisation of agonists is a concept that is also employed in the regulation of other NF-κB signalling cascades. For example, the phosphorylation- and ubiquitylation-dependent degradation of BCL10 following TCR activation has been reported. The positive regulators of NF-κB signalling that have been described to be subject to ubiquitylation and degradation in the pathways activated by interleukin 1 (IL1) or Toll-like receptor (TLR)-stimulation are IL1 receptor-associated kinase 1 (IRAK1) and members of the pellino E3 family. Once activated by phosphorylation, pellino proteins act as agonists of NF-κB activation by mediating the signal-promoting K63-linked ubiquitylation of IRAK1 (Moynagh, 2009). RIG-I (retinoic acid inducible gene-I, also known as DDX58) signalling is also subject to degradation of agonistic components: RIG-I itself is modified with K48-linked chains by RNF125. The same E3 also mediates degradation of the downstream protein mitochondrial antiviral signalling protein (MAVS) (Arimoto et al., 2007).

Overall, K48-linked chains are implicated in both positive and negative regulation of the canonical and non-canonical NF-κB pathway at multiple stages. They act through a number of different mechanisms, some of which are common to all NF-κB-inducing stimuli, whereas others are specific for only some of these stimuli.

The role of K63-linked polyubiquitylation in NF-κB signalling

In contrast to K48-linked chains, K63-linked chains do not target proteins for proteolytic degradation and act mainly in favour of NF-κB activation. Although this type of ubiquitin chain exerts it regulatory effects at different stages of the pathway, its key regulatory functions are in influencing the assembly and stability of IKK-activating complexes and in kinase activation.

K63-linked ubiquitin chains as recruitment platforms in TNF signalling

In the TNFR1-associated signalling complex (TNF-RSC), components that become modified with K63-linked chains include RIP1, TRAF2, cIAP1 and cIAP2 as well as TGF-β-activated kinase 1 (TAK1, also known as MAP3K7) and NEMO (Wajant and Scheurich, 2011) (Table 1, Fig. 3). TRAF2 (Lee et al., 2004; Wertz et al., 2004) and cIAP1 and cIAP2 (Bertrand et al., 2008; Park et al., 2004) have been suggested to be the relevant E3 in RIP1 modification. One model proposes that TRAF2, in the presence of its cofactor sphingosine 1-phosphate (S1P), directly mediates this modification (Alvarez et al., 2010). Another model suggests that TRAF2 is responsible for the recruitment of cIAP1 and cIAP2 to the receptor complex, and the E3 activity of cIAP1 and/or cIAP2, but not of TRAF2, is required for attaching K63-linked chains to RIP1 (Bertrand et al., 2008; Park et al., 2004;

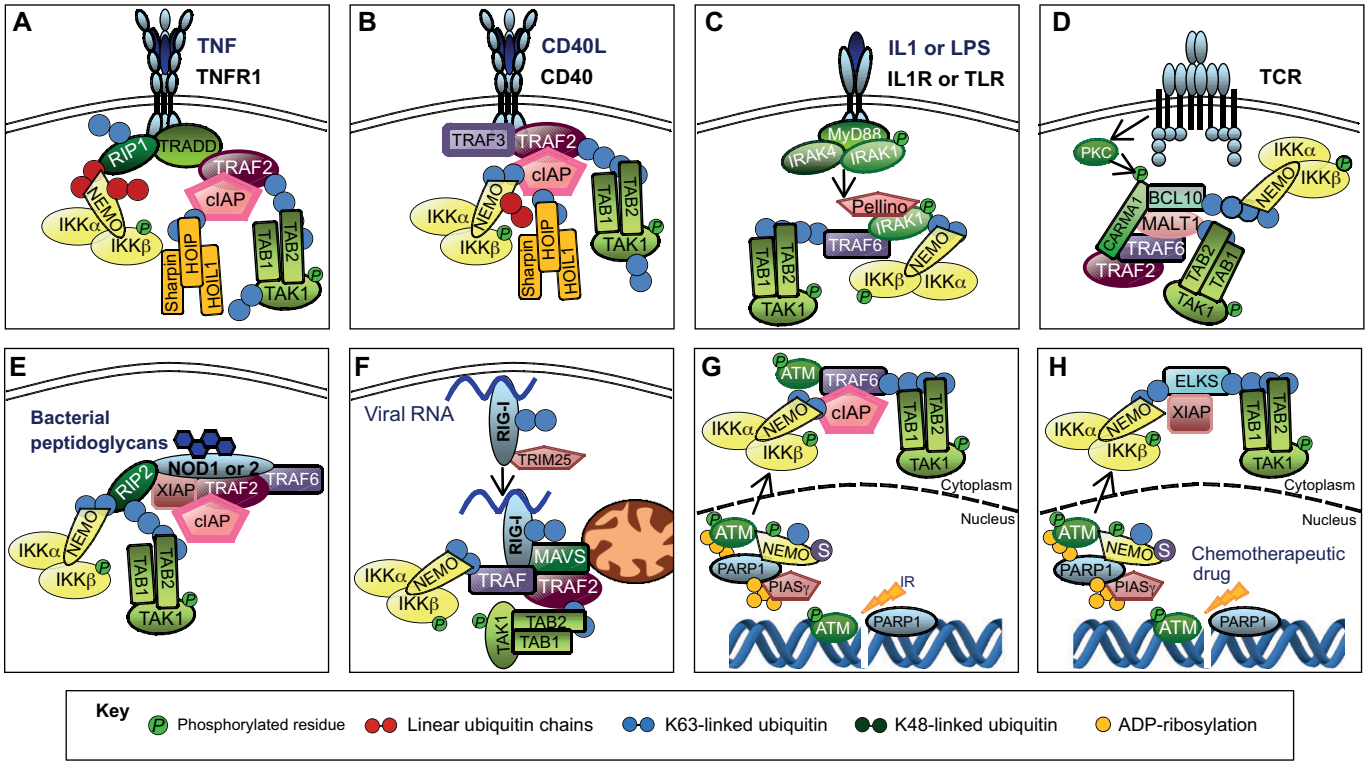


Fig. 2. Composition of IKK-activating complexes. In response to ligand stimulation, receptors that activate NF-κB recruit intracellular proteins to initiate signal transduction. (**A**) TNFR1 recruits TRADD, RIP1, TRAF2, cIAP1 and 2, and LUBAC (which comprises SHARPIN, HOIP and HOIL1). Formation of ubiquitin chains allows the TAB–TAK and IKK complexes to be recruited. (**B**) The CD40-associated IKK-activating complex consists of TRAF3, TRAF2, cIAPs, LUBAC and the TAB–TAK and IKK complexes. (**C**) Stimulation of IL1R or TLRs induces recruitment of MYD88 and the kinases IRAK1 and IRAK4. IRAK4-mediated phosphorylation of IRAK1 causes dissociation of IRAK1 from the receptor and formation of a secondary complex that contains IRAK1, TRAF6 and pellino and the TAB–TAK and IKK complexes. (**D**) Following TCR engagement, activated protein kinase C (PKC) phosphorylates CARMA1. A complex composed of CARMA1, BCL10, MALT1, TRAF2 and TRAF6 is assembled, which recruits the TAB–TAK and IKK complexes. (**E**) Binding of bacterial peptidoglycans to NOD1 or NOD2 results in the assembly of a complex consisting of RIP2, TRAF6, TRAF2, cIAP, XIAP and the TAB–TAK and IKK complexes. (**F**) TRIM25-mediated ubiquitylation of RIG-I allows association with MAVS and recruitment of TRAF6, TRAF2 and the TAB–TAK and IKK complexes. (**G**) DNA double-strand breaks (DSBs) induced by ionizing radiation are sensed by poly(ADP-ribose) polymerase 1 (PARP1) and ATM. This triggers formation of a nuclear complex, phosphorylation and sumoylation of NEMO and the export of ATM to the cytoplasm. Here, a complex containing TRAF6, cIAP and the TAB–TAK and IKK complexes is assembled. (**H**) DSBs induced by chemotherapeutic agents are sensed as in G, but lead to coupled nuclear export of ATM and NEMO and to the formation of an XIAP- and ELKS-containing complex which mediates IKK activation. K63-linked ubiquitylation is shown in blue, with linear chains in red. Green circles marked '*P*' represent phosphorylation, sumoylation is indicated by purple circles marked 'S' and yellow circles in G and H indicate ADP-ribosylation. PIASγ, protein inhibitor of activated STAT protein gamma; IR, ionizing irradiation.

Vince et al., 2009). K63-linked ubiquitin is clearly present on RIP1 (Gerlach et al., 2011; Newton et al., 2008) and it has been reported that it is essential for the recruitment of the IKK- and TAK1-binding protein (TAB)–TAK1-complexes (Ea et al., 2006; Kanayama et al., 2004; Lee et al., 2004; Wu et al., 2006). Especially the TAB–TAK1 complex depends on K63-linked ubiquitin for its recruitment, as its regulatory subunit, TAB2, has been shown to preferentially bind this linkage type (Kulathu et al., 2009). Recruitment of the TAB–TAK1 complex leads to activation of the kinase subunit TAK1. This could be achieved by conformational changes induced by ubiquitin binding or by positioning TAK1 in proximity of an E3 that subsequently mediates K63-linked

Table 1. Ubiquitin linkages present on components of the TNF-RSC

Linkage type	Modified component	Function	References
K11	RIP1	Recruitment platform; degradation and possibly signal termination	(Dynek et al., 2010; Gerlach et al., 2011)
K48	RIP1	Degradation and signal termination	(Gerlach et al., 2011; Newton et al., 2008)
K63	RIP1	Recruitment platform	(Ea et al., 2006; Gerlach et al., 2011; Li et al., 2006; Newton et al., 2008; Wu et al., 2006)
	TRAF2	Recruitment platform	(Li et al., 2009; Shi and Kehrl; 2003)
	TAK1	Kinase activation	(Fan et al., 2010)
M1	RIP1	Recruitment platform; complex stabilisation	(Gerlach et al., 2011)
	NEMO	Recruitment platform; kinase activation; complex stabilisation	(Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011; Tokunaga et al., 2009)

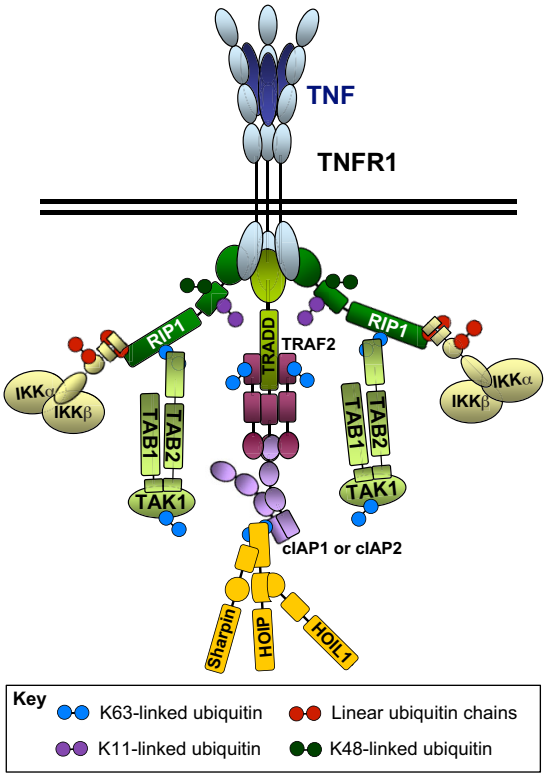


Fig. 3. Different types of ubiquitylation in the TNFR1-associated signalling complex. Schematic representation of the TNF-RSC and differentially linked ubiquitin chains known to modify components of this complex. Please refer to the text for details.

ubiquitylation of the kinase. It has been suggested that ubiquitylation of TAK1 is required for its full activity and that TRAF2 is involved in enabling this modification (Fan et al., 2010).

Like TAB2, NEMO can also bind to K63-linked chains and this binding event promotes IKK activation, again either through conformational changes induced by ubiquitin binding (Laplantine et al., 2009; Wu et al., 2006; Yoshikawa et al., 2009), by placing IKK in a context where it can be phosphorylated by upstream kinases such as TAK1, MEKK2 (MAPK/ERK kinase kinase 2, also known as MAP3K2) or MEKK3 (also known as MAP3K3) (Schmidt et al., 2003; Wang et al., 2001; Yang et al., 2001), or by inducing the clustering of IKK complexes and thereby favouring activation by transautophosphorylation (Hayden and Ghosh, 2008).

K63-linked ubiquitylation of NEMO has been reported to occur in response to a variety of stimuli and to be mediated by different E3s, including TRAF6 (Gautheron and Courtois, 2010). As discussed previously, this modification might induce conformational changes in or clustering of IKK complexes. However, it is also possible that other linkage types are more important both as a binding platform for, and as a modification of, NEMO (see below).

K63-linked ubiquitin chains as recruitment platforms in other signalling pathways

The roles of K63-linked chains in the recruitment and activation of kinase complexes are also relevant in signalling systems other than TNF-induced NF-κB activation. However, the components modified with this chain type vary between signalling pathways. In IL1R and TLR signalling the ubiquitylated proteins required for recruitment of the TAB–TAK- and IKK-complexes are TRAF6 and IRAK1, respectively (Conze et al., 2008; Kishida et al., 2005; Windheim et al., 2008), and the relevant E3 might be TRAF6 in both cases (Conze et al., 2008; Deng et al., 2000). Alternatively, IRAK ubiquitylation could be mediated by pellino proteins following their phosphorylation and activation by IRAK1 or IRAK4 (Ordureau et al., 2008).

Following TCR stimulation, BCL10 and MALT1 are K63-ubiquitylated (Oeckinghaus et al., 2007; Wu and Ashwell, 2008), whereas in the context of nucleotide-binding oligomerization domain 2 (NOD2) signalling, NOD2 itself and RIP2 have been shown to be modified (Abbott et al., 2007; Bertrand et al., 2009; Gautheron and Courtois, 2010; Hasegawa et al., 2008; Yang et al., 2007). In response to genotoxic stress, TRAF6, ELKS (protein rich in glutamate, leucine, lysine, and serine) and RIP1 have been suggested to be the targets of K63-linked ubiquitylation (Hinz et al., 2010; Wu et al., 2010; Yang et al., 2011). In the RIG-I pathway, TRIM25-mediated ubiquitylation facilitates the association of RIG-I with MAVS. In the resulting complex, TRAF2 and/or TRAF6, and potentially cIAP1 and/or cIAP2, could be both generators and acceptors of ubiquitin chains (Damgaard and Gyrd-Hansen, 2011; Gack et al., 2007; Oshiumi et al., 2009). Whereas recruitment of preassembled kinase complexes is an important function of K63-linked chains, these post-translational modifications also serve as binding platforms for other regulators of NF-κB signalling, like the E3 complex LUBAC, which is required for full activation of NF-κB in response to TNF (Gerlach et al., 2011; Haas et al., 2009).

K63-linked chains recruit negative regulators of NF-κB

The final group of proteins that are recruited to the IKK-activating complex downstream of TNFR1, and possibly other receptors, in a ubiquitin-dependent manner are negative regulators of NF-κB activation. These include A20 binding inhibitor of NF-κB (ABIN1) and optineurin (Mauro et al., 2006; Wagner et al., 2008; Zhu et al., 2007). Both proteins, like NEMO, contain a ubiquitin binding in ABIN and NEMO (UBAN) motif that allows recruitment to ubiquitylated proteins (Bloor et al., 2008; Wagner et al., 2008; Zhu et al., 2007). However, owing to the ubiquitin-binding properties of the UBAN domain (i.e. the preferential binding to M1-linked chains), other linkage types might be more important for the recruitment of optineurin and ABIN1 (see below). Furthermore, it has been shown that IL1- but not TNF-induced IKK-activation is blocked by the absence of the ubiquitin-conjugating enzyme UBCH13 (also known as UBE2N) (Yamamoto et al., 2006), an E2 that specifically generates K63 linkages (Deng et al., 2000; Hofmann and Pickart, 1999), and by replacement of wild-type ubiquitin with a K63R ubiquitin mutant that prevents the formation of K63-linked chains (Xu et al., 2009). This suggests that the polyubiquitylation involved in TNF signalling is not restricted to K63-linked chains, and that linear and K63-linked ubiquitylation and potentially other atypical linkages fulfil distinct and non-redundant functions. This view is corroborated by the recent identification of a role for M1- and K11-linked chains in the context of different stimuli (Verhelst et al., 2011).

Linear ubiquitylation in context of NF-κB signalling

Linear ubiquitin linkages are generated when the N-terminal α-amino group on M1, rather than the ε-amino group of a lysine residue, is used as the acceptor site for additional ubiquitin molecules. Hence, the M1-linkage generates a peptide bond,

whereas all other linkage types result in the formation of isopeptide bonds. Nevertheless, M1-linked chains resemble K63-linked chains in the overall structure, as both chain types adopt an apparently similar extended conformation (Komander et al., 2009b). Although these structural similarities suggest a certain degree of redundancy between M1- and K63-linked chains, the conformation of M1-linked chains is more restrained, and it has been shown that some UBDs bind exclusively, or at least with much higher affinity, to one or the other linkage type (Kulathu et al., 2009; Lo et al., 2009; Rahighi et al., 2009).

Currently, the only E3 known to promote generation of M1 linkages is LUBAC (Kirisako et al., 2006; Verhelst et al., 2011). LUBAC is an E3 complex that consists of HOIL1 (for heme-oxidized IRP2 ubiquitin ligase-1), HOIP (for HOIL1-interacting protein) and SHARPIN (for shank-associated RH domain interactor) (Gerlach et al., 2011; Ikeda et al., 2011; Kirisako et al., 2006; Tokunaga et al., 2011). When in complex with SHARPIN, HOIL1 or both in vitro, HOIP exclusively generates linear ubiquitin chains independently of the E2 supplied. M1-linkages were initially shown to mediate proteasomal degradation of a model substrate (Kirisako et al., 2006). This observation was supported by studies that showed that fusion of a non-cleavable linear tetra-ubiquitin chain to a target protein can lead to the degradation of this protein (Prakash et al., 2009) and that linear chains can bind the same proteasomal receptors as K48-linked chains (Thrower et al., 2000). In the context of the RIG-I pathway, LUBAC activity has been linked to the degradation of TRIM25 and hence to suppression of type I interferon induction (Inn et al., 2011).

Linear chains positively regulate NF-κB signalling

A positive regulatory role for linear chains in signalling first became apparent when it was shown that TNF-induced NF-κB activation is impaired when the expression of one or two of the LUBAC components are suppressed by RNA interference (Haas et al., 2009; Tokunaga et al., 2009) and when LUBAC was identified as a functional component of the native TNF-RSC (Haas et al., 2009; Walczak, 2011). Recruitment of LUBAC to the TNF-RSC depends on HOIP (Gerlach et al., 2011) and the E3 ligase activity of cIAP1 and/or cIAP2, which suggests that LUBAC, through HOIP, binds to cIAP-generated ubiquitin chains on a component of the TNF-RSC (Haas et al., 2009). It is probable that further UBDs present in SHARPIN and HOIL1 enable more stable interaction of LUBAC with the TNF-RSC once it has been recruited. Since the initial discovery of a role for LUBAC in signalling pathways, additional studies have revealed that its function is not restricted to signalling downstream of TNFR1 and IL1R but that it also regulates CD40-, lipopolysaccharide (LPS)- and lymphotoxin-β receptor (LTβR)-induced signalling (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011) and the response to genotoxic stress (Niu et al., 2011).

Linear chains in signal activation

In most, if not all, of the pathways involving LUBAC, this E3 ligase seems to act by a di- or possibly a tripartite mechanism. Similar to K63-linked chains, linear polyubiquitin serves as a recruitment platform for NEMO, thereby promoting IKK activation. However, binding of NEMO to K63- and linear chains differs both quantitatively and qualitatively (Lo et al., 2009; Rahighi et al., 2009). Mutations in NEMO that affect its ability to bind linear ubiquitin have been shown to lead to decreased NF-κB activation (Hadian et al., 2011; Rahighi et al., 2009). Mutations in the UBAN domain were also identified in patients suffering from X-linked ectodermal dysplasia and immunodeficiency, indicating that alterations that interfere with the ability of NEMO to bind linear ubiquitin not only inhibit the canonical pathway of NF-κB activation on a molecular level but can also have detrimental effects for the whole organism (Rahighi et al., 2009).

The modification of the regulatory subunit of the IKK complex at K285 and K309 with linear ubiquitin chains (Tokunaga et al., 2009) represents the second leg of the mechanism by which LUBAC could enhance NF-κB signalling. In vitro assays have indicated that NEMO is a direct target of LUBAC-mediated ubiquitylation (Gerlach et al., 2011; Tokunaga et al., 2009). Furthermore, the reconstitution of NEMO-deficient cells with a K285R and K309R double-mutant, in which the ubiquitylation sites are mutated, is not able to rescue the induction of NF-κB signalling by LUBAC or IL1 (Tokunaga et al., 2009). Although the precise role of the attachment of M1-linked chains to NEMO remains unclear, this modification might cause conformational changes or clustering of IKK units, thereby favouring their activation.

In the context of the TNF-RSC, a third aspect of positive regulation by linear chains has been identified. The activity of LUBAC, probably at least partially by modification of RIP1 and NEMO (and by providing a binding site for the latter), leads to an overall stabilisation of the receptor complex (Haas et al., 2009). By retaining RIP1, TRAF2, cIAP and TAK1 in the complex, LUBAC extends the half-life of the TNF-RSC, thereby allowing enhanced IKK activation. This effect of linear ubiquitylation is probably also a result of linear chains being more refractory to cleavage by most of the deubiquitylating enzymes (DUBs) that are present in the receptor complex (Komander et al., 2009b).

Linear chains in signal termination

In addition to positively regulating NF-κB signalling, linear chains might also be required for efficient termination of the NF-κB response, as they serve as recruitment platforms for the UBAN-containing proteins ABIN1 and optineurin. It has been reported that optineurin interferes with TNF-induced NF-κB activation by competing with NEMO for binding to ubiquitylated RIP1 (Zhu et al., 2007) and by assisting the DUB protein cylindromatosis (CYLD) in its negative regulatory role in TNF signalling (see Harhaj and Dixit, 2011). A similar mode of action has been suggested for ABIN1 which mediates the interaction of the DUB A20 with ubiquitylated NEMO, thereby negatively affecting activation of IKKs (Harhaj and Dixit, 2011).

Further studies will be required to clarify which aspects of the mechanism involving linear ubiquitin chains – namely, (1) provision of binding platforms by ubiquitylation of RIP1, NEMO and possibly other factors, (2) kinase activation by conformational changes induced by NEMO ubiquitylation, and (3) stabilisation of IKK-activating complexes – are relevant in the context of a particular stimulus. In addition, it will be interesting to investigate whether other E3s are capable of forming M1-linkages in the context of native signalling complexes.

Atypical polyubiquitin chains and monoubiquitylation

There is accumulating evidence that other polyubiquitylation events, and monoubiquitylation, are also important for efficient and controlled NF-κB activation in different contexts. It was reported recently, that, following TNF stimulation, cIAP1, together with

UBCH5 (also known as UBE2D1), generates K11-linked chains on RIP1 and that NEMO can bind to K11 and K63 linkages with similar affinities, which suggests a role for K11 linkages in promoting NF-κB activation (Dynek et al., 2010). However, considering that K11-linked ubiquitin chains were previously characterised as a degradative signal with roles in cell cycle regulation (Matsumoto et al., 2010), a similar effect on RIP1 and, hence, a negative regulatory role in TNF-signalling could also be envisioned.

Another linkage type that has been reported to influence protein complex assembly in NF-κB signalling is K33-linked ubiquitin chain formation, which has been suggested to negatively regulate TCR signalling by targeting TCRζ and thereby preventing its association with, and phosphorylation by, the downstream kinase zeta-chain associated protein kinase 70 kDa (ZAP70) (Huang et al., 2010). Furthermore, there are reports on the modification of NEMO with different ubiquitin linkage types. This includes K27-linked polyubiquitylation following TLR stimulation (Arimoto et al., 2010), in which TRIM23 is the relevant E3, and the cIAP-mediated attachment of K6 linkages, which was described in the context of TNFR1 signalling (Tang et al., 2003). It is suspected that the role of at least some of the atypical ubiquitin linkages in activating and regulating signalling pathways has so far been underestimated. However, further studies are required to validate this hypothesis and to identify which linkage types are important in which context. Similarly, enzymes involved in generating these linkages and their targets will need to be characterised in more detail.

The assembly of polyubiquitin is not always necessary to mediate signalling effects, as modification with single ubiquitin moieties at one (monoubiquitylation) or several sites (multi-monoubiquitylation) can be sufficient as a signal (Hoeller et al., 2006). Indeed, it has been reported that constitutive processing of p105 to p50 involves monoubiquitylation of p105 (Perkins, 2006). In addition, monoubiquitylation of phosphorylated and SUMOylated NEMO in the nucleus, potentially by cIAP1, leads to its export to the cytoplasm, which represents a crucial step in genotoxic-stress-induced NF-κB activation (Hadian and Krappmann, 2011).

The role of DUBs in NF-κB signalling

In addition to the enzymes that mediate post-translational modifications, proteins that catalyse the reversal of these processes are equally important for the system to work in a stimulus-dependent and regulated manner. In the case of ubiquitylation, this is carried out by a set of cysteine- or metallo-proteases (Harhaj and Dixit, 2011; Komander et al., 2009a), the so-called DUBs. Like E3s, DUBs can exhibit specificity for certain linkage types, which can be mediated by the presence of UBDs in the DUB itself, by ubiquitin-binding adaptor proteins or by selectivity of the catalytic core (Harhaj and Dixit, 2011; Komander, 2010; Komander and Barford, 2008; Komander et al., 2008).

Several DUBs have been implicated in NF-κB signalling. This includes the A20 ubiquitin-editing complex [components of this complex as well as their specific activities are given elsewhere (Harhaj and Dixit, 2011)], CYLD, cezanne (also known as OTUD7B), ubiquitin-specific peptidase 11 (USP11), USP15 and USP21, which all serve as negative regulators of the canonical NF-κB pathway (Bremm et al., 2010; Brummelkamp et al., 2003; Heyninck et al., 1999; Jaattela et al., 1996; Kovalenko et al., 2003; Schweitzer et al., 2007; Sun et al., 2010; Trompouki et al., 2003; Xu et al., 2010).

The DUBs A20, CYLD, cezanne and USP21 work on upstream complexes by removing non-proteolytic ubiquitylation from components such as RIP1, TRAF6, RIP2, NOD2 and MALT1 (Harhaj and Dixit, 2011). These DUBs differ in their linkage specificities (Wertz et al., 2004) and act in a temporally distinct manner, but show overlapping target specificities. For instance, all four DUBs have been shown to remove ubiquitin chains from RIP1 in the context of TNF signalling. The reason for this overlap in target specificity between the different DUBs has not been clarified. However, considering that mass spectrometric analysis of the TNF-RSC revealed the presence of K48-, K63-, K11- and M1-linked ubiquitin on TNF-RSC-associated RIP1 (Gerlach et al., 2011), it is an intriguing possibility that different ubiquitin linkages, attached to specific sites on RIP1, fulfil distinct functions and that their individual removal is mediated by specialised DUBs.

It has been reported that the two other DUBs, USP11 and USP15 negatively regulate NF-κB activation by removing K48-linked chains from IκB, which might serve as a fine-tuning mechanism in NF-κB activation (Harhaj and Dixit, 2011). Whereas DUBs are as important for the regulation of NF-κB signalling as the enzymes that generate ubiquitin chains, our understanding of the specificities and mechanisms of actions of these enzymes is far from complete. Further studies are required to elucidate the complex interplay between different ubiquitin linkages, and to clarify the mechanisms leading to their stimulus-dependent generation on and removal from individual signalling complex components by specific enzymes.

Conclusions and perspectives

Ubiquitylation is a major principle in the regulation of NF-κB activation. The functional outcome of modification with ubiquitin chains does not only depend on the target protein but also on the residues involved in forming the inter-ubiquitin linkage. K48-linked ubiquitin chains mediate both positive and negative regulation of NF-κB signalling by targeting both agonists and antagonists of this signalling cascade for proteasomal degradation. Non-proteolytic chain types, such as K63- and M1-linked chains, exert their effects by providing binding platforms for mediators of NF-κB activation, such as the IKK- and TAK–TAB-complexes, and for protein complexes involved in the termination of the signal, such as DUBs and their adaptor proteins. The roles of atypical chain types are less well understood and further studies are required to characterise the generation of these chains in the context of NF-κB signalling, to identify target proteins and to analyse functional outcomes of modification with certain linkage types.

NF-κB activation is crucial for raising and maintaining an immune response, but also for the appropriate termination of such a response (Ben-Neriah and Karin, 2011). Owing to this role as a central regulator of the immune system, aberrant activation of NF-κB is associated with many acute and chronic inflammatory diseases (Wullaert et al., 2011), and other disorders including cancer have also been shown to be caused or aggravated by dysregulated NF-κB signalling (Ben-Neriah and Karin, 2011; Kirkin and Dikic, 2011). Mutations in components of the NF-κB signal transduction machinery (e.g. NEMO) or in proteins mediating the ubiquitin-dependent regulation of this pathway (e.g. CYLD) (Courtois and Gilmore, 2006; Courtois and Israel, 2011; Shifera, 2010) have been associated with a variety of diseases. This makes the NF-κB pathway an attractive target for therapeutic intervention in a number of diseases. However, because of its role in a multitude of processes required for homeostasis and survival, serious side

effects have to be reckoned with. Therapeutic approaches that target more specific regulatory events within the pathway might therefore be a promising alternative. Disrupting the attachment of a particular type of ubiquitin chain to a specific site in a defined target protein might be sufficient to interfere with certain outcomes of NF-κB signalling, while not affecting others. However, the design of this kind of specific therapy requires a complete knowledge of the events taking place during activation and termination of this pathway and of their direct and indirect effects. Further studies on the specific roles of the different types of ubiquitin linkages, as well as their interplay with other post-translational modifications, are required to truly understand the fascinating mechanisms that regulate the activity of NF-κB.

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