Linear polyubiquitination: a new regulator of NF- κ B activation

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The ubiquitin-conjugation system regulates a vast range of biological phenomena by affecting protein function mostly through polyubiquitin conjugation. The type of polyubiquitin chain that is generated seems to determine how conjugated proteins are regulated, as they are recognized specifically by proteins that contain chain-specific ubiquitin-binding motifs. An enzyme complex that catalyses the formation of newly described linear polyubiquitin chains—known as linear ubiquitin chain-assembly complex (LUBAC)—has recently been characterized, as has a particular ubiquitin-binding domain that specifically recognizes linear chains. Both have been shown to have crucial roles in the canonical nuclear factor- κ B (NF- κ B)-activation pathway. The ubiquitin system is intimately involved in regulating the NF- κ B pathway, and the regulatory roles of K63-linked chains have been studied extensively. However, the role of linear chains in this process is only now emerging. This article discusses the possible mechanisms underlying linear polyubiquitin-mediated activation of NF- κ B, and the different roles that K63-linked and linear chains have in NF- κ B activation. Future directions for linear polyubiquitin research are also discussed.

Keywords: linear polyubiquitin chain; LUBAC; NEMO; NF- κ B; ubiquitin

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See Glossary for abbreviations used in this article.

Introduction

The ubiquitin-conjugation system was initially identified as part of an energy-dependent protein-degradation system (Ciechanover *et al*, 1978). For almost 20 years after the discovery of ubiquitin, the conjugation of ubiquitin chains to a protein was thought to be a signal for degradation by the proteasome, and the polyubiquitin chains that mediated this effect were thought to be linked through K48 (Chau *et al*, 1989). However, ubiquitination was also shown to

have non-proteolytic roles when Spence and colleagues reported that K63 has a crucial role in DNA repair without affecting protein turnover (Spence *et al*, 1995). The seminal discovery that an E2 complex containing UBC13 selectively generates K63-linked chains, made by Hofmann & Pickart (1999), stimulated research into the non-proteolytic functions of ubiquitin and K63 chains, which were subsequently shown to be involved not only in DNA repair but also in signal transduction (Kerscher *et al*, 2006).

Ubiquitination is now recognized to regulate numerous biological phenomena by modifying protein function through several mechanisms. Although monoubiquitination has been shown to have signalling functions—such as in the endocytic pathway (Hicke & Dunn, 2003)—in most cases it is the conjugation of polyubiquitin to proteins that has crucial roles in cell physiology (Glickman & Ciechanover, 2002). The conjugation of ubiquitin to target proteins is mediated through a cascade of reactions catalysed by three enzymes—E1, E2 and E3—although the precise mechanisms of polyubiquitin generation and, in particular, the mechanisms that ensure a particular linkage specificity have not yet been identified (Hochstrasser, 2006). E3 enzymes recognize substrate proteins and facilitate the transfer of ubiquitin from an E2 donor. The members of one class of such enzymes, the HECT E3 ligases, usually function as monomers, and ubiquitin is initially transferred to a conserved cysteine residue of the ligase before it is transferred to substrates. Most other E3s are included in the RING/U-box family of ligases, the members of which seem to function as a scaffold that binds to both an E2 bound to ubiquitin and a substrate, and facilitate the transfer of ubiquitin directly from the E2 to the substrate. RING E3 ligases have been structurally subdivided into those that normally function as multimers such as the Cullin–RING ligases, and those that function as monomers such as c-Cbl and Parkin (Dye & Schulman, 2007).

Polyubiquitin chains were thought to be formed only by the conjugation of the ubiquitin carboxy-terminal glycine to an internal lysine residue of another ubiquitin. The presence of isopeptide linkages at all seven lysine residues of ubiquitin has been reported and the type of conjugated polyubiquitin seems to determine the mode of the regulation of conjugated proteins (Ikeda & Dikic, 2008; Peng *et al*, 2003; Pickart & Fushman, 2004). The roles of other lysine-linked ubiquitin chains are under extensive investigation (Jin *et al*, 2008; Xu *et al*, 2009). However, we have identified a new type of chain—the linear polyubiquitin chain—in which the C-terminal glycine of ubiquitin is conjugated to the α -amino group

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of the amino-terminal methionine of another ubiquitin. Linear polyubiquitin is generated by a unique ubiquitin ligase complex that we named LUBAC (Kirisako *et al*, 2006).

LUBAC and the generation of linear chains

LUBAC is composed of two RING-IBR-RING proteins—HOIL-1L and HOIP, with molecular weights of 58 kDa and 120 kDa, respectively—the primary structures of which are shown in Fig 1. We hypothesize that the LUBAC complex is composed of two or three molecules of each protein, as gel-filtration analyses estimated its molecular weight to be approximately 600 kDa (Kirisako *et al*, 2006). Both HOIP and HOIL-1L have multiple domains, among which the UBA domain of HOIP and the UBL domain of HOIL-1L are involved in LUBAC formation. The subunit composition of LUBAC seems to be unique as structurally both HOIL-1L and HOIP belong to the monomeric RING ligase group, but form a complex through a domain specialized in dimer formation (Kirisako *et al*, 2006). By contrast, the BRCA1 E3 ligase has been reported to form a dimer with BARD1 through the RING domain (Brzovic *et al*, 2001).

LUBAC can generate linear chains with several E2s—including UBCH5s, E2-25K and UBCH7—and cannot generate polyubiquitin with N-terminally tagged wild-type ubiquitin, indicating that it generates only linear (and not lysine-linked) chains. Notably, E2-25K generates exclusively K48-linked chains *in vitro* in the absence of an E3 (Chen & Pickart, 1990), although it generates linear chains in the presence of LUBAC. Therefore, LUBAC—and not E2s—seems to be predominantly responsible for determining linkage specificity. This situation is different from the generation of K63-linked chains, as K63 specificity is determined by an UBC13-containing E2 complex (Hofmann & Pickart, 1999).

Both HOIP and HOIL-1L have NZF domains that are dispensable for the generation of N-terminally linked linear chains, although they have ubiquitin-binding activity (Kirisako *et al*, 2006). Instead, NZFs are crucial for NF- κ B activation by LUBAC through their involvement in NEMO binding (Tokunaga *et al*, 2009).

LUBAC specifically activates NF- κ B

NF- κ B is a dimeric transcription factor composed of the Rel family proteins p65 and p50 (Fig 2). It is activated in response to multiple signals and induces the expression of a wide range of molecules that mediate many different processes, including inflammation and cell survival (Hayden & Ghosh, 2008). In the resting state, most NF- κ B is sequestered in the cytoplasm through its binding to inhibitory proteins such as I κ Bs. Although two NF- κ B-activation pathways exist (Hacker & Karin, 2006), LUBAC has a role only in the canonical pathway (Tokunaga *et al*, 2009), which is the focus of this article (Fig 2). Upon activation by various stimuli, I κ Bs are phosphorylated by the IKK complex and are subsequently ubiquitinated with K48-linked chains by the SCF ^{β TrCP} ligase, which targets them for degradation by the proteasome. Then, the liberated NF- κ B translocates into the nucleus and induces the expression of target genes. The IKK complex is composed of three proteins: IKK α , IKK β and NEMO (IKK γ); IKK α and IKK β have kinase activity, whereas NEMO does not. Instead, NEMO exerts its regulatory function by integrating upstream signals that lead to IKK activation. In the canonical pathway, IKK β has an essential role in the phosphorylation of I κ Bs and its activation is mediated by the phosphorylation of specific serine residues (Hacker & Karin, 2006).

Glossary

ABIN-1	A20 binding inhibitor of NF- κ B-1
BARD1	BRCA1-associated RING domain 1
BRCA1	breast cancer 1
c-Cbl	c-Casitas B-lineage lymphoma
CYLD	cyldromatosis
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin ligase
HECT	homologous to E6-associated-protein carboxyl terminus
HOIL-1L	longer isoform of haem-oxidized iron-regulatory protein ubiquitin ligase 1
HOIP	HOIL-1L interacting protein
I κ B	inhibitor of κ B
IBR	in-between RING
IKK	inhibitor of κ B kinase
JNK	Jun amino-terminal kinase
LUBAC	linear ubiquitin chain assembly complex
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
MEKK3	MAPK kinase kinase 3
NEMO	NF- κ B essential modulator
NF- κ B	nuclear factor- κ B
NZF	novel zinc finger
PKC	protein kinase C
RING	really interesting new gene
RIP1	receptor interacting protein 1
SCF ^{βTrCP}	Skp1-cullin-F box (β TrCP)ubiquitin ligase
TAB	TAK1-binding protein
TAK1	transforming growth factor β -activated kinase 1
TNF- α	tumour necrosis factor- α
TNFR1	TNF- α receptor 1
TRAF	TNF receptor-associated factor
UBA	ubiquitin-associated
UBAN	ubiquitin-binding in ABIN and NEMO
UBC	ubiquitin conjugating enzyme
UBL	ubiquitin-like
U-box	UFD2-homology domain
Uev	ubiquitin E2 variant
XR-MSMD	X-linked recessive Mendelian susceptibility to mycobacterial diseases
ZF	zinc finger

LUBAC has been shown to bind to NEMO in the IKK complex after stimulation with TNF- α , and to conjugate linear chains onto K285 and/or K309 *in vitro*. Wild-type NEMO—but not a NEMO mutant with arginine substitutions at K285 and K309—was linearly ubiquitinated in cells in a signal-dependent manner. Importantly, the involvement of LUBAC in NF- κ B activation was confirmed in genetically modified mice lacking its subunit HOIL-1L; TNF- α -mediated activation of NF- κ B was severely impaired in primary hepatocytes and MEFs from HOIL-1L-null mice. However, JNK activation was not affected, indicating that linear ubiquitination of NEMO is involved specifically in NF- κ B activation. Hepatocytes from HOIL-1L-knockout mice and HOIL-1L-null MEFs undergo apoptosis in response to TNF- α administration owing to impaired NF- κ B activation, strongly indicating that LUBAC-mediated linear ubiquitination of NEMO is involved specifically in NF- κ B activation (Fig 2; Tokunaga *et al*, 2009).

Fig 1 | Schematic structure of LUBAC subunits HOIL-1L and HOIP. The NZF motifs of LUBAC have been shown to have ubiquitin-binding activity, although they are dispensable for ubiquitin-chain formation. HOIL-1L, longer isoform of haem-oxidized iron-regulatory protein ubiquitin ligase 1; HOIP, HOIL-1L interacting protein; LUBAC, linear ubiquitin chain assembly complex; NEMO, NF- κ B essential modulator.

A ubiquitin-binding motif specific for linear chains

Ubiquitin-binding motifs are known to have crucial roles in decoding ubiquitin signalling (Ikeda & Dikic, 2008). The tertiary structure of the linear chains is indeed similar to that of K63 chains because K63 is spatially close to the N-terminal methionine of ubiquitin (Komander *et al*, 2009). For this reason, linear chains have been used historically as models that mimic K63 chains to probe their function. However, two groups have recently reported that linear chains are clearly discriminated from K63 chains by at least some ubiquitin-binding motifs (Lo *et al*, 2009; Rahighi *et al*, 2009). The UBA motif of NEMO—also known as the NUB or CoZi domain—binds preferentially to linear di-ubiquitin, with approximately 100 times stronger affinity than to K63-linked chains (Lo *et al*, 2009). In addition, the NZF domain of TAB2 has been reported to bind to K63 chains but not to short linear chains of four ubiquitin residues (Komander *et al*, 2009), although it can weakly bind to longer linear chains of more than 10 ubiquitins (Tokunaga *et al*, 2009). These results indicate that linear chains transmit signals that differ from the K63 chain, despite their three-dimensional structural similarity. Mutations in UBA amino acids that are specifically required for linear di-ubiquitin recognition by NEMO markedly reduced TNF- α -induced NF- κ B activation, although activation of JNK or p38 was not affected (Rahighi *et al*, 2009). Furthermore, XR-MSMD is caused by hypomorphic mutations of NEMO (Dai *et al*, 2004), and mutations found in XR-MSMD (E315A and R319Q) have also been shown to be crucial for ubiquitin binding by UBA (Rahighi *et al*, 2009). Therefore, defects in linear polyubiquitination and the linear polyubiquitin-binding activity of NEMO might be the cause of these diseases.

The UBA motif is also found in other proteins (Wagner *et al*, 2008), and the motif present in ABIN-1 has been shown to bind selectively to linear di-ubiquitin (Rahighi *et al*, 2009). Although ABIN-1 was identified originally as a binding protein of A20—which is a negative regulator of NF- κ B (Klinkenberg *et al*, 2001)—subsequent studies using ABIN-1-null mice showed that it does not affect TNF- α -mediated NF- κ B activation, but rather suppresses TNF- α -induced apoptosis (Oshima *et al*, 2009). Therefore, the linear chains conjugated to NEMO could be recognized by two proteins, NEMO and ABIN-1, thereby activating two different signals in TNF- α signalling. As UBA motifs are found in several molecules besides NEMO and ABIN-1, it will be of great interest to determine whether other UBAs can bind to linear chains. Furthermore, the identification of other E3s that generate linear ubiquitin chains would broaden the significance of linear polyubiquitination beyond the NF- κ B pathway (Sidebar A).

Different ubiquitin chains in NF- κ B activation

The role of K63-linked chains in NF- κ B activation has been extensively studied and reviewed (Chen & Sun, 2009). Chen and colleagues elegantly demonstrated that an E2 complex composed of UBC13 and Uev1a—which mediates K63-linked chain formation—was involved in TRAF6-induced NF- κ B activation (Deng *et al*, 2000), and further showed that the TAK1–TAB1–TAB2 complex is a crucial component of NF- κ B activation in response to several signals, including antigen stimulation of lymphocytes and TNF- α treatment (Chen & Sun, 2009). TNF- α -induced K63 polyubiquitination of RIP1 is hypothesized to recruit NEMO and TAK1—which phosphorylates and activates IKK, leading to the activation of NF- κ B (Ea *et al*, 2006)—to TNFR1. NEMO and TAB2 have both been proposed to have K63 chain-binding activity, which is thought to be crucial for the recruitment of NEMO and TAK1 to TNFR1 (Ea *et al*, 2006; Wu *et al*, 2006). The importance of K63 chains in NF- κ B activation has been confirmed by the observation that small-interfering RNA-mediated silencing of UBC13 results in defective NF- κ B activation in human embryonic kidney 293T cells and insect cells (Andersen *et al*, 2005; Zhou *et al*, 2005). However, the deletion of UBC13 in mice did not overtly affect the signal-induced activation of NF- κ B, although the generation of K63-linked chains by a compensatory E2 (Chiu *et al*, 2009; Yamamoto *et al*, 2006a) in this setting cannot be ruled out. Furthermore, the UBA motif of NEMO, which was suspected to be a K63-binding motif (Ea *et al*, 2006; Wu *et al*, 2006), shows a substantially higher affinity for linear chains (Lo *et al*, 2009; Rahighi *et al*, 2009), arguing against the hypothesis that K63 chains are indispensable for NF- κ B activation.

The involvement of LUBAC in the TNF- α -induced activation of NF- κ B in UBC13-null MEFs was assessed to discriminate the function of linear chains from that of K63 chains in NF- κ B activation. Under these conditions, LUBAC can still activate NF- κ B (Tokunaga *et al*, 2009), emphasizing that LUBAC-mediated linear ubiquitination of NEMO has a distinct role from K63 chains in TNF- α -induced NF- κ B activation. Understanding the different functions of linear and K63 chains in cell signalling remains an outstanding issue in the field and is now being investigated intensely. The results obtained from knock-out mice provide some clues to the function of linear ubiquitination, although a more detailed analysis is still necessary.

In response to TNF- α signalling, K63 chains are necessary for JNK activation but dispensable for the NF- κ B pathway (Yamamoto *et al*, 2006a), whereas linear chains are necessary for the activation of NF- κ B but not for JNK (Tokunaga *et al*, 2009). The NZF domain of

Fig 2 | The canonical NF- κ B-activation pathway. Upon activation by various stimuli, I κ B α is phosphorylated by the IKK complex. The K48-linked polyubiquitination of phosphorylated I κ B α leads to its degradation. Subsequently, free NF- κ B translocates into the nucleus and induces the expression of target genes. Although signal-induced K63-linked polyubiquitination has been proposed to have important roles in this pathway, the crucial role of the LUBAC-mediated linear polyubiquitination of NEMO in NF- κ B activation is now clear. HOIL-1L, longer isoform of haem-oxidized iron-regulatory protein ubiquitin ligase 1; HOIP, HOIL-1L interacting protein; I κ B α , inhibitor of κ B; IKK, inhibitor of κ B kinase; LUBAC, linear ubiquitin chain assembly complex; NEMO, NF- κ B essential modulator; NF- κ B, nuclear factor- κ B; Ub, ubiquitin.

TAB2 selectively binds to K63 chains; therefore, K63 chains might recruit TAK1 together with TAB2 to a signalling complex. The TNF- α -induced activation of both JNK and NF- κ B is defective in TAK1-null MEFs (Sato *et al*, 2005; Shim *et al*, 2005); therefore, the recruitment of TAK1 to K63 chains could be indispensable for the activation of JNK but not for NF- κ B (Fig 3A). However, as the UBC13-catalysed K63 chain is involved in NF- κ B activation in response to at least some signals (Yamamoto *et al*, 2006b), K63 chains might function upstream of linear chains in some signalling cascades. For example, the K63 chain could be required to recruit LUBAC through the ubiquitin-binding activity of its NZF domains (Fig 3B). Alternatively, K63 and linear polyubiquitination could function independently in distinct signalling cascades (Fig 3C); the transient association of IKK α and IKK β with CD40 has been shown to precede UBC13 association, and K63 polyubiquitination—catalysed by UBC13—is not required for NF- κ B activation in anti-CD40-stimulated B cells (Matsuzawa *et al*, 2008). Therefore, signals that activate NF- κ B might be generated more rapidly than K63 chains under some conditions. K63-linked polyubiquitination of NEMO has been proposed to be crucial for the activation of NF- κ B (Chen *et al*, 2006). In anti-CD40-stimulated B cells, NEMO is ubiquitinated in a UBC13-dependent manner, but the ubiquitinated NEMO is not associated with IKK α / β under these conditions (Matsuzawa *et al*, 2008). However, we have observed that LUBAC can ubiquitinate NEMO in the IKK complex (Tokunaga *et al*, 2009)—which might transmit a different signal—thereby opening the possibility that NEMO is modified with

different polyubiquitin chains depending on whether it is in the IKK complex or free. Further analyses are clearly needed to investigate the various roles of K63-linked and linear chains of polyubiquitin in signalling (Sidebar A).

Mechanisms of LUBAC-mediated NF- κ B activation

Linear ubiquitination of NEMO is involved in the activation of the IKK complex (Tokunaga *et al*, 2009), for which two models have been proposed: trans-autophosphorylation and IKK kinase-mediated phosphorylation (Hacker & Karin, 2006). TAK1 and MEKK3 have been proposed to be the upstream kinases that phosphorylate IKKs. However, the precise mechanism for signal-induced IKK activation has not been resolved conclusively. The fact that the linear ubiquitination of NEMO by LUBAC and the linear ubiquitin-binding activity of NEMO are involved in NF- κ B activation suggests a new mechanism for IKK activation. The residues of NEMO that are conjugated to linear chains are probably K285 or K309 (Tokunaga *et al*, 2009). K309 is within the UBAN motif and its conjugation to a linear chain disrupts NEMO binding to linear polyubiquitin chains, whereas linear ubiquitination of K285 does not affect the linear polyubiquitin-binding activity of UBAN (Sidebar A; Rahighi *et al*, 2009). However, the UBAN motif of a given NEMO molecule is unlikely to recognize short linear chains conjugated to itself; rather, linear chains conjugated onto NEMO are likely to be recognized by another NEMO molecule, thereby inducing the multimerization of the IKK complex and the trans-autophosphorylation of IKKs (Fig 4A). The crystal

Fig 3 | Possible relationships between K63-linked and linear polyubiquitin chains in NF- κ B activation. (A) K63-linked chains generated by UBC13 recruit TAK1, which triggers JNK activation, but not NF- κ B activation, in TNF- α signalling. (B) K63-linked chains generated by UBC13 are involved in both JNK and NF- κ B activation in T-cell receptor signalling. Considering the importance of linear chains in NF- κ B activation, UBC13-catalysed K63-linked chains might be crucial for recruitment of LUBAC. (C) Signals stimulating NF- κ B activation might be generated independently of K63-linked chains because the IKK complex has been proposed to associate with CD40 before UBC13 associates with CD40. HOIL-1L, longer isoform of haem-oxidized iron-regulatory protein ubiquitin ligase 1; HOIP, HOIL-1L interacting protein; IKK, inhibitor of κ B kinase; JNK, Jun amino-terminal kinase; LUBAC, linear ubiquitin chain assembly complex; NEMO, NF- κ B essential modulator; NF- κ B, nuclear factor- κ B; TAK1, transforming growth factor β -activating kinase 1; TNF- α , tumour necrosis factor- α ; Ub, ubiquitin; UBC13, ubiquitin conjugating enzyme 13.

structures of several fragments of NEMO have been solved and it is suspected to have an extended structure (Rushe *et al*, 2008). NEMO is also known to bind to IKKs through its N-terminal region and the UBAN motif is in its C-terminal half. In addition, the recognition of linear di-ubiquitin by NEMO induces a slight conformational change of the UBAN domain (Rahighi *et al*, 2009), which could cause conformational changes in the IKK-binding region and affect the spatial positioning of IKKs, leading to trans-phosphorylation of the kinases (Fig 4B). Alternatively, the linear chains on NEMO might function as a scaffold for upstream IKK kinases (Fig 4C). Notably, decreased but sustained I κ B α phosphorylation is observed in HOIL-1L-null cells (Tokunaga *et al*, 2009). I κ B α phosphorylation normally leads to its degradation (Hayden & Ghosh, 2008), which is an effect that is delayed in HOIL-1L-null cells. Therefore, linear ubiquitination could also have additional roles in NF- κ B activation through the regulation of I κ B α degradation.

Future directions

Linear ubiquitin chains were previously thought not to have physiologically relevant functions, partly because linear polyubiquitin genes exist in genomes (Finley *et al*, 1987)—for example, two polyubiquitin genes are present in the human genome (Ryu *et al*, 2007). Therefore, genetically encoded free linear polyubiquitin could compete with post-translationally generated linear chains to bind to linear chain-specific binding motifs. However, linear polyubiquitins are cleaved into ubiquitin monomers co-translationally by deubiquitinating enzymes before polyubiquitin chains are generated (Turner & Varshavsky, 2000), possibly because ubiquitin folds quickly even in the absence of chaperones (Briggs & Roder, 1992). In addition, the excess ‘free’ linear polyubiquitin that is not cleaved co-translationally and polyubiquitin chains that are released from target proteins have been proposed to be cleaved into ubiquitin monomers by isopeptidase T (Reyes-Turcu & Wilkinson, 2009). Isopeptidase T can specifically disassemble free polyubiquitin—including linear polyubiquitin—by recognizing its free C terminus (Reyes-Turcu *et al*, 2006). It is therefore unlikely that unanchored linear polyubiquitin chains, including those that are newly translated, exist in cells, suggesting that linear polyubiquitin chains that are actively conjugated onto substrate proteins post-translationally are probable modulators of protein function.

Fig 4 | Possible mechanisms underlying linear polyubiquitin-mediated IKK activation. (A) Linear polyubiquitin conjugated onto NEMO might be recognized by the UBAN motif of NEMO in another IKK complex, which induces multimerization of the IKK complex and trans-autophosphorylation of IKKs. (B) Subtle conformational changes in the UBAN motif of NEMO triggered by the recognition of linear chains might affect the spatial position of the IKKs, which leads to trans-phosphorylation of IKKs. (C) Linear polyubiquitination of NEMO might function as a scaffold to recruit an unknown kinase to phosphorylate IKKs. IKK, inhibitor of κ B kinase; NEMO, NF- κ B essential modulator; Ub, ubiquitin; UBAN, ubiquitin-binding in ABIN and NEMO.

The study of LUBAC-mediated linear polyubiquitin assembly also provides a useful tool to analyse the mechanism—and the specificity—of polyubiquitin synthesis. Polyubiquitin chains are hypothesized to be generated by the sequential addition of ubiquitin to the distal ends of a ubiquitin tree by three enzymes: E1, E2 and E3 (Chau *et al*, 1989). In the case of the RING-finger ligases, E3 recognizes both E2 and a substrate, and facilitates the transfer of ubiquitin from E2 to the substrate. As the ubiquitin chain becomes longer, the distal end of ubiquitin is spatially separated from E2 (Hochstrasser, 2006). Therefore, this classical model of polyubiquitin assembly is unlikely to apply in most cases. However, it could apply to linear polyubiquitination because LUBAC has been shown both to recognize ubiquitin and to assemble linear chains (Kirisako *et al*, 2006). In addition, the linkage specificity of LUBAC is strict because it cannot assemble polyubiquitin from N-terminally tagged wild-type ubiquitin. As the NZF domains are dispensable for linear chain generation, identification of the ubiquitin-binding motif in LUBAC that is necessary for the ubiquitination reaction will provide essential information regarding the mechanism of polyubiquitin chain assembly.

Moreover, linear chain-dependent specific activation of the NF- κ B pathway could constitute a useful target for drug discovery. As NF- κ B is involved in many diseases—such as cancer, autoimmune disorders and allergic diseases—the development of agents that specifically inhibit NF- κ B would be beneficial. A drug

that specifically suppresses LUBAC-mediated linear ubiquitination or blocks the binding between the polyubiquitin linear chain and the UBAN of NEMO might provide new targets to inhibit NF- κ B activity specifically.

The identification of an E3 complex that generates linear polyubiquitin chains has opened up a new area of research in ubiquitin biology, although a great deal of work is undoubtedly needed to clarify the roles of the linear chains. For example, TNF- α -induced activation of NF- κ B, although severely suppressed, is not completely abolished in HOIL-1L-deficient cells (Tokunaga *et al*, 2009). This phenomenon is not fully understood at present, but could be due to the presence of small amounts of HOIP in these mice, which might associate with other proteins to mediate linear polyubiquitination (Tokunaga *et al*, 2009). However, it could also indicate the presence of a parallel signalling pathway to that mediated by LUBAC. HOIL-1L—also known as RBCK1—has also been reported to regulate NF- κ B negatively (Tian *et al*, 2007), although this seems unlikely to happen *in vivo*, as the study of HOIL-1L-null mice demonstrated that it has crucial roles in NF- κ B activation (Tokunaga *et al*, 2009). The conflicting results were obtained by overexpressing HOIL-1L in cultured cells, which precludes their extrapolation to a physiological setting (Tian *et al*, 2007).

Although much awaits elucidation with respect to the regulation and physiological functions of linear ubiquitination, some crucial molecules that are implicated in this process have already

Sidebar A | In need of answers

- (i) How can linear NEMO activate NF- κ B? Possible mechanisms for IKK activation by linear chains are discussed in this article, but linear chains might have additional roles in NF- κ B activation.
- (ii) What are the functional differences between K63 of ubiquitin-linked and linear chains in signalling?
- (iii) Are there other ligases that specifically generate linear chains and additional binding motifs that specifically recognize linear chains?
- (iv) Do linear ubiquitin chains have functional roles in systems other than NF- κ B activation?

been identified. *CYLD*—a susceptibility gene for familial cylindromatosis—negatively regulates the NF- κ B pathway through its deubiquitination activity (Brummelkamp *et al.*, 2003; Kovalenko *et al.*, 2003; Trompouki *et al.*, 2003) and has been shown to cleave not only K63 linkages but also linear ubiquitin linkages (Komander *et al.*, 2009). *CYLD* has been shown to bind to NEMO, suggesting that it could hydrolyse NEMO-conjugated linear chains *in vivo*, thereby negatively regulating NF- κ B. In another regulatory twist, LUBAC has been reported to be attenuated by PKC signalling (Nakamura *et al.*, 2006).

Research into the function of linear polyubiquitin in cell signalling has just begun, and we believe that new and unexpected functions of linear polyubiquitination will be revealed in the near future.

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