

Theme Issue Article

Ubiquitylation within signaling pathways in- and outside of inflammation

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Summary

Ubiquitin is a highly conserved 76-amino-acid peptide that becomes covalently attached to lysine residues of target proteins. Since ubiquitin itself contains seven lysine residues, ubiquitin molecules can generate different types of polyubiquitin chains. Lys48-linked polyubiquitylation is well-known as posttranslational tag for targeting proteins for degradation by the 26S proteasome. Recent studies have revealed several new functions of ubiquitin, e.g. activation of protein kinases, control of gene transcription, DNA repair and replication, intracellular traffick-

ing and virus budding. These functions are mainly mediated by Lys63 polyubiquitin chains or attachment of a single ubiquitin molecule to one or several lysine residues within the target protein. Importantly, protein ubiquitylation exhibits inducibility, reversibility and recognition by specialized ubiquitin-binding domains, features similar to protein phosphorylation. In this review we comprehensively describe regulations of protein ubiquitylation and their impact on distinct signaling pathways.

Keywords

Endothelial cells, signal transduction, inflammation

Thromb Haemost 2007; 97: 370–377

Introduction

Posttranslational modifications represent a powerful mechanism to regulate the activity, abundance, stability, and localization of a wide variety of signaling molecules in the cell. In addition to the well-studied events of phosphorylation and acetylation, mechanisms involving ubiquitin and ubiquitin-like (UBL) proteins, such as small ubiquitin-related modifier (SUMO) and interferon-stimulated gene 15 (ISG15), have become of great interest in this context. The covalent ligation of the 76-amino-acid peptide ubiquitin is a highly conserved process that comprises a plethora of enzymes and accessory proteins that are usually homologous across species (1). The conjugation reaction occurs via the sequential action of three enzymes, namely an ubiquitin-activating enzyme E1, an ubiquitin-conjugating enzyme E2 and an ubiquitin-ligase E3. First, in an ATP-dependent reaction, the E1 forms a thioester bond between its active site cysteine residue and the carboxyl-terminal glycine of ubiquitin (2). In a trans-esterification reaction, the activated ubiquitin is subsequently transferred to a cysteine of an E2. In the final step, which is accomplished by the action of E3, ubiquitin is covalently attached via an isopeptide bond to a lysine residue in the target protein (3).

Ubiquitylation is a reversible modification; ubiquitin or ubiquitin chains can be removed from the substrate by members of a large family of enzymes of isopeptidases or DUBs (deubiquitylation enzymes) (4, 5).

Probably all eukaryotes express proteins that are related in sequence to ubiquitin or function in an analogous manner. At least ten UBL-ligation pathways that parallel the ubiquitin pathway in mechanism are known to transfer small protein moieties onto various substrates, among them SUMO, NEDD8 and ISG15. Sumoylation is highly regulated in all eukaryotes and participates in diverse events such as nuclear transport, transcriptional regulation, chromosome segregation, and cell-cycle control (6). Conjugation of NEDD8 to cullins, all of which are subunits of SCF (Skp1/Cullin/F-box protein) complexes, is necessary for the activity and specificity of these ubiquitin ligases (7). Multiple proteins are conjugated to ISG15, among them are the JAK and STAT proteins, suggesting that ISG15 participates in JAK-STAT signaling in response to interferon (8).

The addition of a single ubiquitin is termed monoubiquitylation or, if several substrate lysines are modified in this manner, multiple monoubiquitylation. However, through internal lysine residues, which can also be subjected to isopeptide formation,

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Received August 29, 2006
Accepted after revision December 7, 2006

Prepublished online February 8, 2007
doi:10.1160/TH06-08-0471

ubiquitin can also form different polyubiquitin chains. Generally, all lysines (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) are possibly involved in chain formation *in vivo*, but the best-characterized are linked via Lys48 and Lys63. Lys48 polyubiquitylation is an efficient modification to mark proteins for degradation by the 26S proteasome, whereas Lys63 chains are involved in non-proteolytic functions, e.g. protein trafficking and DNA repair (9). Monoubiquitylation and multiple monoubiquitylation are major regulators of protein trafficking in both the exocytic and endocytic pathways (10).

In eukaryotic cells a single E1 enzyme is responsible for the activation of ubiquitin for conjugation, while many different E2s and E3 enzymes work together to ensure the correct timing, localization and specificity of the ubiquitylation reaction. The coexistence of many E3 ligases in the same cell type implicates the need for a tight selection of appropriate E2s and target proteins. Thus, the most important function of E3s is the ability to specifically recognize substrates.

E3 ubiquitin ligases

Generally E3s can be classified by their domain composition into HECT- (homologous to E6-AP carboxyl-terminus) or RING- (really interesting new gene) containing proteins. The majority of human E3s – in total more than 600 – have been annotated in the human genome, belong to the RING-type E3 ligases which are characterized by the presence of a RING-finger domain or the structurally related U-box (UFD2-homology domain) or PHD (plant homeodomain) domain. They either act as monomers, like Mdm2 which is responsible for the targeting of p53 to the proteasome (11), or are essential components of multi-subunit complexes, like the SCF complex (12). RING-finger proteins facilitate the transfer of ubiquitin to the substrate but lack intrinsic catalytic activity (13).

The second class of E3 ligases, termed HECT, includes a protein domain showing a high degree of identity (about 50%) with the carboxyl-terminal region of E6-AP. The latter was identified as the cellular protein mediating the association between the tumor suppressor protein p53 and the E6 oncoprotein of tumorigenic human papillomavirus (14). The E6-AP-E6 complex was shown to function as an ubiquitin ligase for p53 to initiate p53 proteasomal degradation (15). More than 20 proteins were subsequently identified in the human genome to not only possess substantial homology to the 350 amino acid carboxyl-terminus of E6-AP, but also to display the ability to form thioester bonds with ubiquitin, indicating that the HECT domain-containing proteins are active ubiquitin ligases (16). In contrast to RING E3 ligases, HECT-domain proteins include an active cysteine residue at the very carboxyl-terminal end that is responsible for direct binding of ubiquitin (3). HECT E3s act exclusively as monomeric proteins that contain additionally to the HECT domain at the C-terminus diverse N-terminal motifs, such as calcium-binding C2 modules, which mediate the interaction with phospholipids and proteins, or a series of WW domains that recognize proline-rich PPxY regions (17).

It is well established that protein ubiquitylation is induced in response to a variety of stimuli in cells. Since E3 ubiquitin ligases play a central role in substrate recognition and specificity,

they are tightly regulated by signal-induced cellular responses such as posttranslational modification, protein transport, and degradation and formation of signaling complexes (18, 19).

Ubiquitylation and ubiquitin-binding domains

Ubiquitin is a structurally extended molecule which has a large surface to interact with other proteins. The variability of interactions is even increased by the possibility that ubiquitin can form polyubiquitin chains that are linked via different lysine residues generating different conformations, thus providing structurally different interaction sites (20). Monoubiquitin and polyubiquitin chains can be recognized by several different classes of ubiquitin-binding motifs which are present in a variety of different proteins (21). Among others, these domains include UIM (ubiquitin-interacting motif), UBA (ubiquitin-associated motif), CUE (Cue1-homologous), UEV (ubiquitin E2 variant domain), PAZ (polyubiquitin-associated zinc finger) and NZF (novel zinc finger) (22). UIMs are present in a number of different proteins implicated in endocytosis, such as Eps15 (epidermal growth factor receptor pathway substrate 15), Eps15R, epsins, and Hrs (hepatocyte growth factor [HGF]-regulated tyrosine kinase substrate), where they are critical for function, and are likely to bind monoubiquitinated partners in the cell (23). The UBA domain is a motif of 55 amino acids binding preferentially to polyubiquitin chains (24). Association of the UBA domain to ubiquitin has been demonstrated for proteins of diverse function (25). The CUE domain is structurally significantly related to the UBA domain, but was shown to preferably associate with monoubiquitin (26). The UEV domain is related to the catalytic domain found in E2 enzymes, but lacks the catalytic cysteine residue that is necessary for conjugation to ubiquitin (27).

The diversity and complexity of the different ubiquitin-binding domains allow a high degree of variability to form oligomeric complexes of ubiquitylated proteins. These features enable the ubiquitylation system to not only control protein degradation but also to modulate and control signaling networks induced by extracellular stimuli.

Monoubiquitylation in cellular signaling pathways

Emerging evidence exists that several cellular proteins with diverse functions use monoubiquitin as their target signals. In particular, membrane proteins follow a pathway that not necessarily involves polyubiquitylation and/or proteasomal degradation. Ligation of an ubiquitin molecule to one (monoubiquitylation) or more (multiple monoubiquitylation) lysine residues of ion channels, membrane receptors or junctional complexes determines their regulated internalization and sorting into diverse endocytic compartments. Internalized membrane proteins are either recycled (returned to the plasma membrane) or targeted to multivesicular bodies and lysosomes for degradation (28). In addition to providing internalization or sorting signals, monoubiquitin tags regulate other biological processes such as histone modification, transcription, DNA repair and viral budding. What determines whether a substrate is mono-, multiple mono- or polyubiquitylated is as yet unknown. The observations that the same substrate can undergo all these modifications by distinct E3 ligases, and

the same E3 ligases can mono-, multiple mono- or polyubiquitylate different substrates complicate the examination of the mechanism (29, 30).

A membrane protein that is monoubiquitylated or multiple monoubiquitylated is committed for internalization and entry into the endocytic route. The information needed for internalization apparently resides in the ubiquitin molecule itself, because in frame fusion of ubiquitin to receptors lacking lysine residues or its fusion to heterologous proteins that are normally not internalized can trigger their internalization. The requirement of ubiquitylation for the early steps of receptor endocytosis is still a matter of debate based on the fact that ubiquitin is sufficient to facilitate the removal of receptors from the plasma membrane, but is also dispensable for endocytosis of many transmembrane receptors *in vivo* (31). The E3 RING-finger type ligase Cbl promotes multiple monoubiquitylation of activated receptor tyrosine kinases, ensuring their efficient lysosomal targeting (32). To direct the ubiquitylated cargo in the endocytic pathway several endocytic regulator proteins harboring ubiquitin-binding domains that interact with monoubiquitin molecules are required. Ubiquitin receptors like the UIM-containing proteins Hrs, Eps15 and epsin interact with ubiquitin and clathrin and are therefore crucial for clathrin-mediated endocytosis of ubiquitylated membrane proteins (33–35). However, a clathrin-independent internalization pathway has been described for ubiquitylated TGF α receptor, which involves the interaction of E3 ligase/cargo with lipid rafts and caveolae. In this case the choice of internalization via clathrin or lipid rafts dictates whether the receptor will be recycled or degraded (36).

There is growing evidence that membrane receptors located at endocytic compartments can still transmit signals after internalization and that these might be qualitatively different from those initiated at the plasma membrane (37). It is well established that signaling via neurotrophin receptors occurs after their internalization. For example the receptor tyrosine kinase TrkA promotes neuronal growth factor-induced cell survival when at the cell surface, whereas it induces differentiation when internalized (38). In addition, a mitogen-activated receptor, namely EGFR (epidermal growth factor receptor), can signal subsequent to its endocytosis. Inhibition of clathrin-dependent EGFR internalization significantly blocks activation of mitogen-activated kinase in response to EGF (39). Signaling from endosomes seems to be of physiological relevance, because signal transduction of EGFRs in endosomes is required for cell survival (40). Interestingly, the fate of the EGF receptor, whether it is recycled or degraded, is likely to be controlled by the extracellular concentration of its ligand. The possibility to choose between two different internalization routes provides the means for a ligand-sensing mode of regulation of receptor signaling (41). In agreement with signaling from endosomes is the observation that several adaptor and signaling molecules, including Grb2, Crk, β -arrestin and ras, translocate to endosomes upon activation and endocytosis of cell-surface receptors.

Thus, monoubiquitylation of membrane proteins represents an efficient signal for directing and regulating the subcellular distribution, intracellular trafficking and signaling of receptors by modulating the quality, strength and duration of the signal.

Lys48-linked polyubiquitylation and NF- κ B (nuclear factor- κ B) signaling

NF- κ B constitutes an inducible transcriptional regulator that plays a crucial role in inflammation, innate and adaptive immunity, cell proliferation and survival. Deregulation of NF- κ B activity is associated with a variety of inflammatory and autoimmune diseases, as well as cancer. In mammals, five Rel family members have been identified: RelA/p65, RelB, c-Rel, p50/p105, and p52/p100, which form various NF- κ B homo- and heterodimers (42). The NF- κ B pathway can be activated by many different extracellular stimuli, such as TNF α (tumor necrosis factor alpha), LPS (lipopolysaccharide), IL-1 (interleukin 1), growth factors, UV-light, bacteria and viruses (43). Depending on the stimulus, two different pathways of NF- κ B activation are distinguished, the so-called classical and alternative pathways. The critical event of the classical pathway is the activation of the I κ B kinase (IKK) complex, which ultimately leads to phosphorylation of I κ B. In un-stimulated cells, NF- κ B mostly consisting of p65/p50 heterodimers is sequestered in the cytoplasm through interaction with inhibitory proteins, the I κ Bs. Stimulus-induced phosphorylation of two N-terminal serines in the I κ Bs is mediated by the macromolecular IKK complex termed the signalosome containing the catalytic subunits IKK α /IKK1, IKK β /IKK2, and the regulatory subunit IKK γ /NEMO (NF- κ B essential modifier) (44). This event thereon is essential for Lys48-polyubiquitylation of I κ B molecules by the SCF^{TRCP}-E3 ligase complex (45), which in turn leads to rapid degradation of I κ B by the 26S proteasome complex, thus allowing active p65/p50 NF- κ B dimers to translocate to the nucleus and turning on the expression of its target genes (46). Inhibition of I κ B degradation by proteasome inhibitors prevents NF- κ B nuclear translocation and underlines the crucial role of the ubiquitin-proteasome pathway in NF- κ B signaling (47). The I κ B-independent alternative activation pathway involves inducible proteolytic processing of NF- κ B1/p105 and NF- κ B2/p100 resulting in mature p50 and p52, respectively (47, 48). p105 can be processed either co-translationally or post-translationally. Co-translational degradation is likely to be a constitutive process to create a basal level of p50 in un-stimulated cells. Upon stimulation with phorbol esters or LPS the IKK complex is activated and phosphorylates p105 at the C-terminal domain which leads to subsequent ubiquitylation and partial degradation (49, 50). The processing of p100 is tightly regulated. Activation of certain members of the TNF-receptor family leads to activation of IKK α which in turn phosphorylates p100 at two C-terminal serine residues (51, 52). Thereby SCF^{TRCP} ligase is recruited to polyubiquitylate p100 at a specific lysine residue leading to the degradation of the C-terminal I κ B-like domain by the proteasome, generating the mature p52 subunit (53). The alternative pathway principally results in the release of transcriptionally active RelB/p50 or RelB/p52 heterodimers, which also translocate into the nucleus and regulate various target genes.

Lys63-linked ubiquitin chains play a crucial role in regulating NF- κ B activation

Additionally to Lys48-mediated polyubiquitylation, which dictates I κ B degradation and p105 and p100 processing, the NF- κ B system provided us with one of the first examples for the important role of Lys63-mediated ubiquitin conjugation. TRAF (TNFR-associated factor) proteins constitute RING-type E3-ubiquitin ligases that play a pivotal role in signaling pathways involved in the activation of NF- κ B by many cell-surface receptors, including the TNFR super family, the IL-1R (IL-1 receptor) and TLRs (toll-like receptors) (54). Among the seven human TRAF family members, TRAF2 and TRAF6 are the most intensively studied. Both represent ubiquitin ligases that catalyze the synthesis of a unique polyubiquitin chain linked through lysine 63 of ubiquitin. TRAF2 is recruited to the TNFR complex via binding to the adaptor protein TRADD (TNFR-associated via death domain) (55). Both TRADD and TRAF2 interact with RIP (receptor-interacting protein), which is necessary for TNF α induced NF κ B activation, while its kinase domain is dispensable (56, 57). The connection between TNFR1 signaling and IKK and NF- κ B activation has been described in two recent publications. It was shown that NEMO is a novel ubiquitin-binding protein, which targets Lys63-linked polyubiquitylated RIP *in vivo* (58). RIP recruits NEMO to TNFR1, which, in turn, promotes IKK activation. Mutations in the ubiquitin-binding site of NEMO disrupt IKK action, thus polyubiquitylation of RIP and its recognition by NEMO are required for IKK activation (59).

TRAF6 together with the heteromeric E2 Ubc13/Uev1A is thought to be essential for IKK and JNK (Jun-terminal kinase) activation in IL-1R and TLR pathways. TRAF6 activity itself is dependent on K63-linked polyubiquitylation, which is induced by TRAF6 dimerization or oligomerization (60, 61). Ubiquitylated TRAF6 conjugates are subsequently recognized by the highly conserved C-terminal zinc finger domain of either TAB2 (TAK1-binding protein), or TAB3 which function as scaffold proteins that recruit and activate the protein kinase TAK1 (transforming growth factor β [TGF β]-activating kinase 1) which is critical in the activation of NF- κ B and MAP kinases (61). By mutating the zinc finger domain, TAB2 becomes unable to bind ubiquitin chains, and its ability to activate TAK1 and IKK is lost (62). However, reconstitution studies in TRAF6-deficient cells suggest that the RING-finger domain of TRAF6, which is essential for ligation of K63-linked polyubiquitin chains to target proteins, is dispensable for IL-1R- and LPS-mediated NF- κ B activation (63). The role of Ubc13 in NF- κ B and MAP kinase signaling also remains a controversy. While RNA-silencing experiments result in clear defects in NF- κ B activation (64), expression of a dominant negative form of Ubc13 only marginally affects the NF- κ B signaling pathway (65). In addition, it was shown recently that conditional ablation of Ubc13 has almost no effect on NF- κ B activation, but severely influences MAP kinase activation (66). Therefore, although Lys63-mediated TRAF6 ubiquitylation seems to be crucial for protein kinase activation, the exact mechanism of action remains to be determined. A simplified scheme of the IL-1R/TLR and TNFR signaling pathways resulting in NF- κ B activation is depicted in Figure 1.

NF- κ B activation and non-inflammatory responses

Besides its role in NF- κ B activation in response to inflammatory stimuli and in innate TLR signaling, ubiquitylation is also involved in adaptive immune responses. Recent studies have revealed that polyubiquitylation of the IKK regulatory subunit

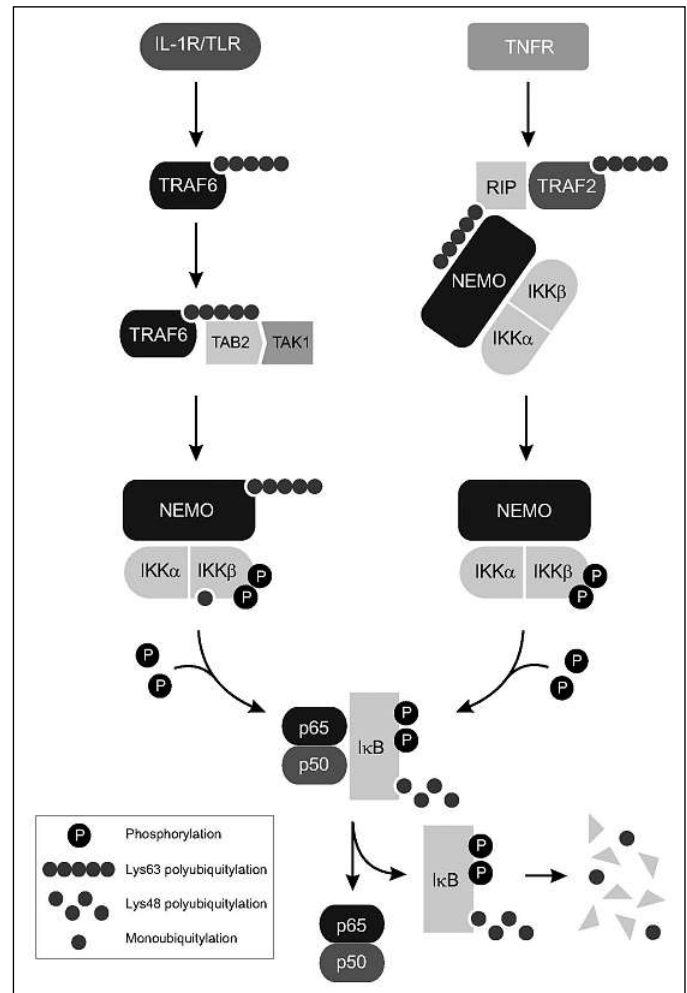


Figure 1: Ubiquitin in the activation of the IKK/NF- κ B pathway. The IKK complex can be activated by cell-surface receptors such as IL-1R, TLR and TNFR. Activation of the E3 ubiquitin ligases TRAF2 and TRAF6 leads to attachment of Lys63 polyubiquitin chains to TRAF2, TRAF6, NEMO and RIP. In the IL-1R/TLR signaling branch polyubiquitylated TRAF6 recruits TAB via binding to the polyubiquitin chain, which then triggers TAK1 activation. Activated TAK1 then phosphorylates the activation loop of IKK α thereby triggering its monoubiquitylation. In the TNFR signaling branch polyubiquitylated RIP recruits NEMO, which interacts with the polyubiquitin chain leading to the activation of the IKK complex. Once activated, IKK mediates serine phosphorylation of I κ B and targets I κ B for Lys48 polyubiquitylation and subsequent degradation by the 26S proteasome. Thus NF- κ B p50/p65 dimers are released and can enter the nucleus where they turn on the transcription of numerous genes implicated in immune and inflammatory responses. I κ B, inhibitor of NF- κ B; IKK, I κ B kinase; IL-1R, interleukin 1 receptor; NEMO, NF- κ B essential modifier; NF- κ B, nuclear factor- κ B; RIP, receptor-interacting protein; TAB, TAK1-binding protein; TAK1, TGF β -activated kinase; TNFR, tumor necrosis factor receptor; TRAF, TNFR-associated factor.

NEMO is also essential for lymphoid cell activation. A protein complex consisting of CARMA1 (caspase-recruitment domain [CARD]-membrane-associated guanylate kinase protein 1), BCL-10 (B cell lymphoma-10) and MALT1 (mucosa-associated-lymphoid-tissue lymphoma translocation gene 1) is central in antigen-receptor signaling that leads to NF- κ B activation. This complex recruits TRAF6, induces oligomerization of TRAF6 and activation of its E3 ligase activity, leading to Lys63-linked polyubiquitylation of NEMO (67). However, there is some discrepancy regarding TRAF6 as the responsible E3 ligase since another report showed that MALT1 directly ubiquitylates NEMO, despite the fact that MALT1 does not contain a known E3 motif (68).

In some cancer cells DNA-damaging agents can activate NF- κ B by a mechanism including ubiquitin and the ubiquitin-like molecule SUMO. The mechanism by which the nuclear DNA damage signal is transduced to the cytoplasmic IKK complex has been reported recently (69). In response to DNA damage, a small fraction of nuclear NEMO is targeted by SUMO, which keeps NEMO sequestered in the nucleus. DNA damage also activates the kinase ATM (ataxia telangiectasia mutant), which phosphorylates NEMO, leading to replacement of SUMO by ubiquitin at two lysine residues. Ubiquitylated NEMO is able to shuttle out of the nucleus and associate with the IKK complex, which results in IKK activation.

Ubiquitylation and inhibition of IKK/NF- κ B activation

The concept of a non-degradative function for Lys63 chains in IKK signaling was strengthened by the identification of DUBs that specifically remove Lys63-linked chains. One of these DUBs is the cylindromatosis tumor suppressor protein CYLD, which inhibits IKK activation by cleaving Lys63-linked polyubiquitin chains on several proteins, including TRAF2, TRAF6 and NEMO without affecting Lys48-modified I κ B (70–72). After stimulation with TNF α , TRAF2 autoubiquitinates itself via attachment of Lys63-linked polyubiquitin chains leading to its activation. TRAF2 activation in turn results in immediate phosphorylation and degradation of I κ B, followed by the release and nuclear translocation of NF- κ B. Recent studies provide a model where CYLD is recruited to and removes Lys63-linked polyubiquitin chains from activated TRAF2. Thereon inhibitors of apoptosis can bind and ubiquitinate TRAF2 by Lys48-linked chains which results in proteasomal degradation of TRAF2 and attenuation of NF- κ B signaling (73). However, results obtained from in-vitro transfection experiments remarkably differ from knock-out studies. Unexpectedly, bone marrow-derived macrophages isolated from *Cyld*^{-/-} mice showed no defect in TNFR-induced NF- κ B signaling (74). Instead, *Cyld*^{-/-} mice contain less peripheral T cells. By effecting the ubiquitination status and thereby the activity of the kinase Lck, which among others is important for proper T cell development, CYLD was shown to regulate proximal T-cell-receptor signaling in thymocytes. Furthermore, loss of CYLD in *Cyld*^{-/-} keratinocytes had no effect on transcriptional regulation involving the classical p65/p50 NF- κ B dimer, but clearly affects Bcl-3-linked p50- or p52-dependent gene regulation (75). The fact that there are differences in

CYLD function in thymocytes and keratinocytes points at receptor-specific and cell-type-specific functions of CYLD.

Another DUB acting in this pathway is A20, a NF- κ B-induced protein that inhibits NF- κ B in a negative-feedback loop (76, 77). A20 is a cytoplasmic protein that contains an N-terminal ovarian tumor (OTU) domain and seven novel zinc-finger structures at the C-terminus (78, 79). Overexpression studies originally showed that A20 inhibits TNF-mediated cell death in addition to NF- κ B activation (80). Moreover, A20-deficient mice developed severe multi-organ inflammation and are extremely susceptible to sub-lethal doses of TNF α (81). This phenotype correlates with enhanced sensitivity to TNF-induced apoptosis and prolonged NF- κ B activation of A20-deficient cells. The failure to downregulate NF- κ B transcriptional activity is due to a constitutively active IKK complex, indicating that A20 interferes with the signal transduction pathway upstream of the complex. The mechanism for IKK inhibition is based on two enzymatic activities of A20. On the one hand the OTU domain exhibits deubiquitylating activity, whereas the zinc finger region possesses E3 ligase activity. The OTU domain disassembles Lys63-linked chains either TNF-dependent from RIP (77) or LPS-dependent from TRAF6 (82), in both cases suppressing IKK activation. Moreover, A20 mediates the assembly of Lys48 ubiquitin chains on RIP after the Lys63 chains have been removed, thereby targeting RIP for degradation by the proteasome, further diminishing IKK activation. Therefore, A20 provides insights into how structurally different polyubiquitin chains can modulate the signaling and degradation of a target protein.

Ubiquitylation in other signaling pathways

It is becoming more and more apparent that ubiquitylation and the regulation of protein ubiquitylation by extracellular stimuli is involved in a panoply of signaling pathways. In this review we will shortly describe how ubiquitylation controls signaling pathways considering TGF β induction as example.

TGF β is a multifunctional cytokine that is implicated in the regulation of various aspects of immune responses. Suppression of the TGF β pathway results in aberrant lymphocyte proliferation and multi-organ inflammation (83, 84). Binding of TGF β to its serine/threonine kinase receptors leads to nuclear translocation of Smad transcription factors (85). Phosphorylation of receptor-activated Smads (R-Smads) is followed by complex formation with common mediator Smads (Co-Smads), translocation into the nucleus, and subsequent regulation of gene transcription. Smad ubiquitylation is regulated by two HECT-type E3 ligases, namely Smurf1 and Smurf2 (Smad ubiquitylation regulatory factor) (86, 87). Smurfs can exert a dual function in the TGF β -signaling pathway. In the first case, Smurf2 exerts an enhancing function by forming a complex with Smad2 and SnoN, the transcriptional co-repressor of Smad (88). The HECT domain of Smurf2 targets SnoN for ubiquitin-mediated degradation. Thus, TGF β -stimulated complex formation plays a positive regulatory role by degrading the transcriptional co-repressor SnoN. However, Smurfs can also inhibit responses to TGF β by direct ubiquitylation of Smads (89) or by binding of Smurf2 and inhibitory Smad7 to the cytoplasmic domain of TGF-receptor which is thereupon ubiquitylated and degraded (90).

Another E3 ligase that promotes TGF β -mediated signaling is the HECT domain protein Itch (91). In contrast to Smurfs, Itch does not seem to influence protein stability of Smad or SnoN, but rather triggers ubiquitylation of Smad2, thereby enhancing TGF β -induced phosphorylation of Smad2. Thus, Itch functions as a positive regulator of TGF β signaling through proteolysis-independent ubiquitylation. It awaits further investigation how these different functions of E3 ligases in the TGF β -signaling pathway are regulated and whether there is cell-type specificity. The enhancing and inhibitory functions of E3 ligases in the TGF β -signaling pathway are schematically depicted in Figure 2.

Conclusions and perspectives

In the past few years a concept in which regulatory ubiquitylation of proteins is used for signal propagation with a complexity equivalent to, or even exceeding, that of phosphorylation has been established. Like most other posttranslational modifica-

tions that are of regulatory importance, protein ubiquitylation, as well as the conjugation of ubiquitin-like polypeptides, is rapidly modulated in response to extracellular signals. The modulation can occur either through modification of substrates or through modification of a component of the ubiquitylation machinery, which in turn can affect the ability of E3 ubiquitin ligases or deubiquitylation enzymes to recognize and interact with their substrates or alter their catalytic activity.

The IKK/NF- κ B pathways are classical examples of signaling that involve kinase-induced ubiquitin-dependent protein destruction as well as destruction-independent polyubiquitylation. The latter mechanism uses ligand-induced oligomerization and activation of E3 ubiquitin ligases, non-destructive addition of Lys63 polyubiquitin chains onto adaptor proteins and subsequent recruitment of protein kinases through polyubiquitin-binding domains. Several issues have to be solved to clarify whether this model can serve as a general scheme for activation and regulation of protein kinases. It will have to be established

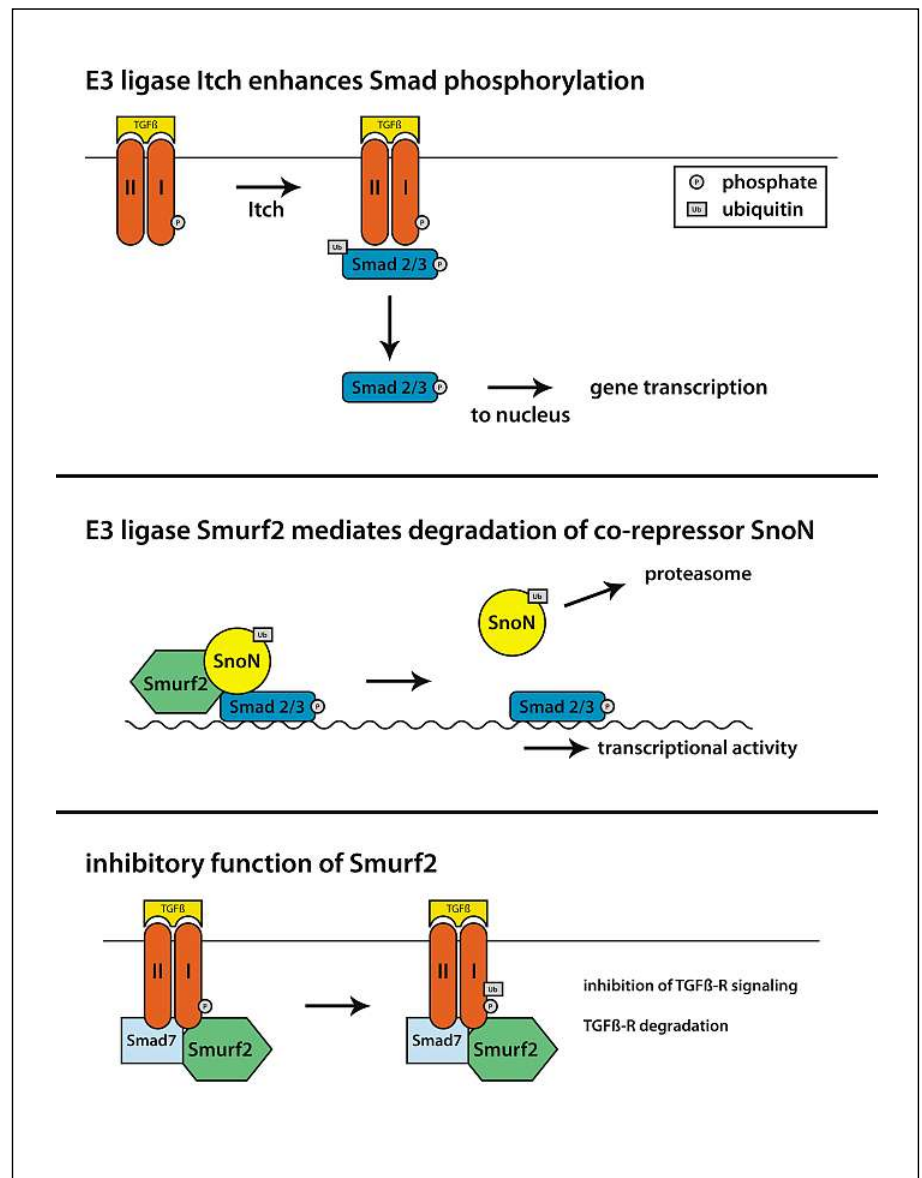


Figure 2: Activating and inhibiting functions of Itch and Smurf2 in the TGF β -signaling pathway. See text for details. TGF β , transforming growth factor β ; I, II, TGF β -receptor I and II; Smad, intracellular transducer of TGF β signals; Smurf, Smad ubiquitylation regulatory factor; SnoN, SKI-related novel protein.

what determines the generation of Lys63 versus Lys48 chains, which structural and functional requirements adapter molecules do exert, what the mechanisms are that trigger-protein kinase activation upon Lys63 chain recognition, what the exact function of NEMO ubiquitylation is, whether there are other lysines in poly-ubiquitin chain formation involved, and what the specific role of deubiquitylating enzymes in the regulation of signaling pathways is.

Alterations of ubiquitylation pathways have been demonstrated to contribute to the pathogenesis of several diseases, such as cancer, immune diseases and neurodegenerative disorders. In

order to understand how the ubiquitin tag is integrated into diverse signaling pathways we have to learn much more about how ubiquitin modification controls protein function, activity and localization, and how the ubiquitin signal is propagated and translated to regulate downstream cellular events.

Acknowledgements

We apologize that we could not cite all the outstanding contributors to this field due to space limitations. We thank Jesse J. Lipp for help with the graphics.

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