

## Review

# CYLD: a tumor suppressor deubiquitinase regulating NF- $\kappa$ B activation and diverse biological processes

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Protein ubiquitination is a reversible reaction, in which the ubiquitin chains are deconjugated by a family of deubiquitinases (DUBs). The presence of a large number of DUBs suggests that they likely possess certain levels of substrate selectivity and functional specificity. Indeed, recent studies show that a tumor suppressor DUB, cylindromatosis (CYLD), has a predominant role in the regulation of NF- $\kappa$ B, a transcription factor that promotes cell survival and oncogenesis. NF- $\kappa$ B activation involves attachment of K63-linked ubiquitin chains to its upstream signaling factors, which is thought to facilitate protein–protein interactions in the assembly of signaling complexes. By deconjugating these K63-linked ubiquitin chains, CYLD negatively regulates NF- $\kappa$ B activation, which may contribute to its tumor suppressor function. CYLD also regulates diverse physiological processes, ranging from immune response and inflammation to cell cycle progression, spermatogenesis, and osteoclastogenesis. Interestingly, CYLD itself is subject to different mechanisms of regulation.

*Cell Death and Differentiation* (2010) 17, 25–34; doi:10.1038/cdd.2009.43; published online 17 April 2009

Ubiquitination involves covalent linkage of ubiquitin molecules to substrate proteins as monomers (monoubiquitination) or polymers (polyubiquitination).<sup>1</sup> Polyubiquitination occurs by linking the carboxy-terminal glycine of one ubiquitin to an internal lysine of another ubiquitin. Among the well-studied polyubiquitin chains are those linked by lysine 48 (K48) and K63. Although the K48-linked ubiquitin chain marks the substrate protein for proteasomal degradation, the K63-linked ubiquitin chain facilitates various non-degradative biological processes, such as protein trafficking and signal transduction.<sup>1–3</sup> The ubiquitination reaction is catalyzed by the sequential actions of three enzymes: the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2), and the ubiquitin ligase (E3). Like phosphorylation, ubiquitination is a reversible reaction being mediated by deubiquitinases (DUBs).

The DUBs form a large family of proteases, which hydrolyzes ubiquitin chains, and thus they are believed to oppose the functions of their counteractive ubiquitinases. The existence of close to 100 DUBs in the human genome<sup>4</sup> implies that DUBs may possess certain levels of substrate specificity and participate in specific biological functions. On the basis of the structure of their catalytic domains, DUBs are classified into five subfamilies: ubiquitin-specific proteases (USPs), ubiquitin carboxy-terminal hydrolases, ovarian tumor-related proteases, Machado–Joseph disease protein domain

proteases, and Jab1/Pab1/MPN domain containing metallo-enzymes.<sup>4</sup> The USPs form the largest subfamily of DUBs, which is composed of more than 50 members in humans. Although the function of most DUBs is yet to be characterized, one USP member, cylindromatosis (CYLD), has been extensively studied in both human patients and animal models. This review will discuss the recent progress on the molecular and functional aspects of CYLD.

## Tumor-Suppressing Function of CYLD

**Tumor suppressor of familial cylindromatosis.** CYLD was originally identified as a gene mutated in familial cylindromatosis (FC), a genetic condition that predisposes patients for the development of tumors of skin appendages, termed cylindroma.<sup>5</sup> Cylindromas are benign tumors that typically appear on the scalp and are thought to be derived from hair follicle stem cells.<sup>6</sup> The cylindromatosis patients carry heterozygous germ-line mutations in the CYLD gene, but the wild-type CYLD allele undergoes loss of heterozygosity (LOH) and occasionally somatic mutations in the tumors, thus emphasizing the tumor suppressor role of CYLD.<sup>5</sup> It is now evident that CYLD gene mutations are also associated with another skin appendage tumor, multiple familial trichoepithelioma (MFT).<sup>7</sup> Owing to their clinical

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**Keywords:** deubiquitination; deubiquitinase; CYLD; NF- $\kappa$ B; tumor suppressor

**Abbreviations:** K48, lysine 48; DUB, deubiquitinase; USP, ubiquitin-specific protease; CYLD, cylindromatosis; FC, familial cylindromatosis; MFT, multiple familial trichoepithelioma; LOH, loss of heterozygosity; NEMO, NF- $\kappa$ B essential modulator; IKK, I $\kappa$ B kinase; RIP1, receptor-interacting protein 1; RANK, receptor activator of nuclear factor  $\kappa$ B; RANKL, RANK ligand; TPA, 12-O-tetra-decanoylphorbol-13 acetate; Plk1, polo-like kinase 1; LPS, lipopolysaccharide; APC, anaphase-promoting complex; IBD, inflammatory bowel diseases

Received 02.2.09; revised 06.3.09; accepted 16.3.09; Edited by G Melino; published online 17.4.09

similarities and involvement of mutations in the same gene, FC and MFT are believed to represent phenotypic variations of the same disease.<sup>8</sup> In fact, patients with the so-called Brooke–Spiegler syndrome have both FC and MFT types of tumors.<sup>8</sup>

The human CYLD gene is located on chromosome 16q12.1 and encodes a protein of 956 amino acids. The C-terminal region of CYLD contains a catalytic domain with sequence homology to USP family members.<sup>5,9</sup> The FC and MFT patients carry heterozygous germ-line mutations in the CYLD gene with LOH occurring in the tumors, a finding that defines CYLD as a tumor suppressor.<sup>5</sup> The mutations of CYLD in FC and MFT appear predominantly in the exons that encode the catalytic domain.<sup>5,10</sup> These are mostly frameshift or nonsense mutations, resulting in generation of predictably truncated CYLD proteins lacking a functional DUB domain. In a few cases, missense mutations cause substitutions of important residues within the DUB catalytic domain of CYLD. Therefore, the DUB activity of CYLD is critical for its tumor suppressor function.

**Regulation of other cancers.** The tumor suppressor function of CYLD seems to be highly tumor-type specific. Other than FC and MFT, no other types of neoplasm have been tightly associated with mutations and LOH of CYLD. However, accumulating evidence indicates that CYLD deficiency may, nevertheless, promote the development of other cancers. Recent studies reveal that CYLD is among the frequently mutated genes in multiple myeloma, a late stage B-cell malignancy that involves deregulated activation of the transcription factor NF- $\kappa$ B.<sup>11,12</sup> Moreover, LOH of chromosome 16q, in which CYLD gene resides, is detected in a large population of multiple myeloma patients and is associated with poor overall survival.<sup>13</sup> Although multiple genes are affected in the multiple myeloma cases with 16q LOH, CYLD is one of the two most likely genes responsible for the poor prognosis of patients with 16q LOH. Comparative genomic hybridization assays also suggest potential genetic abnormalities of CYLD (reduction in copy number) in hepatocellular carcinoma, uterine cervix carcinoma, and kidney cancer.<sup>14–16</sup> In addition to genetic mutations,

suppressed CYLD gene expression may also contribute to tumorigenesis. Indeed, reduced expression CYLD has been detected in colon and hepatocellular carcinomas, as well as in melanoma.<sup>17,18</sup>

**Tumor-suppressing function in animal models.** The tumor-suppressing function of CYLD has also been studied using mouse models. CYLD knockout mice do not spontaneously develop tumors, but they are more sensitive to chemically induced skin tumors than wild-type mice.<sup>19</sup> Loss of CYLD promotes the tumor cell proliferation because of enhanced expression of cyclin D1. Unlike the human cylindromas, which develop from hair follicles, the tumors in this mouse model are derived primarily from epidermal keratinocytes.<sup>19</sup> Nevertheless, this animal study confirms the tumor suppressor function of CYLD and further supports the idea that CYLD deficiency may be associated with different types of tumors. Along the same line, another study shows that CYLD knockout mice are also more susceptible to colon tumor induction by dextran sulfate sodium.<sup>20</sup> As the CYLD deficiency causes aberrant immune and inflammatory responses,<sup>21</sup> future studies should examine the possible contribution of different immune components to the enhanced tumor susceptibility of CYLD knockout mice.

### Negative Regulation of NF- $\kappa$ B by CYLD

The initial clue to the signaling function of CYLD came from an RNAi-based functional screening study, which identified CYLD as a DUB that negatively regulates NF- $\kappa$ B activation.<sup>22</sup> At the same time, yeast two-hybrid screening studies identified CYLD as a protein that binds to NF- $\kappa$ B essential modulator (NEMO), a regulatory subunit of I $\kappa$ B kinase (IKK).<sup>9,23</sup> CoIP assays also revealed the association of CYLD with two IKK regulatory proteins, TRAF2 and TRAF6, and subsequent studies identified additional molecular targets of CYLD (Table 1). Moreover, a long list of putative CYLD-associating proteins has been identified by CYLD immunoprecipitation and mass spectrometry assays, although further validation is required to determine whether CYLD specifically associates with any of these proteins.<sup>34</sup> Among the

**Table 1** Molecular targets and functions of CYLD

Target	Downstream pathways	Biological processes	References
TRAF2	NF- $\kappa$ B, JNK	Apoptosis	9,22,23
TRAF6	NF- $\kappa$ B, JNK, p38	Apoptosis, inflammation	9,22,23
TRAF7	NF- $\kappa$ B, p38	Inflammation	24
NEMO	NF- $\kappa$ B	Apoptosis	9,22,23
TRIP <sup>a</sup>	NF- $\kappa$ B	Apoptosis	25
Bcl3	NF- $\kappa$ B (transactivation)	Proliferation, tumorigenesis	19
Tak1	NF- $\kappa$ B, JNK, p38	T-cell activation	26,27
?	NF- $\kappa$ B	B-cell activation	28,29
Lck	TCR-proximal signaling	Thymocyte development <sup>b</sup>	30
RIP1	NF- $\kappa$ B	Spermatogenesis <sup>b</sup>	31
RIG-I	IKKi/TBK-1	Antiviral response	32,33
Plk <sup>a</sup>	Cell cycle	Mitotic cell entry <sup>b</sup>	34
TRPA1	Calcium signaling	Channel function	35

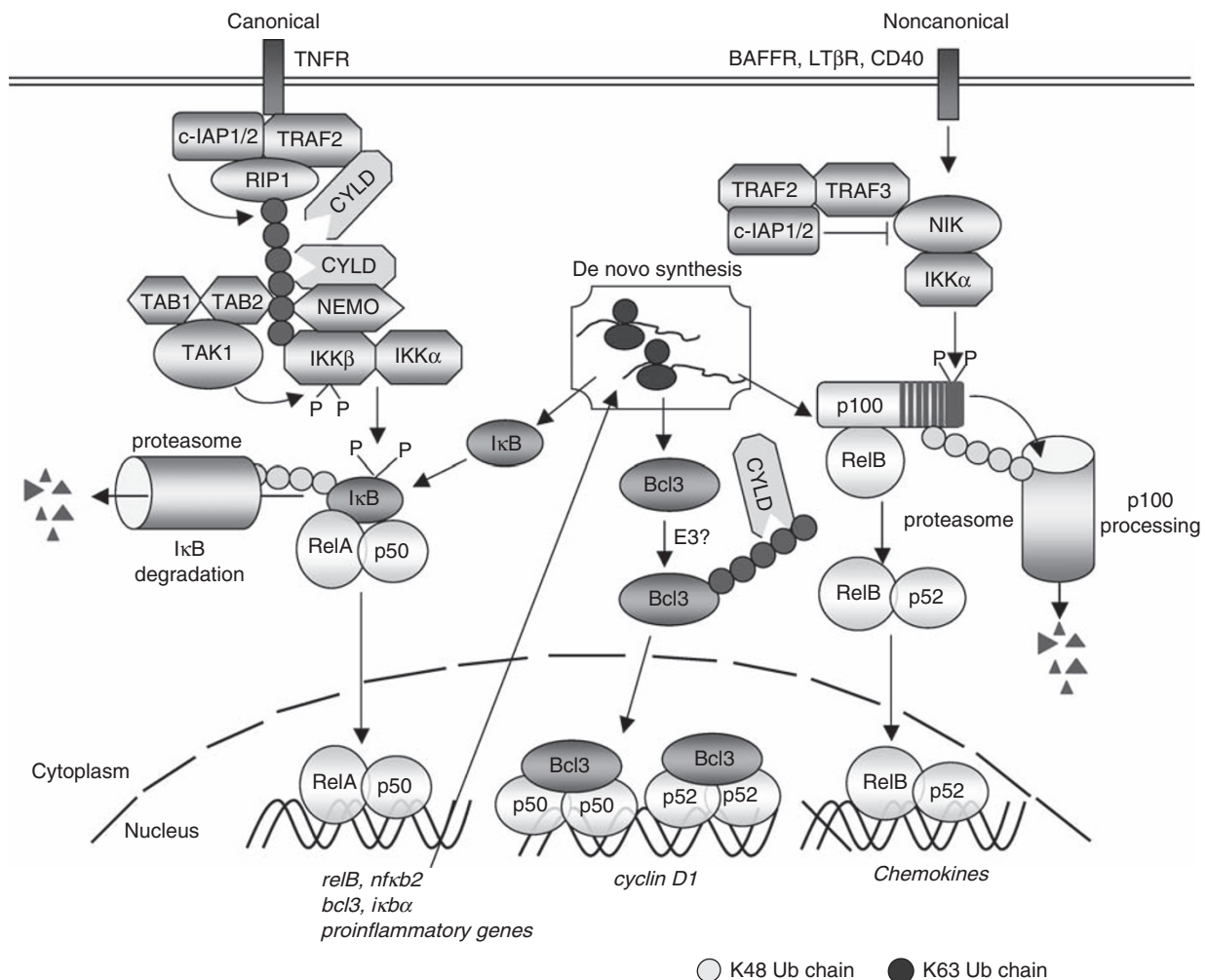
Abbreviations: NEMO, NF- $\kappa$ B essential modulator; Plk, polo-like kinase; RIG-I, retinoic acid-inducible gene 1; RIP1, receptor-interacting protein 1; Tak1, TGF- $\beta$ -activated kinase 1; TBK-1, TANK-binding kinase 1; TRAF, TNF receptor-associated factor; TRIP, TRAF-interacting protein; TRPA1, transient receptor potential channel A1. The question mark indicates that the target molecule has not been identified in B cells. <sup>a</sup>Deubiquitination proposed but not yet examined. <sup>b</sup>These processes are positively regulated by CYLD, whereas all others are negatively regulated by CYLD

characterized CYLD targets, most, although not all, are involved in the signal transduction mediating NF- $\kappa$ B activation (Table 1).

**Deubiquitination of multiple NF- $\kappa$ B signaling components.** A central step in NF- $\kappa$ B signaling is activation of IKK, which phosphorylates NF- $\kappa$ B inhibitory proteins (I $\kappa$ Bs), thereby triggering the degradation of I $\kappa$ Bs and nuclear translocation of NF- $\kappa$ B.<sup>36</sup> Strong evidence suggests that IKK activation by different receptor signals involves conjugation of K63-linked ubiquitin chains to its signaling components, including NEMO and upstream regulatory factors, such as Tak1, TRAF2, TRAF6, and receptor-interacting protein 1 (RIP1)<sup>21,37</sup> (Figure 1). The K63-linked ubiquitin chains seem to facilitate protein–protein interactions in the assembly of signaling complexes, in which

IKK is activated by its upstream kinases, particularly TGF- $\beta$ -activated kinase 1 (TAK1). When transfected into mammalian cells, CYLD deubiquitinates NEMO as well as several IKK upstream regulators, including TRAF2, TRAF6, TRAF7, RIP1, and Tak1.<sup>9,22–24,31</sup> In support of these findings, CYLD knockdown or knockout promotes ubiquitination of these signaling molecules in different cell types.<sup>20,26,31,39,40</sup>

The identification of multiple targets of CYLD in the NF- $\kappa$ B signaling pathway raises the question of how CYLD recognizes different targets. Emerging evidence suggests that the association of CYLD with some of its targets occurs indirectly through adaptors. Two recent studies have shown that the adaptor protein p62 (also named sequestosome 1) binds to CYLD and recruits it to TRAF6.<sup>40,41</sup> Whether p62 also recruits



**Figure 1** Regulation of canonical and noncanonical pathways of NF- $\kappa$ B activation by cylindromatosis (CYLD). NF- $\kappa$ B can be activated by canonical and noncanonical pathways, which rely on I $\kappa$ B degradation and p100 processing, respectively. Noncanonical NF- $\kappa$ B signaling involves receptor-mediated degradation of negative regulatory ubiquitin ligase complex, c-IAP/TRAFF2/TRAFF3, and accumulation of NIK.<sup>36</sup> The canonical pathway involves K63 type of ubiquitination of several signaling components, particularly receptor-interacting protein 1 (RIP1), which is required for the recruitment and activation of I $\kappa$ B kinase (IKK) and its activating kinase, Tak1. This pathway can be stimulated by various immune receptors, including TNFR1 (shown in the figure), IL-1R, TLRs, antigen receptors, etc.<sup>36</sup> (not shown). Activation of IKK by TNFR1 involves RIP1 ubiquitination by the E3 ubiquitin ligases, TRAF2, and cIAP1 and 2 (cIAP1/2).<sup>2,38</sup> Additionally, K63-linked ubiquitination of an NF- $\kappa$ B coactivator, Bcl3, promotes its nuclear translocation and, thereby, enhances NF- $\kappa$ B function. The different NF- $\kappa$ B complexes regulate distinct target genes, although they may function cooperatively in many cases. CYLD deubiquitinates the canonical NF- $\kappa$ B signaling components and Bcl3, and negatively regulates the NF- $\kappa$ B activation and Bcl3 nuclear translocation. Additionally, CYLD may also indirectly inhibit the atypical NF- $\kappa$ B pathways, as the inducible expression of noncanonical NF- $\kappa$ B members, RelB and NF- $\kappa$ B2 p100, and the coactivator, Bcl3, depends on the canonical NF- $\kappa$ B activation

CYLD to other molecules is not known. Another potential adaptor of CYLD is NEMO, which directly binds CYLD and associates with various IKK regulators, such as RIP1 and TRAF2. It will be interesting to examine whether the binding of CYLD to the IKK regulators is dependent on NEMO. Notably, both NEMO and p62 contain a ubiquitin-binding domain, which mediates protein interaction in a ubiquitin-dependent manner.<sup>42,43</sup> The involvement of adaptors for DUB function is not unique to CYLD. The association of another DUB, A20, with its targets also requires specific adaptors, including ABIN1 and Tax-binding protein 1.<sup>44–46</sup> Similar to the CYLD adaptors, ABIN1 and Tax-binding protein 1 both contain a ubiquitin-binding domain.<sup>43,46</sup> It is important to note, however, that the function of these ubiquitin-binding adaptors is not limited to the regulation of NF- $\kappa$ B signaling, as shown for ABIN1 that has an A20-independent anti-apoptotic function.<sup>47</sup> Nevertheless, it is intriguing to propose that ubiquitin-binding adaptors may facilitate the association of CYLD and other DUBs to a specific group of substrate proteins conjugated with ubiquitin chains.

Although CYLD has been firmly established as a DUB that targets NF- $\kappa$ B signaling factors, how CYLD negatively regulates NF- $\kappa$ B activation is not completely understood. Initial studies revealed that CYLD negatively regulates NF- $\kappa$ B activation by different inducers, including TNF- $\alpha$ , IL-1, CD40, and PMA.<sup>9,22,23</sup> However, subsequent studies indicate that the function of CYLD may be dependent on cell types and stimulating receptors.<sup>20,26,48</sup> This mode of action of CYLD may be due to its functional redundancy with other DUBs, particularly A20 that is known to also negatively regulate NF- $\kappa$ B.<sup>49,50</sup> Additionally, the different levels of CYLD expression may also contribute to its cell type-specific function.<sup>40</sup> Along the same line, recent studies suggest that the mechanism of ubiquitin-dependent IKK activation seems to be complex. For example, mutation of a major ubiquitin acceptor site (K319) of NEMO in a knock-in mouse has little effect on the activation of NF- $\kappa$ B by lipopolysaccharide (LPS), mitogens, IL-1, or TNF- $\alpha$ .<sup>51</sup> It is possible that additional lysines could be used as ubiquitin acceptor sites in the absence of K319. Indeed, several other lysines (including K285, K399) are ubiquitinated in response to different stimuli, and K399 ubiquitination seems to be required for NF- $\kappa$ B activation by Bcl10 in the TCR pathway.<sup>52,53</sup> However, it remains to be examined whether these other ubiquitin acceptor sites of NEMO are required for IKK activation *in vivo*. A more recent study suggests that ubiquitination of TRAF6 is dispensable for its signaling function, as a lysine-deficient TRAF6 mutant is fully functional in transducing the IL-1R and RANK (receptor activator of nuclear factor  $\kappa$ B) signals.<sup>54</sup> On the other hand, the RING domain of TRAF6 is critical for its signaling function, suggesting that ubiquitination of certain targets of TRAF6 is required for IKK activation.<sup>54–56</sup> Consistent with this idea, TRAF6-mediated IRAK1 ubiquitination is important for IKK activation by IL-1R.<sup>57</sup> Although both TRAF6 and IRAK1 are conjugated with K63-linked ubiquitin chains upon IL-1 stimulation, only the ubiquitinated-IRAK1 is bound by the IKK regulatory subunit, NEMO.<sup>57</sup> Thus, although multiple signaling factors can be conjugated with ubiquitin chains during the activation of IKK (Figure 1), probably only some of them rely on the ubiquitin chains for their signaling function. It

is also possible that functional redundancy exists among these ubiquitination events. Additional knock-in studies are important for understanding how ubiquitination regulates NF- $\kappa$ B signaling *in vivo*. Such knowledge will also help understand the DUB function of CYLD in NF- $\kappa$ B regulation.

**CYLD versus A20: similarities and differences.** Similar to CYLD, A20 mediates deubiquitination of various signaling molecules in the NF- $\kappa$ B pathway, which contributes to the negative regulation of NF- $\kappa$ B signaling.<sup>58</sup> It is remarkable that A20 and CYLD target the same set of signaling factors involved in the activation of NF- $\kappa$ B.<sup>21</sup> As both A20 and CYLD have been implicated as K63-specific DUBs, the question is raised as to why the same signaling pathway requires two functionally similar DUBs. One possible answer is that CYLD and A20 may act in different phases of NF- $\kappa$ B activation. CYLD seems to be a constitutively active DUB that prevents spontaneous ubiquitination of its targets. CYLD knockdown by siRNA results in constitutive ubiquitination of TRAF2.<sup>39</sup> Constitutive ubiquitination of several other CYLD targets have also been detected in CYLD KO cells.<sup>31,26,28</sup> Furthermore, CYLD mutations seem to contribute to the constitutive activation of NF- $\kappa$ B in multiple myeloma cells.<sup>11,12</sup> Additional evidence suggests that CYLD is functionally inactivated during signal-induced NF- $\kappa$ B activation. In response to mitogens and TNF- $\alpha$ , CYLD is rapidly and transiently phosphorylated, which seems to attenuate its DUB function.<sup>39</sup> Interestingly, the phosphorylation of CYLD is mediated by IKK,<sup>39</sup> implying that CYLD may not be a major factor involved in feedback control of NF- $\kappa$ B activation.

In contrast to the constitutive action of CYLD, the function of A20 depends on its inducible expression.<sup>59</sup> Furthermore, the DUB activity of A20 is stimulated by the NF- $\kappa$ B stimuli, which involves its phosphorylation by IKK.<sup>60</sup> Thus, A20 functions inducibly and is required for termination of the signal-induced NF- $\kappa$ B activation,<sup>49,61</sup> as opposed to the constitutive action of CYLD in the control of spontaneous NF- $\kappa$ B activation. These findings suggest that CYLD and A20 may regulate the initial and resolving phases of NF- $\kappa$ B activation, respectively.

**Regulation of atypical mechanism of NF- $\kappa$ B activation.** K63-linked ubiquitination of Bcl3 has been implicated as an atypical mechanism of NF- $\kappa$ B activation.<sup>19</sup> Bcl3 is a homolog of I $\kappa$ B $\alpha$  that preferentially associates with the DNA-binding subunits of NF- $\kappa$ B, p50, and p52.<sup>62</sup> In contrast to I $\kappa$ B $\alpha$ , Bcl3 does not inhibit the DNA-binding function of p50 and p52, but rather functions as a transcriptional coactivator of these NF- $\kappa$ B members. CYLD physically interacts with Bcl3 through a specific region that is different from the domain that mediates binding to TRAF2 and NEMO.<sup>19</sup> CYLD inhibits K63-linked ubiquitination and nuclear translocation of Bcl3, thus suggesting a role for ubiquitination in the regulation of Bcl3 nuclear expression (Figure 1). Loss of CYLD in keratinocytes sensitizes the induction of Bcl3 nuclear translocation by 12-O-tetradecanoylphorbol-13 acetate (TPA) and UV light, which is associated with enhanced sensitivity of CYLD<sup>-/-</sup> mice to TPA-stimulated skin tumor development.<sup>19</sup> In support of these early findings, a recent study shows that

downregulation of CYLD in melanoma cells by the ubiquitin ligase, Snail, causes constitutive nuclear expression of Bcl3, which seems to promote tumor progression.<sup>18</sup>

Constitutive nuclear expression of Bcl3 also occurs in B cells derived from a knock-in mouse that harbors a CYLD splice variant (CYLD<sup>ex7/8</sup>) lacking exons 7 and 8.<sup>29</sup> Notably, CYLD<sup>ex7/8</sup> is competent in Bcl3 binding and deubiquitination, although it is defective in targeting TRAF2 and NEMO.<sup>29</sup> This finding suggests that, in addition to deubiquitinating Bcl3, CYLD may regulate the nuclear expression of this NF- $\kappa$ B coactivator through additional mechanisms. In this regard, B cells from the CYLD<sup>ex7/8</sup> knock-in mice overexpress Bcl3, which likely contributes to its nuclear translocation as shown with Bcl3 transgenic mice.<sup>63</sup> Although how CYLD regulates Bcl3 expression is unclear, one possibility is suggested by the finding that the CYLD<sup>ex7/8</sup> B cells display constitutive NF- $\kappa$ B activity and overexpress several NF- $\kappa$ B target genes, including RelB, NF- $\kappa$ B2, and TRAF2.<sup>29</sup> As Bcl3 is also encoded by an NF- $\kappa$ B target gene, it is possible that its overexpression in CYLD<sup>ex7/8</sup> B cells is mediated by NF- $\kappa$ B-induced expression (Figure 1). However, real-time PCR did not reveal RNA level alterations of Bcl3 or the other NF- $\kappa$ B targets, suggesting the potential involvement of a post-transcriptional mechanism.<sup>29</sup> On the other hand, other studies did reveal overexpression of at least some of the NF- $\kappa$ B target genes at the RNA level in CYLD-knockout B cells and T cells.<sup>26,28</sup> Whether such a discrepancy is due to experimental variations or differences between the mouse models remains to be investigated.

### Physiological Functions of CYLD

The critical role of CYLD in NF- $\kappa$ B regulation suggests the involvement of this DUB in important biological processes. This possibility has been tested using animal models. Several groups have generated CYLD knockout mice,<sup>19,20,26,30,64</sup> and one group created a knock-in mouse selectively expressing a CYLD splicing variant (CYLD<sup>ex7/8</sup>) lacking exons 7 and 8.<sup>29</sup> Although certain discrepancies exist among the different studies, the general message obtained from these studies is that CYLD possess diverse physiological functions.

**Immune response and inflammation.** One prominent function of CYLD is regulation of immune response and inflammation.<sup>21</sup> CYLD knockout mice display abnormalities in thymocyte development and T-cell activation, which is associated with colonic inflammation and autoimmune symptoms.<sup>20,26,30</sup> Similarly, the CYLD deficiency causes spontaneous B-cell activation and hyperplasia,<sup>28</sup> and this phenotype is even more striking in the CYLD<sup>ex7/8</sup> knock-in mice.<sup>29</sup> An important role of CYLD in regulating innate immunity has also been reported. CYLD negatively regulates the induction of proinflammatory mediators by *Streptococcus pneumoniae* and *Escherichia coli*.<sup>27,65</sup> In addition to negatively regulating NF- $\kappa$ B activation, CYLD inhibits the activation of nuclear factor of activated T cells by *S. pneumoniae* through deubiquitinating an upstream kinase, Tak1.<sup>27</sup> Of note, deregulated Tak1 ubiquitination also contributes to the hyperactivation of NF- $\kappa$ B and JNK in CYLD-deficient T cells,<sup>26</sup> suggesting that Tak1 is a common target of CYLD in the innate and adaptive immune

responses. Another known target of Tak1, p38, is also negatively regulated by CYLD, although the connection with Tak1 in this case has not been shown.<sup>64</sup> Interestingly, CYLD-mediated negative regulation of p38 promotes the mortality caused by *S. pneumoniae*, providing an example for the involvement of CYLD in bacterial pathogenesis.<sup>64</sup> Recently, two studies showed a role for CYLD in negatively regulating virus-induced production of type I interferons.<sup>32,33</sup> This function of CYLD involves its physical interaction with and deubiquitination of an intracellular RNA sensor, RIG-I (retinoic acid-induced gene 1), and thus negative regulation of the IKK-related kinases, IKK $\epsilon$  and TBK1.<sup>32,33</sup> More details regarding the immunoregulatory functions of CYLD are discussed in a recent review.<sup>21</sup>

**Germ cell apoptosis and spermatogenesis.** Spermatogenesis is a tightly controlled biological process that involves sequential differentiation of male germ stem cells to mature spermatozoa.<sup>66</sup> A hallmark of spermatogenesis is an early wave of germ cell apoptosis, which is important to eliminate excess germ cells, and thus keep a proper balance between the germ cells and the supporting Sertoli cells.<sup>67,68</sup> In male CYLD knockout mice, the early wave of germ cell apoptosis is attenuated, resulting in the block of spermatogenesis at a late stage.<sup>31</sup> It seems that the germ cells are constantly exposed to NF- $\kappa$ B stimuli, but the magnitude of NF- $\kappa$ B activation is controlled by CYLD, thereby allowing the programmed cell death to occur. The CYLD deficiency causes marked activation of NF- $\kappa$ B in germ cells, which is associated with heightened expression of anti-apoptotic genes, such as Bcl-2. A major target of CYLD in germ cells seems to be RIP1, as RIP1 is constitutively ubiquitinated in CYLD-deficient germ cells. Of note, K63-linked ubiquitination of RIP1 not only promotes NF- $\kappa$ B activation,<sup>69–71</sup> but also has been proposed to prevent RIP1 from accessing caspase-8 and activating apoptosis.<sup>72</sup> Thus, deregulated RIP1 ubiquitination in CYLD-deficient cells may inhibit apoptosis by both NF- $\kappa$ B activation and inhibition of the caspase signaling pathway (Figure 2).

**Osteoclastogenesis and bone homeostasis.** Bone homeostasis is tightly regulated by the functional balance of the bone-forming osteoblasts and bone-erosive osteoclasts.<sup>73</sup> Deregulated osteoclast activity is the major cause of bone erosion in osteolytic diseases, such as osteoporosis, rheumatoid arthritis, and Paget's disease of bone.<sup>74,75</sup> The development of osteoclasts is driven by signal transduction mediated by a TNFR family member, RANK. Engagement of RANK by RANK ligand (RANKL) activates a K63-specific E3 ubiquitin ligase, TRAF6, which undergoes autoubiquitination and mediates ubiquitination of downstream targets, such as NEMO. These ubiquitination events are important for activation of IKK and NF- $\kappa$ B. CYLD is involved in the feedback inhibition of RANK signaling.<sup>40</sup> RANKL-stimulated osteoclastogenesis potently induces the expression of CYLD, and the accumulated CYLD targets TRAF6 by interacting with the adaptor protein, p62. Thus, CYLD inhibits RANK-mediated signaling function by deubiquitinating TRAF6 or its downstream targets involved in NF- $\kappa$ B activation. Loss of CYLD results in hyper production of

osteoclasts, which is associated with severe bone loss or osteoporosis in mice.

**Cell cycle progression and cell migration.** Studies using cell culture models have revealed a role for CYLD in the regulation of cell cycle progression.<sup>34</sup> During the telophase of cell cycle, CYLD localizes to the interphase and midbody probably through interaction with microtubules, a property that is reminiscent of many regulators of cytokinesis. RNAi-mediated CYLD knockdown prolongs the pro-mitotic stage of cell cycle, thus suggesting a role for CYLD in the control of mitotic entry.<sup>34</sup> Interestingly, this novel function of CYLD is independent of NF- $\kappa$ B, although it requires the DUB catalytic activity of CYLD. CYLD physically interacts with polo-like kinase 1 (Plk1), a serine/threonine kinase with a key regulatory role in mitotic cell division.<sup>76</sup> It was proposed that CYLD positively regulates the function of Plk1, probably by deconjugating K63-linked ubiquitin chains on Plk1 or its upstream regulators.<sup>34</sup> However, as the role of K63 ubiquitination in Plk1 regulation has not been established, further studies are required to understand how CYLD regulates mitosis.

A role of CYLD in regulating microtubule dynamics and cell migration has recently been suggested based on *in vitro* studies.<sup>77</sup> CYLD associates with microtubules by binding to tubulin and seems to promote microtubule assembly and stability. RNAi-mediated CYLD knockdown in HeLa cells delays microtubule regrowth after nocodazole washout. Consistent with this function, CYLD is important for cell migration, as revealed using an *in vitro* wound-healing assay.<sup>77</sup> It will be important to examine whether K63 ubiquitination plays a role in tubulin polymerization, and how CYLD regulates this biological process.

### CYLD Structure and K63-Specific DUB Activity

The original cloning work reveals complex domain structures of the CYLD protein.<sup>5</sup> In addition to the C-terminal USP catalytic domain, CYLD contains three Cap-Gly domains, two proline-rich motifs, and a zinc-finger-like B-box domain located within the USP domain<sup>5,78</sup> (Figure 3). The Cap-Gly domain is known to mediate protein association to  $\alpha$ -tubulin and other microtubule-associated proteins.<sup>79</sup> As discussed above, CYLD indeed has microtubule-association function, which is dependent on the first Cap-Gly domain.<sup>77</sup> This function of CYLD may be important for its regulation of microtubule assembly and cell migration, and possibly also cell cycle progression. The proline-rich repeat typically mediates interaction with proteins containing SH3 or WW domains.<sup>80,81</sup> Whether CYLD interacts with such proteins is unclear. Interestingly, the tertiary structure of the third Cap-Gly domain of CYLD resembles that of a SH3 domain.<sup>82</sup> The presence of both proline-rich motifs and an SH3-like domain in CYLD is intriguing, as it could allow CYLD to interact with targets that contain either SH3 domains or proline-rich motifs. Indeed, the SH3-like domain of CYLD is responsible for its binding to a proline-rich region of NEMO.<sup>82</sup> CYLD also contains TRAF-binding motif known to be responsible for association with TRAF2<sup>9</sup> (Figure 3). In addition to these well-

defined protein domains/motifs, the C-terminal portion of CYLD physically interacts with Bcl3<sup>19</sup> (Figure 3).

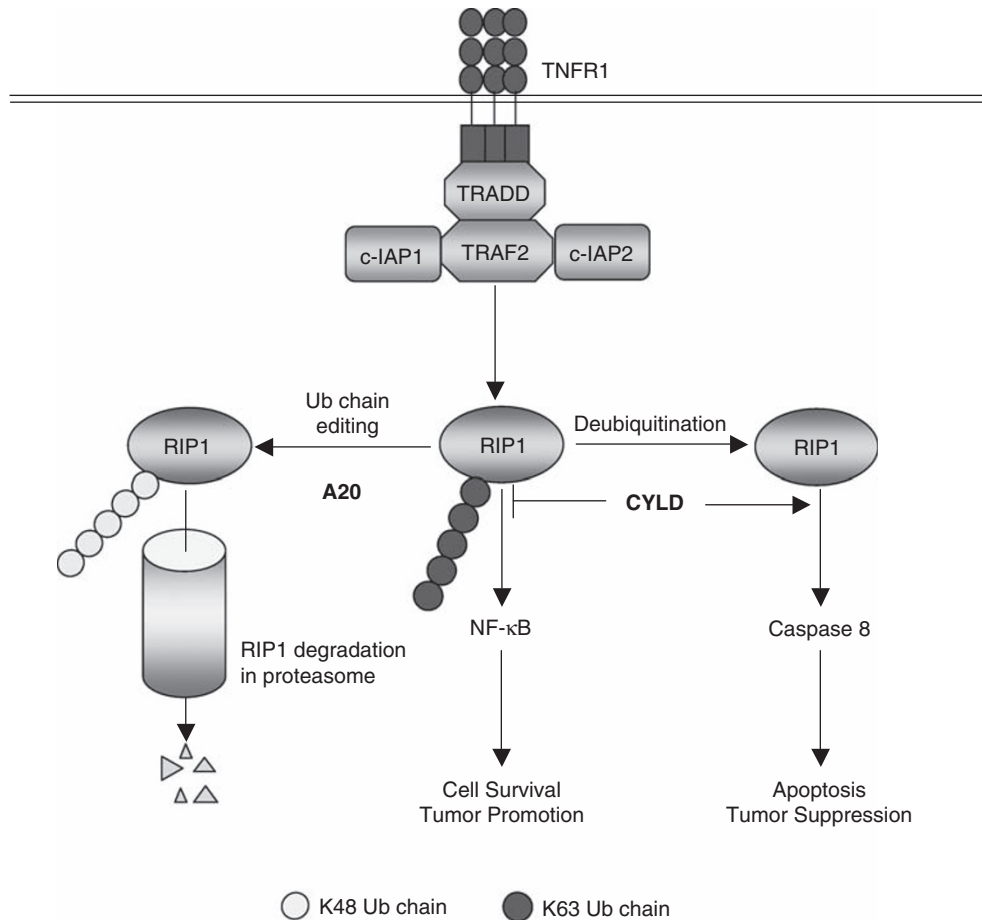
The tertiary structure of the CYLD catalytic domain was recently resolved by crystallography.<sup>78</sup> Similar to the other USP domains so far solved, the USP domain of CYLD contains three typical subdomains: fingers, palm, and thumb. However, CYLD is unique in that it contains an additional USP subdomain, that is, a zinc-binding domain that is similar to a RING or B-box domain (Figure 3, B Box). This subdomain lacks ubiquitin ligase function, but seems to mediate the intracellular localization of CYLD.<sup>78</sup> It is possible that the B box of CYLD is involved in protein-protein interaction. Another unique feature of the CYLD USP domain is the reduced size of its fingers domain. Moreover, sequence alignment with other USP domains reveals that the proximal ubiquitin-binding site of CYLD differs greatly from that of other known USP domains. Accordingly, the CYLD USP domain preferentially digests K63-linked ubiquitin chains as opposed to the K48 specificity of the other USPs.<sup>78</sup> These findings provide structural basis for the K63-specific DUB function of CYLD.

### Mechanisms of CYLD Regulation

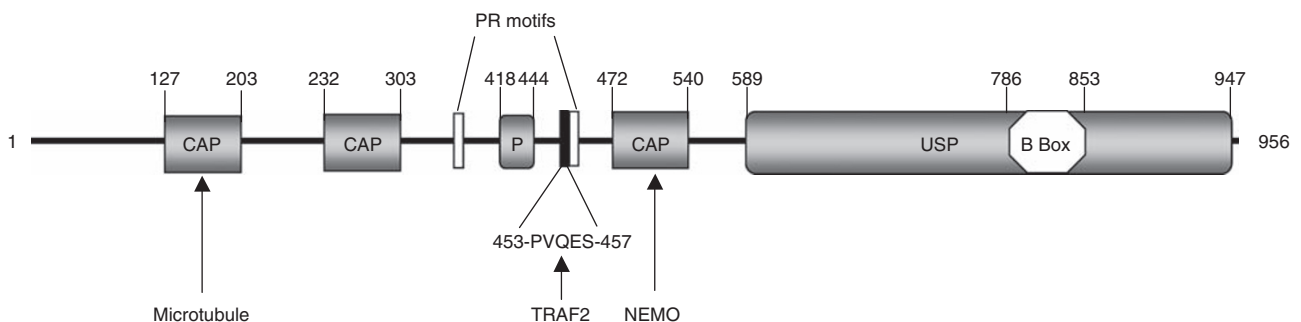
Signaling molecules are often subject to post-translational or transcriptional regulations. To date, very little is known regarding how the function of DUBs is regulated. Given the diversity of DUB functions, it is likely that the regulation of these enzymes involves distinct mechanisms. Recent studies on CYLD suggest the existence of multiple mechanisms in the regulation of this tumor suppressor DUB.

**Phosphorylation.** Initial evidence for post-translational regulation of CYLD came from the finding that CYLD undergoes transient phosphorylation in cells stimulated with NF- $\kappa$ B inducers, such as TNF- $\alpha$ , mitogens, and LPS.<sup>39</sup> The phosphorylation of CYLD occurs in a serine cluster located just upstream of its TRAF2-binding site (amino acid 418–444). Site-directed mutagenesis and phospho-specific immunoblotting analyses reveal that serine-418 and additional serines within this cluster are phosphorylated. When these serines are mutated to alanines, it generates a super-active CYLD mutant that prevents TNF- $\alpha$ -stimulated TRAF2 ubiquitination. Conversely, substitution of the serine cluster to phosphomimetic residues (glutamic acids) creates a CYLD mutant that is defective in TRAF2 deubiquitination. These results imply that the signal-stimulated CYLD phosphorylation negatively regulates its DUB function, thereby allowing temporary accumulation of ubiquitin chains on its targets, such as TRAF2. However, precisely how phosphorylation attenuates the function of CYLD is yet to be investigated. As the phosphomimetic CYLD mutant is competent in TRAF2 binding, phosphorylation of CYLD may not interfere with its association with TRAF2, but probably affects the catalytic activity of CYLD.

The phosphorylation of CYLD may be considered a mechanism of CYLD/IKK mutual regulation. Biochemical and genetic evidence suggests that IKK is a kinase that mediates CYLD phosphorylation.<sup>39</sup> As CYLD physically interacts with the IKK regulatory subunit, NEMO, it is likely that CYLD is recruited to the IKK holoenzyme by NEMO.



**Figure 2** Two mechanisms of apoptosis induction by cylindromatosis (CYLD). Stimulation of TNFR1 triggers lysine 63 (K63)-linked ubiquitination of receptor-interacting protein 1 (RIP1), which in turn leads to activation of the survival factor NF- $\kappa$ B. Deubiquitination of RIP1 by CYLD not only inhibits activation of the survival factor, NF- $\kappa$ B, but also promotes the engagement and activation of caspase 8 by RIP1. A20, a molecule with K63-specific DUB activity and K48-specific E3 ligase activity, mediates ubiquitin chain editing and converts the ubiquitin chains of RIP1 to the proteasome-targeting K48 type



**Figure 3** Structural domains of cylindromatosis (CYLD). The C-terminal portion of CYLD contains a ubiquitin-specific protease (USP) catalytic domain, with an inserted a zinc-binding B-box domain. The N-terminal portion of CYLD has three CAP-Gly domains (CAP), the third of which forms the NF- $\kappa$ B essential modulator (NEMO)-binding domain, and two proline-rich (PR) motifs. CYLD also contains a TRAF-binding motif (PVQES) and a phosphorylation (P) region. The C-terminal portion of CYLD (amino acids 470–957) is known to bind Bcl-3, although the precise Bcl-3-binding domain has not been defined

Indeed, the presence of NEMO is critical for the phosphorylation of CYLD by IKK catalytic subunits.

**Ubiquitination and degradation.** In a study to define the role of CYLD in regulating cell cycle progression, the steady

level of CYLD was shown to decrease drastically as cells exited mitosis; it remains low in G1, and gets reaccumulated as cells enter S phase.<sup>34</sup> The post-mitotic loss of CYLD seems to be mediated through its degradation, which occurs along with the degradation of several other known cell

cycle-regulatory factors, such as cyclin B and Plk1. Degradation of Plk1 and cyclin B is triggered through their ubiquitination by the anaphase-promoting complex (APC), but how CYLD is degraded is unclear as APC does not induce CYLD ubiquitination.<sup>34</sup> The reduction in CYLD protein level has also been seen when TNF- $\alpha$ -stimulated cells are infected with Sendai viruses, although this study did not determine whether viral infection promotes CYLD degradation or inhibits CYLD gene expression.<sup>32</sup>

Ubiquitin-dependent degradation of CYLD has recently been shown to serve as a mechanism by which human papilloma virus E6 protein promotes NF- $\kappa$ B activation under hypoxia conditions.<sup>83</sup> E6 is one of the two major HPV oncoproteins and is known to promote oncogenesis by targeting ubiquitin-dependent degradation of the tumor suppressor p53.<sup>84</sup> Induction of CYLD degradation and NF- $\kappa$ B activation represents a new oncogenic mechanism of E6, which may contribute to the association of hypoxia with poor clinical outcome of HPV-mediated malignancies, including cervical, head and neck cancers.<sup>83</sup>

**Gene expression.** Negative regulators of signal transduction are often involved in feedback inhibition of their targets. Such a function of CYLD was suggested by the finding that the expression of CYLD is induced by proinflammatory cytokines, TNF- $\alpha$  and IL-1 $\beta$ , and the Gram-negative bacterium *Haemophilus influenzae*.<sup>85–87</sup> Moreover, the induction of CYLD expression is dependent on IKK and NF- $\kappa$ B.<sup>85</sup> A robust induction of CYLD expression has also been shown along with RANKL-stimulated osteoclastogenesis.<sup>40</sup> Similarly, the RANKL-stimulated CYLD expression requires NF- $\kappa$ B, although in this case, the noncanonical NF- $\kappa$ B signaling pathway plays a critical role.<sup>40</sup> It should be emphasized that the inducible expression of CYLD seems to be cell type- and stimuli-dependent. For example, although TNF- $\alpha$  stimulates CYLD expression in HeLa cells, human bronchial epithelial cells, and human aortic endothelial cells,<sup>85–87</sup> it fails to induce CYLD expression in 293 cells.<sup>32</sup> In mouse bone marrow-derived macrophages, CYLD expression is strongly induced by RANKL, but not by TNF- $\alpha$  or LPS.<sup>40</sup> Thus, precisely how CYLD gene is induced requires additional studies. Notwithstanding, gene expression is clearly one of the mechanisms that control the function of CYLD.

Under certain pathological conditions, the expression of CYLD is also subject to negative regulation. One example is the downregulated CYLD mRNA expression in patients with inflammatory bowel diseases (IBD).<sup>88</sup> This finding is interesting, because CYLD knockout mice are sensitive to both spontaneous and chemical-induced colitis.<sup>20,26</sup> Thus, CYLD may have an anti-inflammatory role in the development of IBD. These findings also have implications on the tumor suppressor function of CYLD, as colonic inflammation in IBD patients is a risk factor for colorectal cancer.<sup>89</sup> The potential association of CYLD gene suppression with colon cancer is more directly suggested by a study showing reduced expression of CYLD in colon cancer cell lines and tissue samples.<sup>17</sup> It is currently unknown how the CYLD gene is suppressed in IBD and colon cancer cells. Nevertheless, mechanistic insight of CYLD gene repression has been

provided by studies using other cancer models. CYLD gene expression in melanoma cells is repressed by a zinc-finger transcription factor, Snail, which is associated with nuclear expression of Bcl3, and proliferation and invasion of melanoma cells.<sup>18</sup> Another study using a breast carcinoma cell line suggests that the CYLD gene is directly regulated by BAF57, a component of the hSWI/SNF complex that regulates gene expression through chromatin remodeling.<sup>90</sup> Thus, disruption of the hSWI/SNF complex and induction of Snail may contribute to the repression of CYLD gene expression in cancer cells.

**Alternative splicing.** CYLD gene contains multiple exons (20 in human and 16 in mouse). Alternative splicing of the human CYLD gene occurs in a non-coding exon (exon 3) and a small nine-nucleotide coding exon (exon 7), and the splicing variant lacking exon 7 is expressed in almost all tissues, although its function has not been studied.<sup>5</sup> As discussed above, a functional aspect of CYLD alternative splicing has been shown in an elegant study using a knock-in mouse model, CYLD<sup>ex7/8</sup>.<sup>29</sup>

## Concluding Remarks

Since the discovery of CYLD as a tumor suppressor in year 2000, significant progress has been made towards understanding the anti-tumor and physiological functions of this DUB, and delineating the molecular mechanism of its signaling action. Although genetic deficiency of CYLD is tightly associated with FC and its related skin appendage tumors, it is clear that genetic mutations and repressed expression of CYLD also occur in other types of neoplasm. Given the critical role of CYLD in the control of NF- $\kappa$ B activity, it is plausible that defect in CYLD expression or function will have a profound impact on the growth and survival of different types of cancer cells. Thus, CYLD can be considered a new therapeutic target in the treatment of cancers. However, for further exploring this possibility, it is important to understand how CYLD regulates normal biological processes, and how the expression and function of CYLD are regulated.

Animal models have been instrumental for elucidating the physiological functions, as well as the tumor suppressor action, of CYLD. CYLD knockout mice have been actively studied by several groups of investigators. Although there are discrepancies in the findings, a general consensus is that CYLD has an important role in the regulation of immune and inflammatory responses, development of immune and non-immune cells, apoptosis, and tumorigenesis. On the basis of *in vitro* studies, CYLD may also have a role in regulating mitosis and cell migration. As the *in vivo* functions of CYLD are complex, it is important to generate conditional knockout mice to better understand the function of CYLD in different cell types. Double knockout mice lacking both CYLD and NF- $\kappa$ B signaling components will be useful for further delineating the signaling mechanism of CYLD and determining the contribution of canonical and noncanonical NF- $\kappa$ B pathways to the pathology of CYLD knockout mice. Another important area of animal studies is to investigate the involvement of immune system in the tumor-suppressing function of CYLD.



Characterization of the molecular targets of CYLD represents an important approach to elucidate the molecular mechanism of CYLD function. A list of potential CYLD substrates has been identified, and most of them are involved in NF- $\kappa$ B signaling. These findings establish CYLD as a DUB that has a primary role in the regulation of NF- $\kappa$ B, although additional functions of CYLD do exist. A major challenge in future studies is to show how ubiquitination of the CYLD targets regulates the NF- $\kappa$ B signaling. Recent studies, using knock-in mice that harbor ubiquitination-deficient mutants of NEMO and TRAF6, have led to surprising findings showing dispensable role of their ubiquitination in NF- $\kappa$ B activation. Additional knock-in mouse models are obviously required to examine whether functional redundancy exists in the ubiquitination of different NF- $\kappa$ B signaling factors. It is also important to investigate whether the signaling function of different ubiquitination events is cell type- and stimuli-dependent, as revealed for the function of CYLD.

The findings that CYLD is subject to different mechanisms of regulation not only shed light on its dynamic signaling function, but also indicate its vulnerability to pathological disruption. CYLD may be functionally inactivated through its phosphorylation, degraded by the ubiquitination/proteasome pathway, or repressed at the level of transcription. Any of these modifications could lead to functional defect in the negative regulation of NF- $\kappa$ B, thus contributing to the development of cancers and immunological abnormalities. A better understanding of CYLD regulation is important for exploiting this tumor suppressor as a new anti-cancer therapeutic approach.

**Acknowledgements.** The work performed in my laboratory is supported by the National Institutes of Health Grants AI064639, AI057555, and GM084459.

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