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Review

Tumor suppressor CYLD: negative regulation of NF-κB signaling and more

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Abstract. CYLD is a protein with tumor suppressor properties which was originally discovered associated with cylindromatosis, an inherited cancer exclusively affecting the folicullo-sebaceous-apocrine unit of the epidermis. CYLD exhibits deubiquitinating activity and acts as a negative regulator of NF-κB and JNK signaling through its interaction with NEMO and TRAF2. Recent data suggest that this is unlikely to be its unique function *in vivo*. CYLD has also been shown to control other seemingly disparate cellular processes, such as proximal T cell receptor signaling, TrkA endocytosis and mitosis. In each case, this enzyme appears to act by regulating a specific type of polyubiquitination, K63 polyubiquitination, that does not result in recognition and degradation of proteins by the proteasome but instead controls their activity through diverse mechanisms.

Keywords. CYLD, tumor suppressor, ubiquitination, NF-κB, signaling.

Introduction

Ubiquitination is an evolutionarily conserved mechanism of posttranslational modification that affects myriads of proteins in the cell, controlling their half-life and activity [1, 2]. This process involves the covalent attachment to substrates of a polypeptide of 76 amino acids (aa), ubiquitin, according to a precise sequence of events (Fig. 1A). First, ubiquitin, which is synthesized as an inactive precursor protein, is processed by a specific protease (ubiquitin carboxy-terminal hydrolase) to make the carboxy-terminal diglycine motif available for conjugation. Then, activation of ubiquitin is carried out by an ubiquitin-activating enzyme (E1) which catalyzes the formation of a thioester bond between the carboxy terminus of ubiquitin and its active cysteine, in an ATP-dependent manner. Activated ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2), forming an E2-ubiquitin thioester. Finally, in the presence of a ubiquitin-protein ligase (E3), ubiquitin is attached to the target protein through an isopeptide bond between the carboxy terminus of ubiquitin and the ε-amino group of a lysine residue in the target protein. The role of E3 ligase enzymes, of which there are hundreds of members in humans, is therefore to accelerate ubiquitin attachment in a substrate-specific fashion.

Ubiquitination is a versatile event that affects single or multiple lysines of a given substrate and can result in either the covalent addition of a single molecule of ubiquitin (monoubiquitination) or formation of a chain of polyubiquitin (polyubiquitination). Polyubiquitination is made possible because ubiquitin itself contains several lysine residues (K6, K11, K27, K29, K33, K48 and K63) (Fig. 1B), some of which used to extend the ubiquitin chain.

Until recently, polyubiquitination was mostly viewed as playing a role in controlling the half-life of proteins

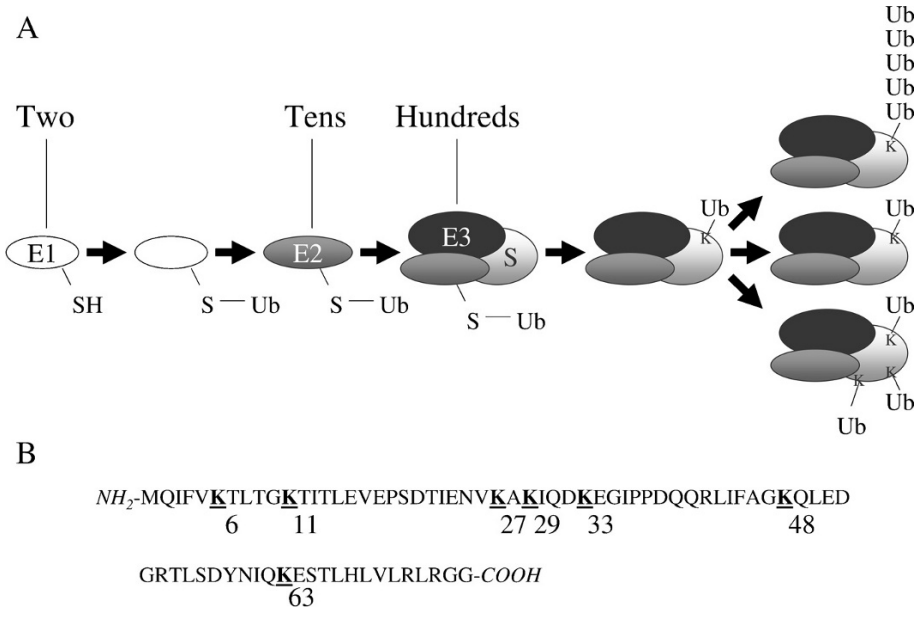

Figure 1. The ubiquitination process. (*A*) The sequence of events involved in protein ubiquitination is shown with the three major components of the ubiquitination process labeled as E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase. Substrates (S) can be monoubiquitinated, multi-monoubiquitinated or polyubiquitinated. (*B*) Primary sequence of ubiquitin. Lysine residues used to extend the chains of polyubiquitin are indicated.

and therefore ensuring the temporal fine-tuning of numerous processes in the cell. Indeed, addition of more than four ubiquitin moieties through K48 covalent linkage provides a tag for recognition by the 26S proteasome and results in proteolysis. Nevertheless, other lysine residues of ubiquitin, such as K29 or K63, can also be used to synthesize polyubiquitin chains and, in these specific cases, modified substrates are not recognized by the proteasome. Instead, their activity is somehow modified, resulting in catalytic activation, formation of protein complexes or internalization through endocytosis [3].

A small family of approximately 100 enzymes exhibiting deubiquitinase activity (DUBs) have been identified in the human proteome and negatively control the ubiquitination processes [4]. In most cases, both their specific substrates and the class of polyubiquitin chains they hydrolyze remain poorly defined. An interesting member of the DUB family, CYLD, has recently been characterized and already provides important insights into the function of this class of enzymes *in vivo*. This review describe how CYLD was initially discovered, how it may regulate important signaling pathways or processes and how its dysfunction has a major impact in cell pathophysiology, including tumorigenesis.

Discovery of CYLD through its association with human genetic diseases

Familial cylindromatosis, also called turban tumor syndrome (OMIM#132700), is a rare inherited cancer which is characterized by the formation of benign tumors, called cylindromas, in hairy parts of the body, mostly the scalp [5]. Cylindromas are considered as originating from a transformation event specifically affecting the folliculo-sebaceous-apocrine unit that produces hair and its associated glands. Early observations established that affected patients are heterozygous at birth for the locus causing the disease, whereas the cylindromas they develop exhibit a loss of heterozygosity (LOH) [6], demonstrating the involvment of a gene coding for a tumor suppressor ('Knudson's two-hit model' [7]). This gene, located at 16q12–13, has been called *CYLD*.

CYLD encodes an evolutionarily conserved protein of approximately 120 kDa (956 aa) which is a divergent member of the DUB family. Like other members of this family of enzymes, CYLD exhibits at the C terminus a catalytic domain composed of two conserved subdomains containing the active cysteine and the active histidine that form the catalytic pocket (Fig. 2). In addition, it contains three CAP-Gly motifs that have been proposed to participate in binding to microtubules [8]. Nevertheless, it remains uncertain whether CYLD directly interacts with microtubules. Most of *CYLD* mutations in familial cylindromatosis are frameshift or nonsense mutations that result in truncation of the catalytic domain [9, 10] (Fig. 2). Very few missense mutations have been reported. All but one (see below) affect essential amino acids of the catalytic pocket.

A genetic disease that shares similarities with familial cylindromatosis, multiple trichoepithelioma (MT; OMIM#601606), has also been shown to be caused by *CYLD* mutations [11–15]. It was known for a while that some individual cylindromatosis patients could

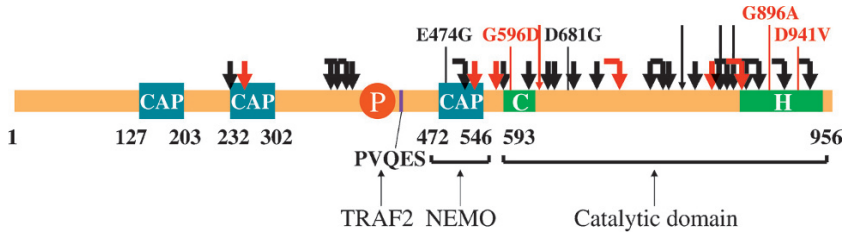

Figure 2. The CYLD protein: structure and mutations in human pathology. The various structural and functional domains of the protein are indicated. Also shown are the mutations affecting CYLD in familial cylindromatosis (Black) and multiple trichoepithelioma (Red), with frameshift mutations represented by a thick arrow, nonsense mutations by a broken arrow and splicing mutations by a thin arrow. The five identified missense mutations are also indicated. CAP, CAP-gly domain; P, phosphorylation sites; C and H, cysteine and histidine boxes of the catalytic domain.

also exhibit trichoepitheliomas, benign tumors originating from the hair follicle, and that affected members of the same family could present a heterogeneous phenotype with intermingled tumors [16]. This provided a rationale for looking at the *CYLD* locus in MT patients. Importantly, mutations of *CYLD* found in MT are not distinct from those generating cylindromatosis (Fig. 2), demonstrating the genetic identity of both diseases and confirming that precursor cells of the folliculo-sebaceous-apocrine unit, which give rise to the hair follicle and its adnexa, are indeed the cells from which tumors develop (Fig. 3).

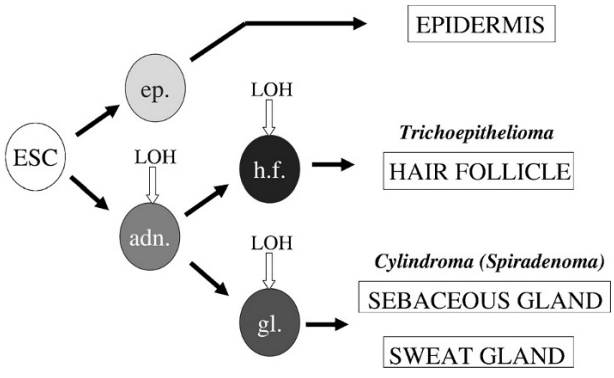

Figure 3. Putative origin of transformed cells in familial cylindromatosis and multiple trichoepithelioma. The loss of heterozygosity (LOH) events that are associated with tumor development are supposed to affect distinct precursor cells generating the skin adnexa. When LOH occurs in the precursors for gland cells (gl.), cylindroma and/or spiradenoma are formed, whereas LOH occuring in hair follicle (h.f.) precursors results in trichoepithelioma formation. LOH affecting the precursor of skin adnexa (adn.) may also result in mixed tumor formation. ESC, epidermal stem cell; ep., epidermis precursor.

CYLD is a negative regulator of NF-κB and JNK signaling

The NF-κB signaling pathway plays a key role in innate and acquired immunity, inflammation and carcinogenesis. NF-κB is a transcription factor, composed of homo- and heterodimeric associations between p50, p52, c-rel, relA and relB subunits, which is located in the cytoplasm of resting cells, associated with the IκB inhibitor. Upon stimulation, IκB is phosphorylated by IκB kinase (IKK) and this modification induces its K48-linked polyubiquitination and degradation by the proteasome, allowing NF-κB to translocate into the nucleus and to regulate the expression of its target genes [17].

Multiple signaling pathways which are induced by pro-inflammatory cytokines, including interleukin-1β (IL-1β) or tumor necrosis factor-α (TNF-α), bacterial components of the outer membrane, including lipopolysaccharide (LPS) or lipoteichoic acid (LPA), viral genetic content and its derived proteins, including double-stranded RNA (dsRNA), Tax from HTLV-1, or Epstein-Barr virus latent membrane protein 1 (LMP-1), and diverse forms of stress are able to activate IKK [18]. This kinase complex is composed of three major subunits. Two of them, IKK-1/α and IKK-2/β, exhibit catalytic activity and share a similar structure, whereas the third one, NEMO (NF-κB essential modulator)/IKKγ, has regulatory functions. NEMO has been shown to be absolutely required for IKK activation in most cases (canonical pathway), whereas it is dispensable for a small number of stimuli that directly target IKK-1 through a kinase called NF-κB-inducing kinase (NIK) (non-canonical pathway) [19].

The molecular mechanism of NEMO-dependent IKK activation has remained quite elusive until recently. It now appears to require a complex set of modifications by polyubiquitination that do not result in degradation by the proteasome. Key participants in these processes are members of the TNF receptor associated factor (TRAF) family of proteins which exhibit ubiquitin ligase activity (E3) and act in concert with Ubc13/Uev1A, a dimeric ubiquitin-conjugating enzyme (E2), to modify substrates through K63-linked polyubiquitination [20, 21]. Among these subtrates are TRAFs themselves, TAK1-binding protein (TAB)2 and TAB3 subunits of IKK kinase TAK (TGF-β-activated kin-

ase) and NEMO. Importantly, TAB2, TAB3 and NEMO also contain ubiquitin-binding domains with affinity for K63-linked polyubiquitin chains. Ubiquitination of both TAK components and NEMO may therefore result in IKK activation through NEMO-dependent recruitment of TAK (Fig. 4) [22].

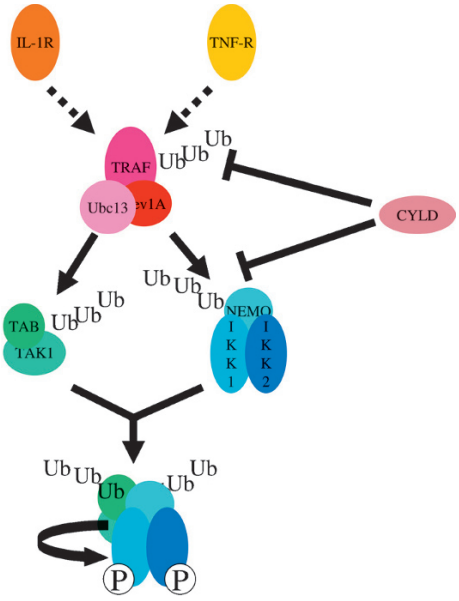

Figure 4. A simplified view of the IKK activation process. The major participants in the canonical pathway of IKK activation are indicated, using, as examples, the signaling pathways triggered by IL-1 and TNF. For clarity, numerous molecules (kinases, adaptors) are not shown above TRAF and are presented by a broken arrow. Proteins that are modified by K63-linked polyubiquitination and represent targets of CYLD are indicated. TRAF, TRAF6 (IL1-R) or TRAF2 (TNF-R); TAB, TAB2/3; Ub, ubiquitin; P, phosphate. See text for more details.

CYLD has been identified as a participant in NF-κB signaling following three distinct strategies. First, using an shRNA library designed to target the various members of the human DUB family, Brummelkamp et al. [23] have shown that shRNAs specifically targeting CYLD mRNA were the only ones able to significantly affect NF-κB activation by TNF in Hela cells. In this study, inhibition of CYLD resulted in increased NF-κB signaling. Second, during a two-hybrid screening in yeast aimed at identifying new proteins interacting with NEMO, Kovalenko et al. [24] and Trompouki et al. [25] also identified CYLD. A Pro-rich sequence located near the N terminus of NEMO and the third CAP-Gly motif of CYLD are involved in this interaction [26]. Worth noting is that a missense mutation of *CYLD* associated with cylindromatosis (E474G) may affect the NEMO-binding domain of CYLD (Fig. 2). Finally, a search for CYLD partners by Regamey et al. [27] identified a TRAF-interacting protein (TRIP), which had previously been proposed as regulating NF-κB activation by TNF [28].

In all these studies, overexpression of CYLD has been shown to negatively regulate NF-κB activation by multiple stimuli, including IL-1, TNF and LPS, but not by NIK, suggesting that this DUB may represent a general negative regulator of the canonical NF-κB signaling pathway. Importantly, this inhibitory effect requires the catalytic activity of CYLD and the affected step(s) is (are) located above or at the level of IKK, since CYLD is unable to deubiquitinate IκB. It has been shown that NEMO may be a *bona fide* target of CYLD since it is ubiquitinated upon cell stimulation (see above) and binds CYLD. Alternatively, or in addition, TRAF2 may also be a target for CYLD DUB activity *in vivo* since CYLD directly interacts with this E3 protein through a PXQXS/T-like motif [24]. Finally, an interaction between CYLD and TAK1 has been recently reported (see below), pointing to another level at which CYLD might act. In relation to these various putative substrates, CYLD has been shown to preferentially hydrolyze K63-linked polyubiquitinated chains, confirming the idea that this kind of posttranslational modification is crucial for IKK activation.

The intricate relationship between CYLD and NF-κB signaling has been further demonstrated by showing that CYLD synthesis is itself regulated by NF-κB [29]. *CYLD* mRNA induction by TNF and the Gram-negative bacteria *Haemophilus influenzae* was impaired in *ikk1* −/−, *ikk2* −/− and *nemo* − MEFs indicating that the canonical pathway of NF-κB activation, which is targeted by CYLD, was involved. Moreover, although very little is known about the regulation of CYLD catalytic activity, this DUB may represent a target of IKK [30]. Indeed, upon stimulation by TNF, LPS or phorbol 12-myristate 13-acetate (PMA)/ionomycin, which mimicks T cell receptor (TCR) signaling, CYLD is transiently phosphorylated at sites located between aa 415–445 and this modication requires NEMO. The exact function of this phosphorylation remains nevertheless unclear. In addition to its role in controlling the activation of NF-κB, CYLD also negatively regulates the c-Jun kinase (JNK) signaling pathway, a mitogen-activated protein kinase (MAPK) pathway that participates in diverse cellular programs including proliferation, differentiation and apoptosis. It has been shown that JNK activation was modulated either by overexpressing CYLD or by inhibiting its expression by RNA interference [31]. This suggests that TRAFs may indeed represent primary targets of CYLD since they are used by stimuli such as IL-1, TNF, LPS or the CD40 ligand to activate both IKK and JNK.

Analysis of CYLD functions in vivo

A series of *cyld* knockout (KO) mice has been recently engineered. They have confirmed the view that CYLD is indeed a negative regulator of NF-κB and JNK signaling pathways and allow a better understanding of what happens *in vivo* when these two pathways are up-regulated. In addition these studies have provided interesting insights into new putative functions of CYLD.

CYLD functions in immunity and infection

Mice with *cyld* KO in the whole animal are viable but exhibit several abnormalities in the immune system, according to the studies from Sun and collaborators [32–34]. For example, *cyld* −/− mice displayed lymphoid organ abnormalities and B cell hyperplasia. Mesenteric lymph nodes were enlarged whereas lymph nodes or Peyer's patches were not affected. B cell development was not modified in the bone marrow but it was in the marginal zone. Spontaneous activation of B cells was observed in *cyld* −/− mice, indicating a role for CYLD in maintaining the naive phenotype of these cells. Upon stimulation by anti-IgM, antigen exposure or LPS, B cells were hyperresponsive. These two features were correlated with increased basal and induced NF-κB activation. This dysregulation may result in constitutive CD21/CD23 expression, bypassing the normal requirement of B-cell-activating factor BAFF, an essential survival factor for B cells [33].

T cells from *cyld* −/− exhibited hyperresponsiveness to TCR stimulation. They were hyperproliferative and synthesized much more interferon γ (IFN-γ) than wild-type T cells after exposure to anti-CD3/CD28 antibodies. A constitutive activation of NF-κB and JNK was observed in both naive and memory *cyld* −/− T cells. Interestingly, TAK1 was constitutively ubiquitinated and activated in these cells. An interaction between CYLD and TAK1 was demonstrated, suggesting that this kinase may also be a target of CYLD deubiquitinase activity. The T cell dysfunction observed in *cyld* −/− mice resulted in autoimmune symptoms and colonic inflammation with features of human inflammatory bowel disease [34].

Cyld −/− mice also expressed fewer peripheral T cells than wild-type mice, due to a defect in double-positive to single-positive transition [32]. This may result from abnormal TCR signaling in the thymocyte at the level of lymphoid-restricted non-receptor tyrosine kinase (Lck)/Zeta chain-associated protein kinase of 70 kD (ZAP70) interaction. This interaction, which is negatively regulated by c-Cbl-dependent ubiquitination of Lck, appears to be under CYLD control. By binding to activated Lck, CYLD would counteract the effect of c-Cbl and favor the phosphorylation and activation of ZAP70 by this kinase.

Perturbation in NF-κB signaling was also observed in another *cyld* KO mouse strain [35]. Splenic *cyld* −/− B and T cells treated with anti-IgM or anti-CD40 and anti-CD3, respectively, exhibited elevated NF-κB stimulation. In addition, *cyld* −/− macrophages displayed receptor-specific enhancement of NF-κB and JNK activation resulting in increased cytokine production. In this latter cell type, basal TRAF2 ubiquitination appeared increased, confirming the *in vivo* targeting of this E3 by CYLD.

CYLD participation in pathogen-induced inflammation, through NF-κB regulation, has been demonstrated by Lim et al. [36]. In this study, middle ear and lung inflammation caused by exposure to non-typeable *H. influenzae* was analyzed in *cyld* −/− mice. An enhanced inflammation process, associated with leukocyte infiltration and pro-inflammatory cytokine synthesis, was observed. The perturbed signaling pathway was identified as Toll-like receptor 2 (TLR2)/myeloid differentiation primary response gene 88 (MyD88)/TRAF6/TRAF7/NF-κB, CYLD acting as a negative regulator of TRAF6 and TRAF7 ubiquitination.

An effect of CYLD on another signaling pathway, the p38 MAPK pathway, has also been uncovered through the analysis of the same *cyld* KO [37]. In this case, the authors tried to define the factors responsible for acute lung injury (ALI) and lethality following *S. pneumoniae* infection. In this system, MKK3/p38-dependent expression of plaminogen activator inhibitor-1 (PAI-1) was shown to protect the mice from *S. pneumoniae*-induced ALI. Remarkably, *cyld* −/− mice were completely protected against *S. pneumoniae* exposure and exhibited hyperactivation of p38 and PAI-1. Since pneumolysin, the virulent factor of *S. pneumoniae*, is recognized by TLR-4, a membrane receptor controlling various signaling pathway through TRAF6, including the p38 pathway, this E3 may be the target of CYLD in this specific case [38].

CYLD functions in tumorigenesis

With respect to tumor suppressor function of CYLD and tumorigenesis, two studies have been published showing that *cyld* invalidation impacts upon cell proliferation and transformation. When *cyld* KO mice described above [35] were treated by azoxymethane/dextran sulfate sodium, a combination of a DNA-alkylating agent with an inflammation-inducing product, chronic colonic inflammation was observed

associated with increased tumor induction. Most of the mice did not survive the treatment after developping broad-based adenocarcinoma in the colon. Although not directly demonstrated, this tumorigenesis process may result from enhanced NF-κB activity, since inflammation observed in this setting has been shown to be TLR dependent [39], a pathway activating NF-κB, and to be associated with up-regulation of cyclo-oxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS), two genes under NF-κB control.

In another study, the role played by CYLD in the epidermis was more specifically addressed with the hope of gaining insights into the cellular and molecular abnormalities characterizing human pathologies caused by *CYLD* mutations (see above) [40]. *Cyld* −/− mice did not exhibit abnormalities of the epidermis and did not spontaneously develop any tumors. Nevertheless, after treating their skin with 7,12-dimethylbenz(a)anthracene and 12-O-tetradecanoylphorbol-13 acetate, which provide tumor initiation and promotion, respectively, they developed papillomas with increased frequency, number and size, as compared to wild type mice. Moreover, TPA-induced proliferation of *cyld* −/− primary keratinocytes was increased. This may be due to higher induction of cyclin D1 expression, caused by NF-κB up-regulation. Interestingly, such NF-κB activation has been shown to be triggered by nuclear accumulation of Bcl3, a known co-activator of p50 and p52 subunits, suggesting that, in this setting, CYLD indirectly, rather than directly, influences NF-κB transcriptional activity [40]. Indeed, K63-linked polyubiquitination of Bcl3 may regulate its shuttling between the cytoplasm and the nucleus and would be under CYLD control. Bcl3 deubiquitination by CYLD may require a direct interaction between the two proteins.

Conditional KOs of *cyld*

Importantly, the various phenotype modifications reported above were all observed with mice lacking CYLD protein expression, owing to their normal viability. In contrast, when mice were engineered to conditionally produce a catalytically inactive version of CYLD, they died at birth, despite exhibiting no major morphological abnormalities [41, 42]. Although the cause of death is still unknown, this demonstrates that the absence of CYLD in mice can be somehow compensated by another DUB, possibly A20 that also negatively regulates NF-κB signaling [43], to control a vital function whose nature will be interesting to determine.

This function does not appear to require TRAF2 or NEMO binding, suggesting that it may not be NF-κB related. Indeed, a mouse strain ($cyld^{ex7/8}$) expressing, after Cre-driven deletion, a CYLD protein lacking the amino acid sequence encoded by exons 7 and 8, which encompasses the binding sites for both TRAF2 and NEMO, survived normally when housed under a pathogen-free environment [44]. In contrast, B cell homeostasis was shown to be severely perturbed in $cyld^{ex7/8}$ mice. In particular, a dramatic accumulation of mature B lymphocytes in all secondary lymphoid organs was observed. This accumulation was a consequence of increased survival, maybe through Bcl2 up-regulation, rather than increased proliferation. It was shown to be B cell intrinsic through the crossing with mice expressing Cre under CD19 control. At the molecular level, expression of $CYLD^{ex7/8}$ in B cells resulted in the accumulation of TRAF2, TRAF3, p100 and RelB proteins, suggesting a dysregulation of the non-canonical NF-κB signaling that may explain Bcl2 up-regulation. Importantly, the same study also showed that the deleted protein synthesized by the $cyld^{ex7/}$ mutant mouse is naturally produced by alternative splicing in wild-type animals, suggesting that this shorter version of CYLD, called sCYLD, performs some specific regulatory function *in vivo*.

CYLD functions in Drosophila

As discussed above, CYLD is an evolutionary conserved protein which has an ortholog, dCYLD, in the fly *Drosophila melanogaster*. Interestingly, a pathway similar to the mammalian NF-κB pathway, the imd pathway, also exists in this organism and plays a critical role in innate immune defense [45]. A recent study has analyzed the function of dCYLD and tested the consequence of its loss on fly immunity [46]. It has been demonstrated that dCYLD was able to interact with kenny, the fly homolog of NEMO. In addition, expression of the antimicrobial peptide diptericin following infection with bacteria appeared defective in a mutant fly strain exhibiting a deletion of *dCYLD*, resulting in increased lethality. More unexpectedly, the same strain displayed abnormal fat body morphology in the adult, accumulation of triglycerides and increased resistance to food starvation. An analogous participation of CYLD in the regulation of fat storage/metabolism remains to be demonstrated in mammals. An independent study revealed additional functions of dCYLD *in vivo* [47]. Flies with *dCYLD* deletion (*dCYLD* −/−) exhibited a shortened lifespan, which was associated with impaired oxidative stress tolerance. Importantly, oxidative stress tolerance in *D. melanogaster* has been shown to be controled by Basket (Bsk), the fly ortholog of JNK. Overexpression of Bsk in *dCYLD* −/− rescued both lifespan and

oxydative stress resistance defects, suggesting a role of dCYLD in the *Drosophila* JNK pathway. This role was further demonstrated by showing participation of dCYLD catalytic activity in Eiger (Egr)-induced cell death. Egr, a TNF-related protein, induces cell death in the developping *Drosophila* eye by interacting with its receptor Wengen (Wgn) and activating dTAK1, the kinase regulating Bsk, through dTRAF2, a TRAF6 ortholog. In *dCYLD* −/− flies, Egr-induced apoptosis was impaired, indicating an unexpected positive role of dCYLD in JNK activation. In the Egr pathway, dCYLD would stabilize dTRAF2 by diminishing its ubiquitination-dependent degradation, suggesting that CYLD, or at least dCYLD, may also control some proteasome-dependent events.

Other putative targets and functions of CYLD

Studies summarized in the previous section have confirmed the role played by CYLD in controlling the activation of NF-κB and MAPK signaling pathways. They have also suggested that this DUB may regulate the ubiquitination state of proteins such as Lck and Bcl3. The extent to which CYLD may impact upon global K63-linked polyubiquitination in the cell remains unknown, but recent publications indicate that its targets are likely to be more numerous than originally suspected.

First, CYLD may regulate the trafficking and activity of cell surface receptors. A study aimed at defining the molecular events triggered by nerve growth factor (NGF) after binding to its receptor, tropomyosin-related kinase A (TrkA), has revealed that TrkA was a substrate for TRAF6-dependent ubiquitination and that this modification was required for receptor internalization and signaling [48]. Moreover, since K63-linked polyubiquitination was detected, the authors looked for a putative participation of CYLD and found that this enzyme was indeed able to deubiquitinate TrkA and to inhibit its internalization.

CYLD may also target components of the cell cycle machinery. Two whole-scale searches for proteins controlling the cell cycle, through the use of shRNA libraries, have identified CYLD as a positive regulator of entry into mitosis [49, 50]. This effect was independent of the NF-κB signaling pathway and appeared to involve binding to polo-like kinase-1 (Plk-1), which is degraded as cells exit from mitosis, thereby helping to reset the cell cycle from the mitotic to the G1 state [51]. Importantly, the catalytic activity of CYLD was shown to be necessary for restoring entry into mitosis, although the targeted ubiquitination event(s) was (were) not identified.

Concluding remarks

The wide range of processes that are controlled by degradative and non-degradative ubiquitination needs to be tightly regulated. One class of proteins that directly participates in this fine-tuning is the DUB family of enzymes. Unfortunately, very little is still known regarding their specific targets, the precise kind of ubiquitination they recognize and the way they are themselves regulated. CYLD provides one of the first examples of the prominent role that DUBs play *in vivo* and demonstrates, through the genetic diseases that result from its mutations, how their deregulation may have devastating effects.

One of the main targets of CYLD is likely to be the NF-κB signaling pathway which regulates diverse key physiological processes. Remarkably, activation of NF-κB heavily depends on ubiquitination, both degradative (the degradation of the IκB inhibitor) and non-degradative (the activation of IKK). CYLD appears to negatively regulate this latter type of ubiquitination and, most likely, helps keep in check an activation pathway that, upon up-regulation, ends up with disturbed immune response/autoimmunity, chronic inflammation or tumor development. It remains, nevertheless, important to understand fully how CYLD exactly operates. For example, although *CYLD* is itself a target gene of NF-κB it is unlikely to act posttranscriptionally since CYLD protein is already present in cells before stimulation. Therefore, it will be crucial to determine whether the catalytic and/or binding activity of CYLD is regulated and, if it is, how it is turned on and off upon cell stimulation. Moreover, defining whether CYLD represents a general negative regulator of NF-κB activation or only controls a subset of pathways that activate this transcription factor remains another important issue, especially considering the very restricted phenotype associated with cylindromatosis. Nevertheless, given the other putative functions of CYLD, the hypothesis that cylindroma development is solely caused by NF-κB dysregulation needs to be carefully re-evaluated. In particular, a defective JNK signaling pathway or perturbed mitosis entry may also contribute to the tumorigenesis process, although in the latter case, CYLD lack-of-function mutations should stop cell proliferation rather than enhance it.

Understanding how CYLD mutations impact on cylindroma formation should benefit from the analysis of mice modeling the disease. Unfortunately, the complete KO of *cyld* in the mouse does not result in spontaneous tumor development. Moreover, when the skin is treated with pro-carcinogenic agents, tumors that occur do not resemble cylindromas. This may indicate that mice are either unable to develop a

'cylindromatosis-like' pathology, due to differences between their skin and human skin, or will only develop it after invalidation in the adequate cell type/compartment. Conditional *cyld* KO mice may therefore be instrumental in solving this question.

Finally, it will be crucial to determine whether the tumor suppressive effect of CYLD is operant in cell types or tissues distinct from those affected in cylindromatosis or MT. A disparate collection of studies suggests that this may indeed be the case. For example, an analysis of various classes of tumors originating from skin adnexa, including apocrine hydrocystomas, eccrine spiradenomas and sebaceous adenoma, have identified LOH at the *CYLD* locus [52]. CYLD expression has also been shown to be down-regulated in liver, kidney and uterus tumors [53–55]. In addition, reintroduction of BRG1-associated factor of 57 kDa (BAF57), a subunit of hSWI/SNF chromatin-remodeling complexes, in the transformed breast cancer cell line BT549, down-regulated its proliferation and induced its apoptosis. Among the transcriptional targets of BAF57 was CYLD, which was able by itself to induce BT549 apoptosis and appeared to be required for BAF57-induced cell death [56]. Last, but not least, two independent studies aimed at analyzing genetic alterations affecting components of the NF-κB pathway in multiple myeloma (MM) have identified on several occasions *CYLD* inactivation, through deletion or mutation [57, 58]. This observation is in accordance with a report claiming that LOH at the *CYLD* locus may be a useful criterion in determining clinal outcome in MM [59].

In conclusion, a growing body of evidence suggests that CYLD deubiquitinase represents a new kind of tumor suppressor that acts not only by negatively regulating the NF-κB activation pathway but also controls other processes that may impact upon cell proliferation and survival, all events sharing a requirement for K63-linked polyubiquitination. Deciphering the various functions of CYLD will be challenging but should help for a full understanding of how its dysregulation results in inflammation and tumorigenesis, consequently suggesting new therapeutic avenues to treat CYLD-associated pathologies.

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