CYLD Alterations in the Tumorigenesis and Progression of Human Papillomavirus–Associated Head and Neck Cancers

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ABSTRACT

Genetic alterations of *CYLD lysine 63 deubiquitinase* (*CYLD*), a tumor-suppressor gene encoding a deubiquitinase (DUB) enzyme, are associated with the formation of tumors in CYLD cutaneous syndrome. Genome sequencing efforts have revealed somatic *CYLD* alterations in multiple human cancers. Moreover, in cancers commonly associated with human papillomavirus (HPV) infection (e.g., head and neck squamous cell carcinoma), *CYLD* alterations are preferentially observed in the HPV-positive versus HPV-negative form of the disease. The CYLD enzyme cleaves K63-linked polyubiquitin from substrate proteins, resulting in the disassembly of key protein complexes and the inactivation of growth-promoting signaling pathways, including pathways mediated by NF-κB, Wnt/β-catenin, and c-Jun N-terminal kinases. Loss-of-function *CYLD* alterations lead to aberrant activation of these signaling pathways, promoting tumorigenesis and malignant transformation. This review summarizes the association and potential role of *CYLD* somatic mutations in HPV-positive cancers, with particular emphasis on the role of these alterations in tumorigenesis, invasion, and metastasis. Potential therapeutic strategies for patients whose tumors harbor *CYLD* alterations are also discussed.

Implications: Alterations in *CYLD* gene are associated with HPV-associated cancers, contribute to NF-κB activation, and are implicated in invasion and metastasis.

Introduction

Cylindromatosis gene

CYLD lysine 63 deubiquitinase (*CYLD*), the cylindromatosis gene, was first discovered by Biggs and colleagues during their identification of causative genes of familial cylindromatosis, an autosomal-dominant hereditary disease (1, 2). Cylindromatosis is characterized by multiple skin appendage tumors, called cylindromas, that are clinically benign and occur primarily on the head and neck. Three distinct tumor syndromes, namely, familial cylindromatosis [Online Mendelian Inheritance in Man (OMIM) 132700], multiple familial trichoepitheliomas (OMIM 601606), and Brooke–Spiegler syndrome (OMIM 605041), comprise the condition referred to as CYLD cutaneous syndrome (hereafter, CYLD syndrome; ref. 3). *CYLD* is the only tumor suppressor gene (TSG) whose loss or mutation has been found to be associated with CYLD syndrome (4). Using polymorphic microsatellite markers and linkage analysis of two families with familial cylindromatosis, Biggs and colleagues first mapped germline alteration of the *CYLD* locus to chromosome 16q12-q13, with LOH of the *CYLD* locus from the unaffected parent, suggesting *CYLD* as a TSG (1, 2, 5). Tumors associated with CYLD syndrome are typically benign, although approximately 5% to 10% of tumors in cases of Brooke–Spiegler syndrome are reported to be malignant (6). Emerging evidence implicates somatic *CYLD* alterations in multiple forms of cancer, including cancers of the head and neck, uterus, stomach, colon, and lung. In this review, we describe the characteristics and impact of somatic *CYLD* alterations in cancer, with particular emphasis on the role of these alterations in invasion, metastasis, and progression of human papillomavirus (HPV)–associated cancers. Finally, opportunities for therapeutic intervention in patients with cancer harboring *CYLD* alterations will be discussed.

Functions of CYLD

The *CYLD* gene (Gene ID: 1540) encodes a 956-amino acid protein consisting of three functional domains: (i) three N-terminal cytoskeleton–associated protein-glycine-rich (CAP-Gly) domains, (ii) two conserved proline-rich motifs located between the second and third CAP-Gly domains, and (iii) a C-terminal catalytic ubiquitin-specific protease domain (**Fig. 1**; refs. 3, 7). The first two CAP-Gly domains bind to tubulin and/or microtubules, ensuring the stability and polymerization of microtubules that are essential for cell activities like migration (8). The third CAP-Gly domain of CYLD has been shown to bind directly to the proline-rich sequence of NF-κB essential modulator (NEMO; an IκB kinase adaptor protein; ref. 9). Biochemical assays have determined that CYLD is a deubiquitinase (DUB) enzyme, which cleaves ubiquitin from substrates including NEMO and tumor necrosis factor–associated factor 2 (TRAF2; refs. 10, 11).

Ubiquitination and deubiquitination are reversible posttranslational modifications that modify substrate proteins and are important for cellular processes including proliferation and survival (12). DUB enzymes reverse the process of ubiquitination, which occurs via conjugation of the 76-amino acid ubiquitin (Ub) peptide to substrate proteins. Monoubiquitination involves covalent linkage of the C-terminal glycine residue (G76) of Ub to a lysine (K) on the substrate protein. During polyubiquitination, the G76 of free Ub moieties are covalently linked to lysine residues on already attached Ub moieties. Ub contains seven lysine residues (K6, K11, K27, K29, K33, K48, and K63), and polyubiquitination can occur at any of these residues. K48- and K63-polyubiquitination have been the most extensively studied, whereas a paucity of reagents has limited investigation and understanding of linkages occurring via K6, K11, K27, K29, and

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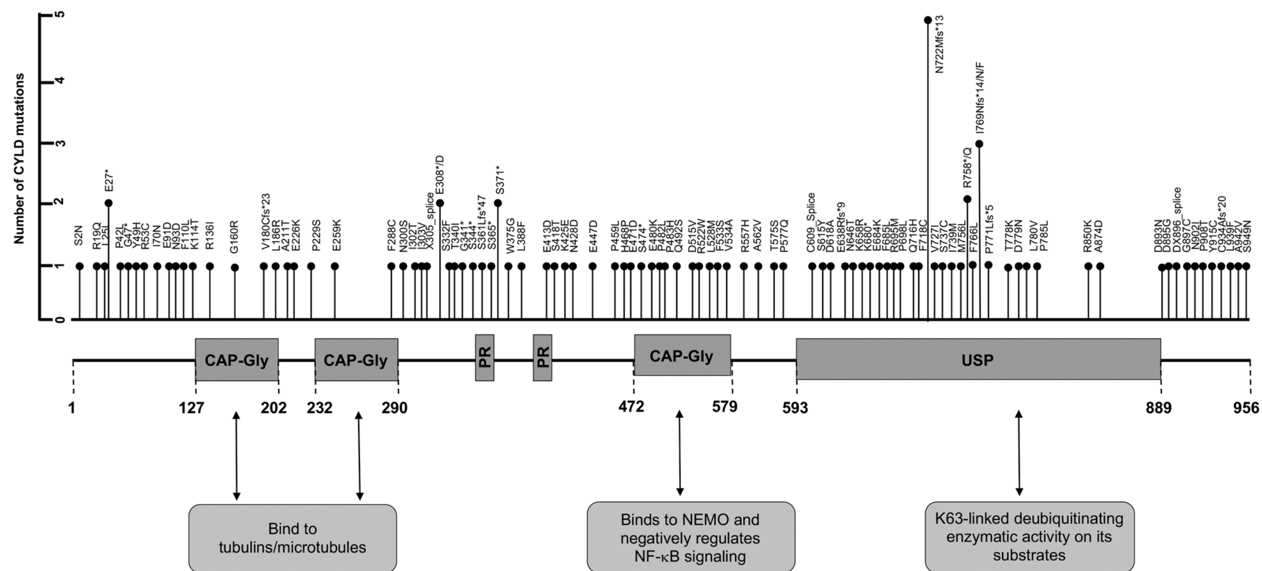

Figure 1.

Functional domains of CYLD WT protein. The first two N-terminal CAP-Gly domains function to bind to tubulin and/or microtubules, ensuring the stability and polymerization of microtubules that are essential for cell activities like migration. The third CAP-Gly domain of CYLD binds directly to NEMO, enabling the recruitment of CYLD to components of the NF-κB signaling pathway. The C-terminal ubiquitin-specific protease domain enables CYLD to deconjugate K63-linked ubiquitin chains from its substrate proteins. As described by TCGA, the figure indicates the location of mutations found in the 5 cancers with the most frequent occurrence of CYLD mutations (uterine corpus endometrial carcinoma, stomach adenocarcinoma, skin melanoma, HNSCC, colorectal adenocarcinoma). The vertical axis indicates the number of different tumors in TCGA (above 5 cancers) with the indicated specific mutation.

K33. Many DUB enzymes, including several associated with the proteasome, act to unlink bonds involving K48 conjugation (13). By contrast, CYLD deconjugates K63-linked ubiquitin chains (14). In so doing, CYLD disrupts key protein–protein interactions that are important for the growth-promoting activities of signaling pathways including the NF-κB, Wnt/β-catenin, and c-Jun N-terminal kinase (JNK) pathways (15–17).

The involvement of CYLD in the regulation of NF-κB signaling has been rigorously defined. The five known members of the NF-κB protein family (p65, RelB, c-Rel, p105/p50, and p100/p52) exist as heterodimers or homodimers in the cytoplasm and are bound to inhibitory proteins called IκBs (**Fig. 2A**; refs. 18, 19). Release from IκBs and signaling via NF-κB are initiated by a variety of ligands that engage cell surface receptors such as Toll-like receptors (TLR), tumor necrosis factor receptors (TNFR) and interleukin-1 receptor (1L1R; ref. 20). Following stimulation of these receptors, IκBs are phosphorylated by IκB kinase complex (IKK complex), comprised of NEMO, IKKα, and IKKβ, resulting in the degradation of the inhibitor IκB (**Fig. 2A**). With the degradation of IκB, NF-κB dimers (p65/p50 being the most abundant in most cell types) are released and translocate to the nucleus, where they bind to the promoters of target genes inducing their transcription (18, 21). K63-linked polyubiquitination is crucial to the process of phosphorylation and degradation of IκBs by IKK complex. For example, TNF binding to its receptors allows recruitment of the proteins TNFR-associated death domain (TRADD), receptor-interacting protein 1 (RIP1), and the E3 ligases TRAF2, TRAF5, and cellular inhibitor of apoptosis 1/2 (cIAP1/2; **Fig. 2B**; ref. 22). TRADD and RIP1 undergo K63-linked polyubiquitination by TRAF2, TRAF5, and cIAP1/2 (**Fig. 2C**), and provide a molecular scaffold to recruit downstream complexes such as TAK1 kinase complex (comprised of TAK1, TAB2, and TAB3) and IKK complex (comprised of NEMO, IKKα, and IKKβ; **Fig. 2D**; refs. 7, 23). The recruitment of these complexes occurs via binding of the polyubiquitin chains on RIP1 to the ubiquitin-binding adaptors TAB2/TAB3 and NEMO, respectively, and facilitates TAK1 kinase–mediated phosphorylation and activation of IKKβ. Activation of IKKβ and the IKK complex results in the phosphorylation and degradation of IκB, and the resulting activation of NF-κB (**Fig. 2E**). As a negative regulator, wild-type (WT) CYLD DUB enzyme acts to hydrolyze K63-linked polyubiquitin chains on TRADD, RIP1, TAK1, and NEMO, resulting in disassembly of the aforementioned complexes and the inactivation of NF-κB signaling (**Fig. 3**; refs. 7, 23).

In addition to activation of NF-κB via K63-linked polyubiquitination, the process of M1-linked polyubiquitination also plays a key role in NF-κB activation. In M1-linked ubiquitination, the C-terminal glycine residue of a free ubiquitin monomer is conjugated to the N-terminal methionine (M1) of a substrate-attached ubiquitin in a head-to-tail linear fashion (24). Linear ubiquitin chain assembly complex (LUBAC), an E3 ligase complex, functions to conjugate M1-linked polyubiquitin chains onto lysine residues of substrate proteins, including TRADD, RIP1, and NEMO (25–27), or more commonly, onto the pre-existing K63-linked polyubiquitin chain, generating hybrid K63/M1 chains (**Fig. 2C**; ref. 28). The K63-linked and K63/M1-linked polyubiquitin chains of TRADD and RIP1 facilitate recruitment and activation of the TAK1 and IKK complexes, ultimately promoting activation of NF-κB (**Fig. 2D** and **E**; ref. 29). CYLD has the capacity to cleave both K63-linked and K63/M1-linked polyubiquitin chains attached to TRADD and RIP1, disrupting formation of complexes in this signaling pathway and downmodulating NF-κB activation (**Fig. 3**; refs. 7, 27). OTU deubiquitinase with linear linkage specificity (OTULIN), an alternative DUB, removes M1-linked polyubiquitin chains from NEMO, similarly promoting NF-κB downmodulation (**Fig. 3**; refs. 7, 30).

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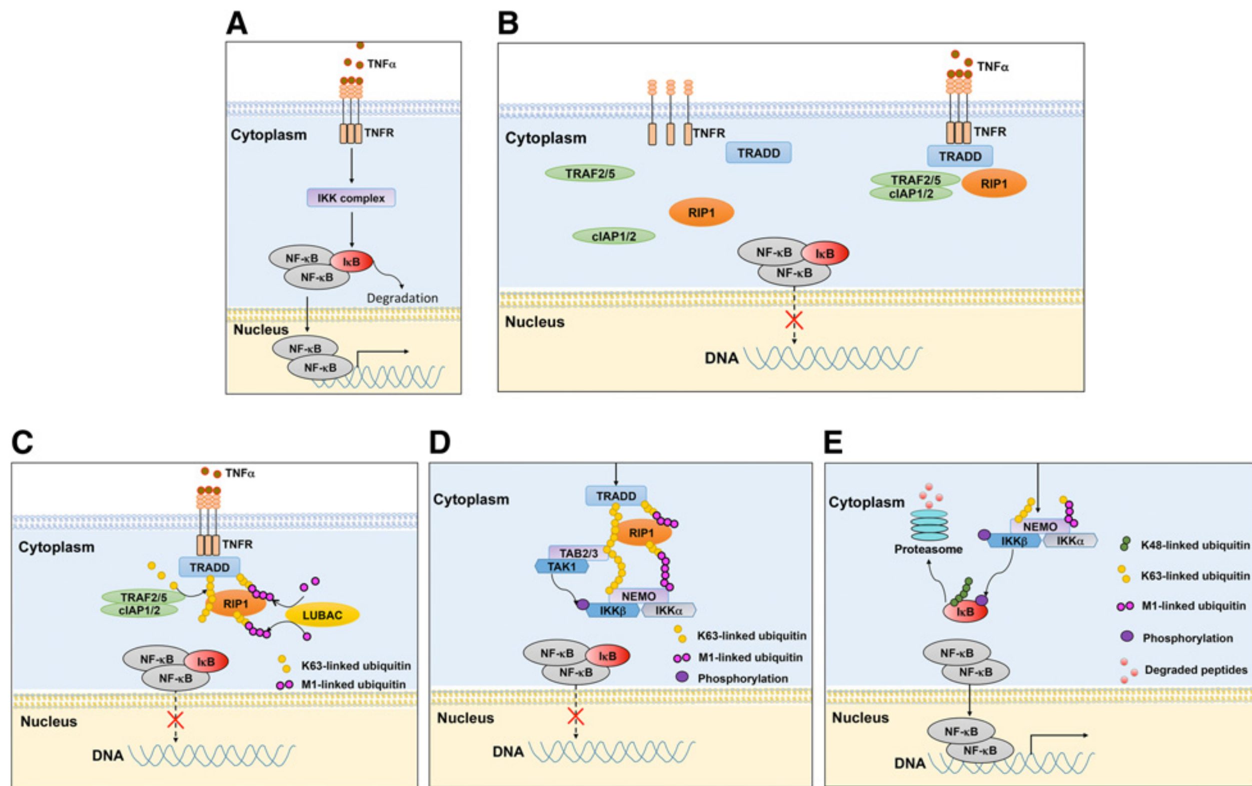

Figure 2.
Ubiquitination is critical to the activation of NF-κB signaling. The initiation of NF-κB signaling is triggered by ligand binding to cognate cell surface receptors (TLRs, TNFR, 1L1R). **A,** The NF-κB protein family (p65, RelB, c-Rel, p105/p50, and p100/p52) exist as heterodimers or homodimers and are retained in the cytoplasm by binding to IκBs. Following ligand stimulation, IKK complexes are recruited and activated, leading to the degradation of IκBs. NF-κB dimers are released and translocate to nucleus and bind to consensus DNA sequences to activate transcription of NF-κB target genes. **B,** Ligand binding results in the recruitment of TRADD, RIP1, and the E3 ligases TRAF2, TRAF5, and cIAP1/2. **C,** TRADD and RIP1 undergo K63-linked polyubiquitination by TRAF2, TRAF5, and cIAP1/2. LUBAC promotes M1-linked and K63/M1-linked polyubiquitination of TRADD, RIP1, and NEMO. **D,** TAK1 kinase complex (TAK1, TAB2, and TAB3) and IKK complex (NEMO, IKKα, and IKKβ) are recruited via binding of the polyubiquitin chains on RIP1 to the ubiquitin binding adaptors TAB2/TAB3 and NEMO, respectively. Recruitment of the complexes leads to TAK1 kinase–mediated phosphorylation and activation of IKKβ. **E,** IκBs are phosphorylated by IKKβ and undergo proteasome-mediated degradation, resulting in the release and activation of NF-κB dimers.

Regulation of CYLD expression and activity

CYLD is constitutively expressed in most normal tissues (1), and the expression and catalytic activity of CYLD are tightly controlled. During inflammation, cytokines (e.g., TNFα and IL1β) and bacterial pathogens induce the upregulation of *CYLD* mRNA and protein, and this transcriptional induction is dependent on activation of NF-κB signaling (31), revealing a negative feedback loop of CYLD/NF-κB signaling regulation. Notably, the transcriptional repressor SNAIL1, which triggers epithelial–mesenchymal transition (EMT) and is a marker for cancer cell malignancy, is recruited to the *CYLD* promoter and downregulates its expression in melanoma (32). In addition, miRNAs were recently reported to bind to the 3′ untranslated region of *CYLD,* silencing CYLD expression in multiple types of cancer (33–38).

A recent study has shown that genetic loss of suppressor with morphogenetic effect on genitalia (SMG7) correlates with loss of CYLD expression in human cancer cell lines and renal carcinoma tumors (39). SMG7 plays a role in the degradation of aberrant cellular RNAs. Loss of SMG7 leads to dysregulated expression of specific long noncoding RNAs and corresponding decreased expression of CYLD (39). Cells with loss of SMG7 exhibit heightened NF-κB activation following treatment with TNFα.

At the posttranslational level, the IKK complex has been shown to mediate phosphorylation at serine 418 of CYLD (although this remains controversial; ref. 40), downmodulating its DUB activity following treatment with TNFα (41). Phosphorylation of CYLD on tyrosine 15 occurs following stimulation of the EGFR, a key target for anticancer therapy in head and neck squamous cell carcinoma (HNSCC; ref. 42). Tyrosine phosphorylated CYLD recruits the ubiquitin ligase Cbl-b, facilitating close proximity of Cbl-b to EGFR that results in ubiquitination and proteasomal degradation of EGFR (42). Thus, loss of CYLD function would be expected to release EGFR from this negative regulatory mechanism. Interestingly, downregulation of CYLD has been reported in pulmonary adenocarcinoma cells with resistance to the EGFR tyrosine kinase inhibitor gefitinib (43).

CYLD Somatic Alterations in Sporadic Cancer

Somatic CYLD alterations in cancer

The Cancer Genome Atlas (TCGA) contains data on somatic genetic alterations from approximately 11,000 tumors in 32 different types of cancer (44, 45). Alterations in *CYLD* were detected in 1.4% of

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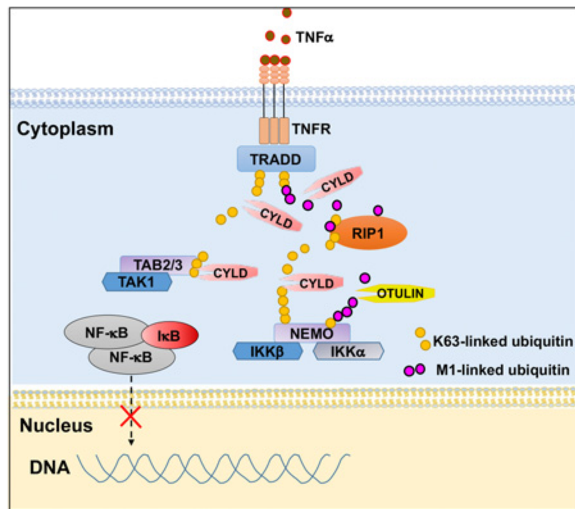

Figure 3.

CYLD negatively regulates NF-κB signaling. The deubiquitinases CYLD and OTULIN cleave K63-linked, M1-linked, and K63/M1-linked polyubiquitin chains on substrate proteins, resulting in the dissembling of the TAK1 and IKK complexes and the inactivation of NF-κB signaling. The reversible processes of polyubiquitination and deubiquitination tightly regulates the activation of NF-κB signaling.

all tumors, with the highest degree of alteration detected in uterine corpus endometrial carcinoma (6%), stomach adenocarcinoma (3.4%), skin melanoma (3.4%), HNSCC (2.9%), colorectal adenocarcinoma (2.8%), lung squamous cell carcinoma (2.7%), thymoma (2.4%), and esophageal adenocarcinoma (2.2%; **Fig. 1**). Other types of cancer exhibit *CYLD* alterations at a frequency lower than 2%. *CYLD* mutations in the TCGA tumors do not exhibit a "hotspot" pattern of mutation. Instead they are widely dispersed throughout all domains of the protein, consistent with a TSG phenotype. Roughly 75% of *CYLD* mutations reported in the TCGA are missense mutations, with the remainder primarily nonsense truncation mutations. Two novel *CYLD* gene fusions, with the *UNGP1* and *FAM49B* genes, have also been detected.

Based on reported correlations of *CYLD* gene deficiency and tumorigenesis, as well as analyses using databases such as OncoKB (17, 46, 47), all *CYLD* nonsense mutations in the TCGA are predicted to sensitize cells to transformation. For example, CYLD-S371*, which has been reported as a germline mutation contributing to the development of hereditary CYLD syndrome (1, 48–54), has also been detected in HNSCC, thymoma, and uterine carcinoma (TCGA PanCancer Atlas). However, little is known about the functions of missense *CYLD* mutations, which comprise the majority of *CYLD* alterations reported in TCGA. Whether these mutations play important roles in promoting tumor development, or are simply passenger mutations, merits further investigation.

In addition to mutations of the *CYLD* gene, copy-number variation also commonly occurs. Analysis of copy-number alterations in 149 HPV-positive and 335 HPV-negative HNSCC tumors revealed more frequent deletion of *CYLD* in HPV-positive (23.5%) versus HPV-negative (5.1%) HNSCC (55). RNA-sequencing analyses supported this finding. In addition, 25% of HPV-positive HNSCC tumors were found to contain both mutations and copy-number variation of *CYLD* (55).

CYLD mutation and HPV-associated cancers

CYLD alterations have been reported to be associated with HPV-related cancers (**Table 1**), particularly HPV-positive HNSCC (11, 55–59). Persistent infection with the high-risk HPV subtypes HPV-16 and HPV-18 is a leading etiologic cause of HNSCC (primarily oropharyngeal cancers), as well as cervical and anal cancers (60, 61). HPV-positive HNSCC is a distinct disease entity from HPV-negative HNSCC, and typically carries a more favorable clinical prognosis (62). The precise role of *CYLD* gene alterations in HPV-associated cancers is an area of active investigation.

Among 515 HNSCC tumors reported in the TCGA, 2.9% harbor *CYLD* mutations, which include 8 truncation mutations, 6 missense mutations, and 1 gene fusion with *UNGP1*. HPV status has been annotated in 487 of the 515 tumors; 72 being HPV-positive and 415 being HPV-negative. Alterations in *CYLD* were more common in HPV-positive HNSCC, with 8 *CYLD* alterations among the 72 HPV-positive tumors (11%), and only 7 *CYLD* alterations among the 415 HPV-negative tumors (1.7%; **Table 1**). Chi-square statistical analysis indicates that the association with HPV-positive HNSCC versus HPV-negative HNSCC is highly significant ($P = 0.0004$). Both HPV-positive and HPV-negative HNSCC tumors harbor truncation and missense mutations, whereas the lone fusion gene (*CYLD-UNGP1*) was found in an HPV-positive tumor sample.

Studies of additional HNSCC cohorts have confirmed an enrichment of *CYLD* alterations in HPV-positive HNSCC (**Table 1**). Gillison and colleagues analyzed a cohort of 149 HPV-positive HNSCCs and 335 HPV-negative HNSCCs and observed *CYLD* alterations in 7.4% of HPV-positive tumors, but only 0.6% of HPV-negative tumors (55). Seiwert and colleagues analyzed a cohort of 120 HNSCC tumors including 51 HPV-positive cases, and found a higher incidence of *CYLD* mutations in HPV-positive (6%) compared with HPV-negative (1%) cancers (63).

In the TCGA, 3 cases of *CYLD* mutation were observed among 278 cervical squamous cell carcinomas, a cancer that is strongly associated with HPV infection. In addition, Hirai and colleagues established two HPV-18–positive cell lines derived from uterine cervical carcinoma (glassy cell carcinoma), both of which demonstrate copy-number loss of *CYLD* locus at chromosome 16q12–13, suggesting

Table 1. Association of *CYLD* mutations with HPV-positive cancer.

Cohort	HPV (+)	HPV (−)	HPV (+) with *CYLD* mutations	HPV (−) with *CYLD* mutations
487 HNSCC (TCGA)	72 (14.8%)	415 (85.2%)	8 (11.1%)	7 (1.7%)
484 HNSCC [Gillison et al. (55)]	149 (30.8%)	335 (69.2%)	11 (7.4%)	2 (0.6%)
120 HNSCC [Seiwert et al. (63)]	51 (42.5%)	69 (57.5%)	3 (5.8%)	1 (1.4%)
574 anal cancers [Williams et al. (64)]	515 (89.7%)	59 (10.3%)	75 (14.5%)	0 (0%)

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loss of function of CYLD may associate with a subset of HPV-positive cervical cancers (56). In addition, the GENIE cohort study determined that *CYLD* is mutated in 10% of patients (8 of 83) with anal cancer, an HPV-associated cancer. Analysis of another cohort of 574 patients with anal cancer revealed 13% of the cases (75 cases) harbored a *CYLD* mutation (64). Seventy of the 75 cases with *CYLD* mutation also had detectable HPV-16 (**Table 1**; ref. 64). In both of these anal cancer cohorts, the majority of the *CYLD* alterations were found to be truncating mutations (75% and 67%, respectively).

Potential Roles of *CYLD* Alterations in HPV-Positive Cancers

Role of CYLD in NF-κB signaling in HPV-positive cancers

The enrichment of *CYLD* alterations in HPV-positive HNSCC suggests a role for these alterations in the development and progression of HPV-positive tumors. Recent evidence indicates that HPV proteins and *CYLD* alterations work in concert to promote tumor growth via effects on the NF-κB signaling pathway.

Aberrant activation of NF-κB signaling is known to promote carcinogenesis via upregulation of several types of NF-κB target genes, including those encoding proinflammatory cytokines (i.e., IL1, IL6, IL12, IL23, IL33) that foster a protumor microenvironment, antiapoptotic proteins (FLIP, c-IAP1/2, and XIAP) that facilitate the evasion of cancer cell death and cell-cycle regulators (cyclins D1, D2, D3, and E, and c-MYC), and proteins that modulate invasiveness (ICAM-1, E-selectin, matrix metalloproteinases, and VEGF; refs. 65–67).

Activation of NF-κB signaling by the HPV E6 oncoprotein has been reported. In cervical keratinocytes, HPV E6 was found to upregulate expression of the p50 NF-κB isoform and increase the DNA-binding capacity of NF-κB (68). Another group has reported that E6-mediated activation of NF-κB in cervical cancer cells occurs via a pathway involving the GTPase Rac1 (69). In yet another study, E6 protein enhanced the nuclear binding activity of p52-containing NF-κB complexes, resulting in upregulation of NF-κB target genes that protected cells from TNF-induced apoptosis (70). Despite these findings, the mechanisms whereby NF-κB signaling is activated by E6, and how this interplays with HPV viral infection, integration, and replication to drive cell transformation remain incompletely understood.

Efficient activation of the NF-κB signaling pathway by HPV may require, or be enhanced by, genetic deletion or functional inactivation of *CYLD*. As described above, the higher prevalence of *CYLD* alterations in HPV-associated cancer suggests that selection for genetic loss or mutation of the *CYLD* gene likely occurs during HPV-mediated cellular transformation. An and colleagues have provided evidence of E6-mediated loss of CYLD, resulting in NF-κB activation in hypoxia-exposed HPV-positive cells (HeLa and SiHa; ref. 57). They found that E6, which binds E6-associated protein (an E3 ubiquitin ligase), promoted K48-linked ubiquitination of CYLD, leading to proteasomal degradation of the CYLD protein. Loss of CYLD prevented deubiquitination of K63-linked TRAF proteins and enabled TRAF-mediated ubiquitination and activation of the IKK complex, with subsequent activation of NF-κB (**Fig. 4**; ref. 57). It should be noted that E6-mediated degradation of CYLD was specific to cells exposed to prolonged hypoxia, where the physical interaction of E6 and *CYLD* might be stabilized through posttranslational modifications (57).

Other possible interactions of CYLD and HPV

As discussed above, loss of CYLD expression or function is important for NF-κB activation in HPV-positive cancers. Genome sequencing data on HPV-positive cancers have revealed that genetic alterations of other ubiquitin modifying proteins may also play a role in HPV-positive cancers. Data from the TCGA show that the TNF receptor-associated factor 3 (*TRAF3*) gene is exclusively mutated in HPV-positive HNSCC versus HPV-negative HNSCC (71). TRAF3 encodes a ubiquitin ligase that negatively regulates alternative pathways of NF-κB activation (72). Similar to *CYLD* alterations, loss of *TRAF3* facilitates the activation of NF-κB in HPV-positive cancers (73). Hajek and colleagues found mutually exclusive inactivating mutations of *TRAF3* (25%) and *CYLD* (11%) in HPV-positive HNSCC tumors in both the TCGA and a different patient cohort (58, 59). In their studies, 54% of the HPV-positive tumors that had *TRAF3 or CYLD* alterations had no evidence of HPV integration, suggesting a selective advantage for *TRAF3*/*CYLD* deficiencies in tumors harboring episomal HPV (58). Surprisingly, further analysis of 34 HPV-positive tumors indicated that *TRAF3/CYLD* alterations correlated with better overall survival (58). These findings are at odds with other reports demonstrating that loss of CYLD function contributes to invasion and metastasis, and further studies are needed to resolve this discrepancy.

Another unique difference between HPV-negative and HPV-positive HNSCC is the mutational status of the *TP53* TSG. The vast majority of HPV-negative HNSCC tumors harbor mutated TP53. By contrast the majority of HPV-positive HNSCC tumors harbor WT *TP53*, although the p53 protein is degraded via HPV E6–dependent ubiquitination/proteasomal degradation. In the TCGA, all HPV-positive HNSCC tumors with *CYLD* alterations also contain WT *TP53*. Fernandez-Majada and colleagues (74) have demonstrated that CYLD can deubiquitinate p53, attenuating proteasomal degradation of p53. Hence, loss of CYLD function in HPV-positive cells likely leads to reduced expression and tumor-suppressor activity of p53.

Activation of PI3K/AKT/mTOR1 signaling plays an important role in HPV viral replication and may work in concert with *CYLD* alterations to drive HPV-mediated tumorigenesis. Phosphorylation and activation of AKT usually require activation of the catalytic subunit of PI3K, encoded by the *PIK3CA* gene (75). In addition, K63-linked ubiquitination of AKT by the E3 ligases TRAF6 or Skp1–Cul1–F-box-protein (SCF) can promote activation of AKT in response to treatment with growth factors such as insulin-like growth factor-1 or EGF (76, 77). CYLD-mediated deubiquitination of K63-ubiquitinated AKT serves to regulate and attenuate AKT activity. When CYLD is mutated or lost, aberrant hyperactivation of AKT may occur (76). Oncogenic activating mutations in *PIK3CA*, which result in activation of AKT, have been shown to occur with higher frequency in HPV-positive versus HPV-negative HNSCC (55). Intriguingly, *PIK3CA* and *CYLD* mutations are largely mutually exclusive in HPV-positive HNSCC tumors in the TCGA. Hence, aberrant activation of the PI3K/AKT/mTOR signaling pathway in HPV-positive HNSCCs may occur via either *PIK3CA* oncogenic mutation or inactivating alterations of *CYLD*.

Role of *CYLD* Alterations in Tumorigenesis

CYLD loss of function promotes tumorigenesis

Emerging evidence from mouse models indicates that deletion of *CYLD* promotes tumorigenesis. Mice with homozygous deletion of CYLD ($CYLD^{-/-}$) developed larger and more papillomas than were seen in WT mice following treatment with the chemical carcinogens 7,12-dimethylbenz[a]anthracene (DMBA) and 12-O-tetradecanoyl-phorbol-13-acetate (TPA; ref. 78). Histologic examination revealed

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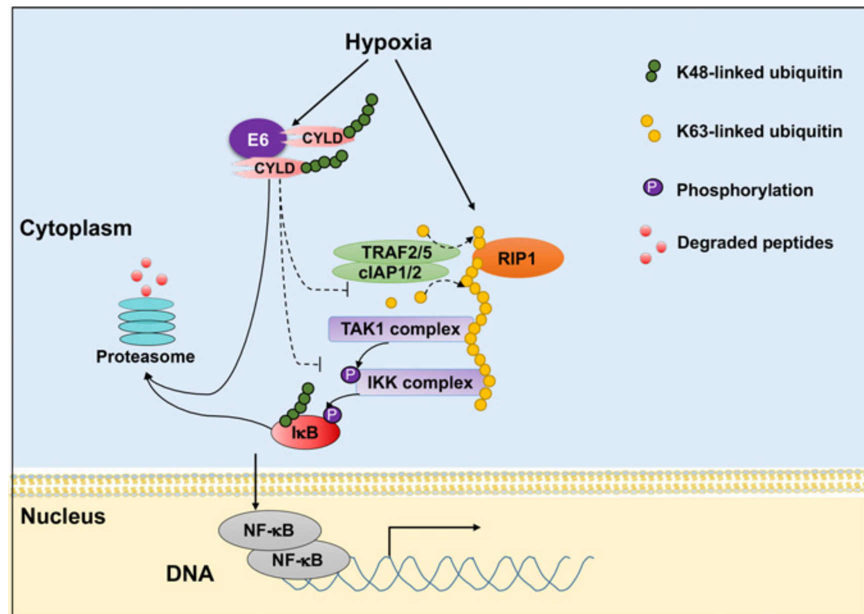

Figure 4.
HPV-16 E6 degrades CYLD to activate NF-κB signaling. When HPV-positive cancer cells are exposed to prolonged hypoxia, E6 promotes K48-linked ubiquitination of CYLD, leading to CYLD degradation by the proteasome. Reduced expression of CYLD results in reduced deubiquitination of K63-linked TRAF proteins, enabling TRAF-mediated ubiquitination and activation of the IKK complex and NF-κB signaling (109, 110).

that the papillomas were composed of hyperplastic squamous epithelium without signs of malignancy (78). In another study, $CYLD^{-/-}$ mice exhibited chronic colonic inflammation and colon tumor formation following treatment with azoxymethane and dextran sulfate sodium (79). These findings demonstrate that *CYLD* plays an important role in suppressing chemically induced neoplasms.

Although $CYLD^{-/-}$ mice do not manifest aberrant phenotypes at birth, adult $CYLD^{-/-}$ mice exhibit pathologic features including lymphoid hyperplasia in the thymus, impaired maturation of $CD4^{+}$ and $CD8^{+}$ T cells, and lymphoid inflammatory infiltration of the liver, spleen, lungs, and salivary glands (79, 80). This implies that germline loss of *CYLD* in traditional knockout mice may exert effects on the immune system that contribute to the process of chemically induced benign tumor formation. To more directly investigate the impact of somatic *CYLD* alterations on tumorigenesis and progression to malignancy, investigators have employed tissue-specific promoters to drive expression of *CYLD* mutants in transgenic models.

CYLD loss of function promotes transformation to malignant phenotype

A role for *CYLD* alterations in skin tumor formation has been demonstrated by transgenic, tissue-specific expression of a mutant human CYLD protein using the epidermis-specific keratin 14 (K14) promoter (81). In this model (K14-$CYLD^{m}$ mice), the mutant CYLD lacked 21 C-terminal residues and was functionally inactive. Following DMBA/TPA treatment, 100% of K14-$CYLD^{m}$ mice exhibited skin tumor formation by week 13, whereas WT mice did not reach 100% tumor incidence until week 21. The K14-$CYLD^{m}$ mice also exhibited a greater number of tumors per mouse than WT mice (81). In addition, 66% of tumors from the K14-$CYLD^{m}$ mice, but only 25% of tumors from WT mice, displayed histologic features of squamous cell carcinoma. Tumors from the K14-$CYLD^{m}$ mice showed malignant features including local invasion to the dermis and increased numbers of atypical cells, as well as evidence of EMT, as indicated by reduced expression of E-cadherin and increased expression of N-cadherin and vimentin (81). Similarly, another study involved transgenic expression of a C601S murine CYLD mutant under the control of the epidermis-specific keratin 5 (K5) promoter. The C601S missense mutation is located in the DUB catalytic domain and generates a functionally inactive enzyme with dominant-negative activity (46, 82). K5-$CYLD^{C/S}$ mice developed spontaneous malignant tumors of diverse origin at the age of 8 months, including skin tumors (squamous cell carcinoma and trichofolliculoma), pulmonary adenocarcinomas, hepatocellular carcinomas, and gastric carcinomas (83).

Jin and colleagues assessed the roles of *CYLD* in epidermal tumorigenesis by generating tissue-specific, knock-in expression of a mutant CYLD under the control of the endogenous *CYLD* promoter (84). By crossing K14-controlled Cre recombinase-expressing mice with $CYLD^{fl9}$ mice, where exon 9 of *CYLD* is flanked by loxP sites, the resulting $CYLD^{E\Delta9/\Delta9}$ mice exhibited tissue-specific genomic deletion of *CYLD* exon 9 and expression of a truncated CYLD protein. The $CYLD^{E\Delta9/\Delta9}$ mice developed multiple sebaceous adenomas and basaloid tumors following treatment for 20 weeks with DMBA/TPA. The developing tumors were all histologically benign and resembled human cylindromas and trichoepitheliomas (84).

It remains unclear why different phenotypes have been obtained in different mouse models of *CYLD* alterations. Although germline deletion of *CYLD* or epidermis-specific expression of $CYLD^{E\Delta9/\Delta9}$ resulted in only benign tumors, epidermis-specific expression of the mutants $CYLD^{m}$ (lacking 21 C-terminal amino acids) or $CYLD^{C/S}$ resulted in malignant tumors. It is possible that the $CYLD^{m}$ and $CYLD^{C/S}$ mutant proteins exert a dominant-negative function, in addition to loss of catalytic function, that helps to drive oncogenesis. Further studies with additional models of the *CYLD* alterations that occur in human cancers are needed to determine the roles of these alterations in promoting tumor development in specific tissues.

CYLD alterations in cancer metastasis and invasion

Clinically, although tumors of hereditary CYLD syndrome are usually benign, malignant tumors arise from the pre-existing benign neoplasms in roughly 5% to 10% of the reported cases (6, 85). How *CYLD* alterations contribute to tumor progression and transformation in hereditary syndromes is not well understood.

K14-$CYLD^{m}$ mice developed skin cancers with local invasion, revealing a potential role for *CYLD* loss of function in transformation to a malignant phenotype (81). de Jel and colleagues assessed the role of

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CYLD in melanoma progression by crossing *CYLD* germline knockout mice ($CYLD^{-/-}$ mice) with *Grm1* transgenic mice (78, 86). In *Grm1* mice, a melanocyte-specific promoter (Dct) drives the expression of oncogenic GRM1 (glutamate metabotropic receptor 1) resulting in spontaneous cutaneous and uveal melanoma formation. Melanoma onset in Tg(*Grm1*) $CYLD^{-/-}$ mice was found to occur earlier (10 weeks after birth) than in Tg(*Grm1*) $CYLD^{+/+}$ mice (18 weeks). Moreover, primary cultures of melanoma cells from Tg(*Grm1*) $CYLD^{-/-}$ mice exhibited significantly enhanced migration compared with cells cultured from Tg(*Grm1*) $CYLD^{+/+}$ mice. Vasculogenic mimicry was observed in all primary cultures of Tg(*Grm1*) $CYLD^{-/-}$ cells, but only one of the six Tg(Grm1) $CYLD^{+/+}$ primary cultures formed vascular structures (86). In addition, immunofluorescence staining for the lymphatic endothelial marker LYVE-1 showed a higher number of lymphatic vessels in melanoma tissues from Tg(*Grm1*) $CYLD^{-/-}$ mice compared with melanomas from Tg(*Grm1*) $CYLD^{+/+}$ mice (86). In a related study, Ke and colleagues found that exogenous expression of WT CYLD in A2058 cells, a highly invasive melanoma cell line with low levels of endogenous WT CYLD, markedly reduced cell migration, when compared with A2058 expressing exogenous LacZ control (87). Tail vein injection of LacZ/A2058 cells resulted in development of melanoma nodules in the lungs, whereas none of the mice injected with WT CYLD/A2058 cells developed lung nodules (87). Collectively, these findings indicate that CYLD suppresses tumor cell invasion and metastasis, with loss of CYLD expression or function contributing to these steps toward advanced malignancy.

An impact of CYLD deficiency on metastasis has also been observed in nonmelanoma skin cancer. Tail vein injection of squamous cell carcinoma cells (PDVC57) engineered to express the catalytically inactive $CYLD^{C/S}$ mutant resulted in rapid formation of lung tumors, with 90% of lung areas occupied by metastatic tumors. By contrast, injection of PDVC57 cells engineered for expression of empty vector exhibited only 10% of lung area occupancy by metastatic tumors (46). Additional *in vitro* studies over the past 5 years have revealed that downregulation of CYLD by siRNA or oncogenic miRNAs results in reduced migration of cell lines representing a variety of cancer types, including breast cancer, hepatocellular carcinoma, HNSCC, bladder cancer, cervical cancer, glioma, and gastric cancer (34–37, 88–90).

Currently, the mechanisms whereby *CYLD* deficiency contributes to tumor cell invasion or metastasis are incompletely understood. Repression of CYLD expression might be an important step during cell migration or invasion associated with different oncogenic processes. For example, SNAIL1, a zinc-finger transcription factor that critically regulates EMT, is reported to mediate melanoma cell migration by binding to the *CYLD* gene promoter and transcriptionally repressing CYLD expression (32). Further, Ke and colleagues showed that CYLD negatively regulates signaling by β1-integrin and JNK, which are essential for melanoma cell attachment and migration (87). Another study found that knockdown of *CYLD* resulted in stabilization and upregulation of TGFβ receptor I (ALK5) in HNSCC, which led to increased phosphorylation of SMAD3. The enhanced EMT and invasiveness observed in the HNSCC cells following *CYLD* knockdown was determined to be dependent on activation of ALK5/TGFβ signaling (91).

Potential Therapeutic Strategies for CYLD-Deficient Cancers

The development of therapeutic strategies for *CYLD*-deficient tumors has primarily focused on targeting signaling pathways that are activated by the loss of CYLD expression or function. Summarized below are efforts from different groups to identify and target key signaling proteins and pathways in the context of CYLD loss (**Table 2**).

Targeting NF-κB signaling

CYLD loss of function leads to NF-κB signaling activation (11). Targeting NF-κB signaling may provide a promising strategy to treat cancers with *CYLD* alterations. In a pilot clinical study, Oosterkamp and colleagues treated patients with CYLD syndrome with salicylic acid, an aspirin metabolite that inhibits activation of IKK (92). Following topical application of salicylic acid for 6 weeks, all 12 patients exhibited stable disease. After continuous treatment for another 18 weeks, 2 patients showed a complete response, with remission for over 1 year with sporadic use of salicylic acid. The remaining patients exhibited only partial responses to the therapy, which may have been due to insufficient potency of salicylic acid against the IKK in those patients (92). In addition, a case report revealed that a patient with CYLD-deficient multiple familial trichoepitheliomas demonstrated shrinkage of facial papules following combined treatment with aspirin and subcutaneous adalimumab (Humira) for 8 months. Adalimumab, an FDA-approved agent used for rheumatoid arthritis, is a monoclonal antibody targeting TNFα that suppresses TNFα activation of NF-κB signaling (93). Aspirin alone also inhibits NF-κB signaling by preventing IκB degradation (94). Therefore, the combined use of adalimumab and aspirin merits further investigation as a treatment strategy for CYLD-deficient cancers (95).

An *in vitro* study in oral squamous cell carcinoma has shown that siRNA-mediated knockdown of *CYLD* induced hyperactivation of NF-κB signaling and rendered cancer cells resistant to cisplatin treatment (96). Treatment with the proteasome inhibitor bortezomib inhibited degradation of IκB, suppressing NF-κB signaling and abolishing cisplatin resistance caused by CYLD downregulation in the cancer cells (96). Hence, the impact of bortezomib, or second-generation proteasome inhibitors such as carfilzomib, on CYLD-deficient cancers may yield new insights.

Tropomyosin receptor kinase as a target for CYLD-defective tumors

Repeated resection is currently the only treatment to control the multiple tumors of CYLD syndromes. Cho and colleagues (97)

Table 2. Potential therapeutic strategies for CYLD-deficient diseases.

Drug	Target	Application	Ref.
Aspirin derivatives	Inhibit NF-κB signaling	Topical use in patient with CYLD syndrome	(92, 107)
SP600125	Inhibit JNK signaling	Treatment of liver cancer in *CYLD*-deficient mice model	(104, 108)
Aspirin plus adalimumab	Inhibit TNFα and NF-κB signaling	Treatment of multiple familial trichoepitheliomas	(95)
Pegcantratinib	Inhibit Tropomyosin receptor	Treatment of inherited *CYLD*-defective skin cancer	(99, 100)
Vismodegib	Inhibit Hedgehog signaling	Treatment of multiple familial trichoepitheliomas	(101)

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analyzed a cohort of resected tumors containing 121 oropharyngeal HNSCCs and 275 nonoropharyngeal HNSCCs and found elevated expression of tropomyosin receptor kinase (TRK) in the oropharyngeal, p16-positive (a marker of HPV) subset, relative to the nonoropharyngeal subset. A different exploratory clinical study analyzed *CYLD*-mutant tumors using unbiased array comparative genomic hybridization and gene expression microarray analysis, and aimed to identify candidate molecular biomarkers in the CYLD-defective tumors that could be therapeutically targeted (98). TRK was found to be overexpressed in all the CYLD-mutated tumors studied relative to its expression in adjacent unaffected skin tissues (98). Based on these results, 15 patients with CYLD syndrome, each with 10 evaluable tumors (5 matched tumors on each body side; a total of 150 tumors analyzed), were recruited and treated with the TRK inhibitor pegcantratinib on the left side of the body and placebo on the right side (99, 100). Disappointingly, only 2 of 75 tumors topically treated with pegcantratinib showed size reduction, as compared with 6 tumors treated with placebo (99). The failure of the trial might be due to the administration of an inadequate concentration of pegcantratinib, and further escalation studies with higher doses are needed.

Targeting Hedgehog signaling in CYLD syndromes

Although the interaction of CYLD and hedgehog signaling has not been clearly defined, a clinical study has provided evidence that targeting hedgehog signaling might be a promising therapeutic strategy for patients with CYLD syndrome. Baur and colleagues reported a case of multiple familial trichoepitheliomas wherein mRNA for the hedgehog signaling effector Gli1 was markedly overexpressed in tumors with a *CYLD* heterozygous germline mutation in exon 17 (101). The patient was treated with the hedgehog pathway inhibitor vismodegib for 2 months and exhibited significant reduction in the size and number of trichoepitheliomas (101). Interestingly, SNAIL1, which is a transcriptional repressor of CYLD, is a key target of Hedgehog/Gli1 signaling (32, 102). Elevated expression of Gli1 has also been reported in HPV-positive cervical cancers (103).

Targeting JNK signaling

Hyperactivation of JNK signaling has been observed in CYLD-associated pathologic conditions (81, 87). Mice with CYLD deficiency ($CYLD^{-/-}$ mice) were found to be highly susceptible to liver cancer development following treatment with the chemical carcinogen diethylnitrosamine, and this susceptibility was dependent on JNK signaling (104). Loss of CYLD in liver cancer cells was determined to stabilize ubiquitination of TRAF2, resulting in phosphorylation of JNK1 and activation of the JNK signaling pathway (104). Notably, injection of $CYLD^{-/-}$ mice with the JNK-specific inhibitor SP600125 prior to diethylnitrosamine treatment markedly reduced hepatocyte proliferation, as assessed by Ki67 staining. This suggests that JNK inhibition has potential for suppressing malignant progression in CYLD-deficient cancers (104).

Targeting Wnt/β-catenin signaling

CYLD also plays a role in negatively regulating protumor signaling by Wnt/β-catenin. Stimulation of cells with Wnt ligand results in formation of a receptor complex consisting of frizzled (Fz) and LRP5/6, followed by Fz recruitment of Disheveled (Dvl) and subsequent phosphorylation of LRP5/6 (105). Phosphorylated LRP5/6 recruits axin, which prevents axin-mediated degradation of β-catenin, liberating β-catenin to induce Wnt-responsive genes. The key role of Dvl in this process is dependent on K63-linked ubiquitination of the Dvl protein. CYLD negatively regulates Wnt/β-catenin signaling by promoting deubiquitination of Dvl (106). Notably, cylindroma skin tumors that harbor CYLD mutations exhibit hyperactive Wnt/β-catenin signaling (106). Thus, targeting the Wnt/β-catenin signaling pathway may be a useful therapeutic strategy in cancers characterized by loss of CYLD function.

Modulating CYLD expression

In addition to genetic alterations, the expression levels of CYLD can be downregulated in cancers through transcriptional repression by SNAIL (32). Hence, direct targeting of SNAIL, a marker of cancer stem cells, may be an effective approach for upregulating CYLD expression. Similarly, a number of miRNAs have been shown to downregulate expression of CYLD via binding to the 3′ nontranslated region of *CYLD* mRNA (33–38). Selective targeting of these miRNAs may be useful for achieving CYLD upregulation.

Summary

Germline alterations of the *CYLD* gene underlie cylindromatosis syndromes. Somatic *CYLD* alterations have been identified in specific cancers, with a higher prevalence in cancers associated with HPV infection, including HPV-positive HNSCC. These loss-of-function alterations lead to altered signaling in the tumor that appears to mediate increased migration, invasion, and metastasis. However, the precise relevance of *CYLD* alterations in therapy selection for patients with cancer remains unknown. Increased understanding of the role of CYLD loss of expression or function in cancer development and progression may identify therapeutic targets. This is particularly relevant for HPV-positive cancers where alterations of *CYLD* are found with greater frequency.

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