

Deubiquitinases in the regulation of NF- κ B signaling

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Nuclear factor-kappa B (NF- κ B) is a critical regulator of multiple biological functions including innate and adaptive immunity and cell survival. Activation of NF- κ B is tightly regulated to preclude chronic signaling that may lead to persistent inflammation and cancer. Ubiquitination of key signaling molecules by E3 ubiquitin ligases has emerged as an important regulatory mechanism for NF- κ B signaling. Deubiquitinases (DUBs) counteract E3 ligases and therefore play a prominent role in the downregulation of NF- κ B signaling and homeostasis. Understanding the mechanisms of NF- κ B downregulation by specific DUBs such as A20 and CYLD may provide therapeutic opportunities for the treatment of chronic inflammatory diseases and cancer.

Keywords: deubiquitinases; NF- κ B; IKK; A20; CYLD

Cell Research (2011) 21:22-39. doi:10.1038/cr.2010.166; published online 30 November 2010

Introduction

Nuclear factor-kappa B (NF- κ B)/Rel represents a eukaryotic transcription factor family that is a master regulator of genes that control innate and adaptive immune responses [1]. NF- κ B proteins all share an approximately 300-amino-acid Rel homology domain (RHD) that regulates DNA binding, nuclear localization and dimerization [2]. NF- κ B family members form homo- and heterodimers, including p65/RelA, c-Rel, RelB, p105/p50 and p100/p52 [3]. NF- κ B proteins are sequestered in the cytoplasm as latent complexes by inhibitory proteins, or I κ Bs, that prevent NF- κ B nuclear translocation and DNA binding [4]. Members of the I κ B family, consisting of I κ B α , I κ B β , I κ B ϵ , NF- κ B1, NF- κ B2, I κ B ζ (also known as MAIL) and Bcl-3, all contain a series of ankyrin repeat domains that mediate binding with NF- κ B subunits [3]. Whereas the majority of I κ Bs serve as inhibitors of NF- κ B, the I κ B ζ and Bcl-3 isoforms instead potentiate NF- κ B transactivation in the nucleus [5, 6]. The NF- κ B1 and NF- κ B2 proteins, also known as p105 and p100, re-

spectively, are precursor forms of the p50 and p52 NF- κ B subunits [7].

There are two unique NF- κ B signaling pathways, termed canonical and noncanonical, that have distinct biological roles [8]. In the canonical NF- κ B pathway, proinflammatory cytokines such as TNF- α or IL-1 β , or pathogen-derived components such as bacterial lipopolysaccharide (LPS), trigger the rapid nuclear translocation of NF- κ B subunits [1]. The canonical NF- κ B pathway plays an important role in many physiological processes such as innate and adaptive immunity and cell survival. A wide variety of stimuli activate canonical NF- κ B signaling, all of which converge at the I κ B kinase (IKK), consisting of catalytic subunits IKK α and IKK β and the regulatory subunit IKK γ (also known as NEMO) [9-11]. The IKK β and IKK γ subunits serve essential and non-redundant roles in the activation of NF- κ B in the canonical pathway [12, 13]. IKK phosphorylates I κ B proteins at two amino (N)-terminal regulatory serine residues, triggering their ubiquitination and degradation by the proteasome, thus allowing NF- κ B to enter the nucleus and activate stimulus-specific gene programs [14]. In response to specific stimuli, such as proinflammatory cytokines, NF- κ B is activated transiently due to a series of tightly regulated negative feedback loops including the induction of inhibitory proteins such as I κ B α that downregulate NF- κ B signaling [15]. Terminating an NF- κ B response is essential to prevent persistent NF- κ B activation that may

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lead to chronic inflammation and/or tumorigenesis.

In the noncanonical pathway, NF- κ B2 is processed by the proteasome to generate p52, which together with RelB regulates a distinct subset of target genes controlling B lymphocyte survival and lymphoid organogenesis [8]. The noncanonical pathway is triggered by a subset of TNF superfamily ligands, such as BAFF, LT- β and CD40L [16-19]. The MAP 3 kinase MAP3K14, also known as NF- κ B inducing kinase or NIK, is a central player in the noncanonical pathway [20]. NIK is predominantly regulated post-translationally with extremely low levels of NIK in most cell types [21]. Low levels of NIK are maintained by persistent degradation of NIK by a multi-subunit E3 ubiquitin ligase complex consisting of TNF-receptor-associated factor (TRAF) 2, TRAF3, cIAP1 and cIAP2 [22-24]. BAFF-R, CD40 and LT- β R ligation all trigger the degradation of TRAF3, resulting in the inactivation of the TRAF/cIAP E3 ubiquitin ligase complex and the concomitant stabilization of NIK [24]. NIK, in turn, phosphorylates IKK α , which phosphorylates NF- κ B2 to trigger its processing by the proteasome [25]. A recent study indicates post-translational regulation of NIK function via its K63-linked polyubiquitination by the zinc finger protein 91 (ZFP-91) [26]. NIK ubiquitination promotes its stabilization through an unknown mechanism.

Ubiquitination is a reversible post-translational modification involving the covalent attachment of one or more ubiquitin monomers to a protein substrate, best known as a targeting signal for proteasome-mediated degradation. However, recent studies have demonstrated numerous nonproteolytic functions of ubiquitination including protein trafficking and activation of kinases and phosphatases [27]. Therefore, ubiquitination plays a central role in the regulation of many signaling pathways. Ubiquitination consists of a three-step enzymatic cascade initiating with the activation of ubiquitin by a ubiquitin-activating enzyme (E1) followed by transfer of the activated ubiquitin to the active-site cysteine of a ubiquitin-conjugating enzyme (E2) and finally, with the assistance of an E3 or ubiquitin ligase, transfer of the ubiquitin to a lysine on a substrate protein to form an isopeptide bond [28]. There are two E1s, approximately 50 E2s and 600 E3s encoded in the human genome [29]. The majority of E2 enzymes have been linked to K48-linked protein ubiquitination and degradation. However, the dimeric E2 complex Ubc13/Uev1a is specific for the synthesis of K63-linked polyubiquitin chains [30, 31]. The E3s provide specificity for ubiquitination of substrates and consist of three groups: RING, HECT and F-box [32]. RING domain and the closely related F-Box E3 ligases catalyze the transfer of ubiquitin from E2s to substrates, whereas

HECT domain E3 ligases directly transfer ubiquitin to substrates [32].

Ubiquitination of a substrate may proceed with a single ubiquitin (monoubiquitination) or a chain of covalently linked polyubiquitin molecules (polyubiquitination) [28]. Ubiquitin contains seven internal lysine residues (K6, K11, K27, K29, K33, K48 and K63) that can each support polyubiquitination chains [28]. Indeed, isopeptide linkages involving every lysine residue in ubiquitin have been detected by mass spectrometry of total protein lysates [33]. K48-linked polyubiquitin chains are recognized by the 26S proteasome, leading to protein degradation. K63-linked polyubiquitin chains do not trigger protein degradation but instead have nonproteolytic functions such as protein trafficking or kinase and phosphatase activation [29, 34]. In addition, ubiquitin chains can also be linked in a linear fashion, whereby the C-terminal glycine is attached to an N-terminal methionine resulting in head-to-tail polyubiquitination [35]. Two RING-type E3 ligases, HOIL1 and HOIP, specifically assemble linear polyubiquitin chains that play an important role in NF- κ B regulation [36]. Other types of ubiquitin modifications have also been observed, including K11-mediated polyubiquitination. A novel K11-linkage-specific antibody was used to demonstrate that K11-linked ubiquitin chains are highly upregulated during mitosis and are linked to protein degradation [37]. There have also been reports indicating that K27-linked polyubiquitin chains regulate the targeting of Jun to lysosomes [38]. In addition, AIP4/Itch catalyzes the formation of K29-linked polyubiquitin chains on Notch receptor to induce its lysosomal-mediated degradation [39]. However, the biological roles of these noncanonical ubiquitin linkages remain poorly understood.

Ubiquitination is reversible and is counter-regulated by a family of deubiquitinases (DUBs) of which there are nearly 100 encoded in the human genome [40]. There are five families of DUBs characterized by specific structural domains: ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTUs), Josephins and JAB1/MPN/MOV34 metalloenzymes (JAMMs). UCHs, USPs, OTUs and Josephins function as cysteine proteases, whereas JAMMs are zinc-dependent metalloproteases [41]. The USPs compose the largest subfamily of DUBs with over 50 members [41]. Many DUBs harbor ubiquitin-binding domains (UBDs) that coordinate recognition and recruitment of ubiquitinated substrates. Conversely, as will be discussed later, a number of DUBs rely on ubiquitin-binding adaptor molecules to confer specificity. In this review, we will describe the roles of DUBs in the regulation of NF- κ B signaling.

Ubiquitin-dependent activation of NF- κ B signaling

Engagement of the TNFR or IL-1R/TLR4 pathways leads to recruitment and activation of specific TRAFs. TRAFs 2-7 all have N-terminal RING domains that confer E3 ligase activity and TRAF domains that mediate homo- and heterotypic protein interactions [42]. TRAF1 lacks a RING domain and functions as a negative regulator of TNF signaling and NF- κ B activation [43]. TRAF6 activates IKK in a K63-ubiquitin chain-dependent manner that requires the dimeric E2 enzyme complex Ubc13/Uev1a as well as the transforming growth factor receptor- β -activated kinase 1 (TAK1) and its regulatory subunits TAB1 and TAB2 [31]. Genetic studies in mice support essential roles for TRAF6 and TAK1 in the activation of NF- κ B by multiple stimuli [44-46]. Surprisingly, genetic ablation of Ubc13 does not affect activation of NF- κ B by multiple stimuli in B cells, macrophages or MEFs [47]. Rather, Ubc13 appears to play an important role in MAP kinase activation, partly through NEMO ubiquitination [47]. Contrary to these results, haploinsufficient *Ubc13*^{+/-} mice, generated by an independent group, are resistant to LPS-induced lethality [48]. Ubc13-deficient macrophages and splenocytes derived from these mice are impaired in the induction of proinflammatory cytokines by LPS [48]. Furthermore, *Ubc13*^{+/-} macrophages display defects in NF- κ B, p38 and JNK activation as a result of impaired LPS-induced TRAF6 ubiquitination [48]. It is unclear why the two different models of Ubc13 deficiency result in distinct phenotypes and conclusions, possibly due to the different strategies used to generate the knockout mice.

Binding of IL-1 to IL-1R triggers the recruitment of Myd88, IRAK1, IRAK4 and TRAF6 to form a receptor-associated complex [49]. TRAF6 undergoes oligomerization, activation and K63-linked polyubiquitination that leads to the recruitment of the TAB1-TAB2-TAK1 complex and subsequent activation of TAK1, which, in turn phosphorylates IKK β [50]. However, the role of TRAF6 autoubiquitination is unclear since reconstitution of TRAF6-deficient MEFs with a TRAF6 lysine-deficient mutant rescues IL-1- and RANK-induced NF- κ B activation similar to wild-type TRAF6 [51]. The RING domain of TRAF6 is important for downstream NF- κ B signaling, indicating that TRAF6-catalyzed ubiquitination of other substrates is more important than TRAF6 autoubiquitination. Indeed, IRAK1 undergoes TRAF6-dependent K63-linked polyubiquitination upon IL-1 stimulation that regulates binding to NEMO [52, 53]. A recent study also suggests that unanchored polyubiquitin chains synthesized by TRAF6 and Ubc13/Uev1a are key regulators of NF- κ B signaling by directly activating TAK1 via binding

to TAB2 [54]. TRAF6 also functions together with UbcH5c to synthesize unanchored polyubiquitin chains that activate IKK [54]. Unanchored polyubiquitin chains also regulate antiviral signaling [55], and may indeed play a pervasive role in regulating signaling pathways.

TNF- α binding to TNFR promotes the rapid formation of a TRADD, TRAF2, cIAP1, cIAP2 and RIP1 receptor-associated complex [56]. RIP1 undergoes K63-linked polyubiquitination on lysine 377 (K377) in response to TNF- α stimulation [57, 58]. Although TRAF2 was originally thought to ubiquitinate RIP1, TRAF2-deficient MEFs exhibit only a mild defect in TNF- α -mediated NF- κ B signaling [59]. This may be due to the functional redundancy with TRAF5, since combined deletion of TRAF2 and TRAF5 completely impairs TNF-mediated NF- κ B activation [60]. More recent studies suggest that cIAP1 and cIAP2 serve as direct E3 ubiquitin ligases for RIP1, suggesting that TRAFs may instead serve as adaptors for RIP1 [61]. RIP1 ubiquitination plays a critical role in the recruitment of the TAK1 complex, which is responsible for the phosphorylation and activation of IKK [62, 63]. RIP1 is therefore a key target of DUBs that downregulate TNF- α -induced NF- κ B signaling.

A20 function and the mechanisms of NF- κ B inhibition

A20 (also known as TNFAIP3) was originally identified as a TNF-inducible gene in human umbilical vein endothelial cells (HUVEC) [64]. In most cell types A20 is expressed at very low levels but can be rapidly induced by proinflammatory cytokines or mitogens [64]. Conversely, thymocytes and peripheral T lymphocytes express high levels of A20 that are subsequently downregulated upon stimulation with T cell receptor agonists [65]. A20 contains an N-terminal OTU domain and seven zinc finger domains (C₂H₂) in the C-terminus (Figure 1). A20 harbors DUB activity mediated by the OTU domain [66, 67]. However, A20 does not exert a global effect on ubiquitinated proteins, suggesting specificity for its substrates. Recently, great progress has been made in understanding how A20 regulates inflammatory signaling pathways, as well as its novel role as a tumor suppressor that will be discussed later.

A20 was first described as a zinc finger protein that protects cells from TNF cytotoxicity [68]. Subsequent studies, based mainly on overexpression, implicated A20 as a negative regulator of NF- κ B signaling in multiple pathways [69-71]. Generation of A20-deficient mice (*Tnfaip3*^{-/-}) confirmed the anti-apoptotic and NF- κ B inhibitory functions of A20. A20-deficient mice die prematurely due to multi-organ inflammation and cachexia as

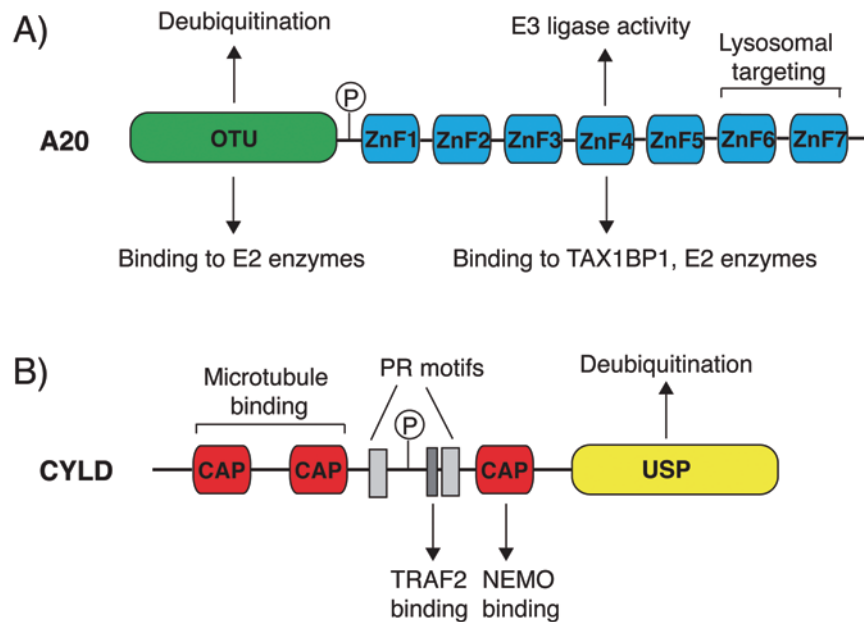


Figure 1 Structural domains in A20 and CYLD. **(A)** A20 contains an N-terminal OTU domain responsible for the DUB activity of A20. The catalytic cysteine residue Cys103 is also important for binding to the E2 enzymes Ubc13 and UbcH5c. There is an IKK β phosphorylation site at Ser381 downstream of the OTU domain. A20 contains seven zinc finger domains (C₂H₂) in its C-terminus. ZnF4 confers A20 E3 ligase activity and is also involved in TAX1BP1 interactions. ZnF6 and ZnF7 are important for targeting A20 to lysosomes. In addition, A20 zinc fingers have been implicated in A20 oligomerization and A20 binding to ABINs and NEMO. **(B)** CYLD contains a C-terminal USP domain responsible for its DUB activity. The N-terminus of CYLD contains three CAP-Gly (CAP) domains and two proline-rich (PR) motifs. The first two CAP domains mediate binding to microtubules whereas the third CAP domain regulates NEMO interactions. CYLD also contains a TRAF2 binding site (PVQES) and a phosphorylation site cluster between CAP domains 2 and 3.

a result of dysregulated NF- κ B signaling [72]. *Tnfaip3*^{-/-} mice are also hyper-responsive to proinflammatory stimuli *in vivo* and succumb to sub-lethal doses of TNF- α or LPS [72]. Consistently, A20-deficient MEFs exhibit persistent TNF- α and IL-1-induced NF- κ B signaling as observed by prolonged IKK activation and I κ B α degradation [72]. MEFs deficient in A20 are also sensitized to TNF-mediated cell death, illustrating the anti-apoptotic function of A20 [72].

The uncontrolled inflammation in *Tnfaip3*^{-/-} mice is independent of the adaptive immune response, since *Tnfaip3*^{-/-} mice on a *Rag1*^{-/-} background lacking T and B lymphocytes are not rescued from premature lethality [72]. Although A20 was initially described as a negative regulator of TNFR signaling, the spontaneous inflammation and cachexia in *Tnfaip3*^{-/-} mice still occurs in both *Tnfaip3*^{-/-} *Tnfa*^{-/-} and *Tnfaip3*^{-/-} *Tnfr1*^{-/-} double-mutant mice, indicating that A20 also restricts TNF-independent signaling pathways *in vivo* [73]. All Toll-like receptor pathways use the common adaptor Myd88 to activate NF- κ B and MAP kinase pathways to induce expression

of proinflammatory cytokines [74]. *Tnfaip3*^{-/-} *Myd88*^{-/-} double-mutant mice are rescued from lethal inflammation, suggesting that TLR signals drive the spontaneous inflammation in the absence of A20 [75]. Consistent with these observations, treatment of A20-deficient mice with broad-spectrum antibiotics rescues the severe inflammation and lethality, indicating that a dysregulated response to commensal intestinal flora is responsible for the constitutive TLR signaling in *Tnfaip3*^{-/-} mice [75]. Therefore, A20 plays an essential role in restricting TLR signaling and maintaining immune homeostasis in the intestine.

The spontaneous inflammation and premature lethality of *Tnfaip3*^{-/-} mice has precluded the use of these mice in experimentally induced inflammatory disease models. Therefore, conditional gene targeting was used to specifically delete A20 in intestinal epithelial cells (IECs) by crossing with Cre transgenic mice driven by the IEC-specific promoter villin [76]. The A20 IEC conditional knockout (A20^{IEC-KO}) mice develop normally, appear healthy and do not develop spontaneous colitis [76].

However, these mice are more susceptible to experimentally induced colitis after injection with dextran sulphate sodium (DSS), and experience pronounced colon shortening, crypt loss and immune cell infiltration [76]. The increased DSS-induced colitis susceptibility of A20^{IEC-KO} mice is triggered by enhanced TNF-mediated apoptosis of IECs, leading to a breakdown of the intestinal barrier and systemic inflammation [76]. Crossing A20^{IEC-KO} mice to TNFR1-deficient mice ameliorates colitis symptoms, indicating that uncontrolled TNFR1 signaling contributes to the intestinal pathogenesis [76]. These findings also underscore the anti-apoptotic function of A20 in the intestinal epithelium in a pathophysiological setting.

In addition to IECs, A20 has also been selectively deleted in B lymphocytes by crossing with CD19-Cre transgenic mice [77]. Although A20 is constitutively expressed in B cells, stimulation with agonistic CD40 antibodies further upregulates A20 expression via NF-κB [78]. B cell-specific A20 knockout (A20^{CD19-KO}) mice develop normally, but have increased numbers of immature (CD19⁺IgM^{hi}) and germinal center (GC) B cells [77]. Furthermore, splenic B cells from A20^{CD19-KO} mice exhibit enhanced proliferation and upregulation of co-stimulatory molecules upon stimulation with α-CD40,

LPS or CpG [77]. CD40-induced IκBα phosphorylation and degradation as well as p100 phosphorylation are all enhanced in splenic B cells from A20^{CD19-KO} mice [77], indicating the importance of A20 in inhibiting canonical and noncanonical NF-κB signaling downstream of CD40 ligation in B cells. As a result of accumulated immature and GC B cells, A20^{CD19-KO} mice develop symptoms of autoimmunity, including the presence of autoantibodies [77]. In this regard, it is interesting that polymorphisms in the vicinity of the human A20 gene have been linked to development of systemic lupus erythematosus (SLE) [79]. Furthermore, splenic B cells from A20^{CD19-KO} mice are resistant to Fas-mediated programmed cell death due to enhanced expression of the antiapoptotic molecule Bcl-x [77]. This result is somewhat surprising given the known anti-apoptotic effects of A20 in TNFR signaling. Nevertheless, the collective results firmly establish A20 as an essential regulator of B cell homeostasis.

A20 functions as a negative feedback inhibitor of NF-κB in multiple innate immune signaling pathways including TNFR and Toll-like receptor pathways (Figure 2). In the TNFR pathway, A20 expression is rapidly upregulated in an NF-κB-dependent manner and targets ubiquitinated RIP1 for inactivation in a two-step sequential pro-

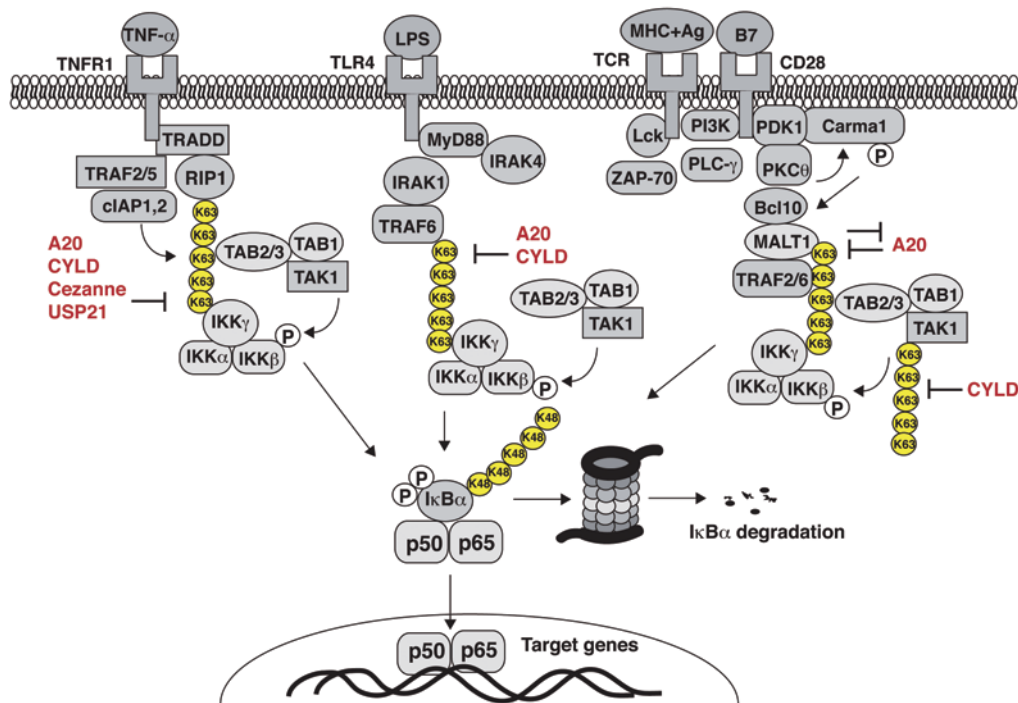


Figure 2 NF-κB signaling pathways downstream of TNFR1, TLR4 and TCR/CD28. TNFR1, TLR4 and TCR/CD28 engagement trigger the K63-linked ubiquitination of RIP1, TRAF6 and MALT1, respectively. Each of these ubiquitinated signaling molecules are targeted for deubiquitination by either A20, CYLD, Cezanne or USP21, as indicated. MALT1 cleaves A20 to inactivate its function. CYLD also removes polyubiquitin chains from TAK1 in the TCR pathway.

cess [64, 67]. A20 first removes K63-linked polyubiquitin chains from RIP1 in an OTU-dependent manner, then catalyzes formation of K48-linked polyubiquitin chains onto RIP1 to trigger proteasome-mediated degradation [67]. Remarkably, recombinant A20 functions as an E3 ligase despite the lack of a RING or HECT domain. The E3 ligase activity of A20 is dependent on the fourth zinc finger domain (ZNF4), suggesting a novel type of E3 ligase domain [67]. Thus, A20 functions as a unique dual function ubiquitin-editing enzyme that inactivates RIP1 via sequential DUB and E3 ligase activities. In addition, A20 may also target substrates for degradation via the lysosomal pathway. A fraction of cellular A20 localizes to lysosomes in a zinc finger-dependent manner and promotes the degradation of TRAF2 in lysosomes [80, 81].

A20-deficient mice exhibit spontaneous multi-organ inflammation due to persistent activation of Toll-like receptor signaling. A20 expression is rapidly induced by LPS in bone marrow-derived macrophages (BMDMs) [73]. A20-deficient macrophages produce more proinflammatory cytokines in response to LPS stimulation, suggesting that A20 is an important negative regulator of TLR4 signaling in macrophages. Indeed, LPS-induced NF- κ B activation is elevated and persistent in *Tnfaip3*^{-/-} macrophages. A20 downregulates NF- κ B signaling upstream of IKK at the level of TRAF6. Overexpression of A20, but not a catalytically inactive DUB mutant C103A, inhibits TRAF6 ubiquitination, and A20-deficient MEFs also exhibit persistent LPS-induced TRAF6 ubiquitination [73]. Although A20 clearly antagonizes TRAF6 ubiquitination, it appears that the mechanisms of TRAF6 downregulation are distinct from those of RIP1 in TNFR signaling since TRAF6 is not degraded upon IL-1 or LPS stimulation [82].

Recently, a new mechanism of A20 inhibition of NF- κ B signaling has emerged. At early times after LPS stimulation, A20 disrupts the binding of TRAF6 with the E2 enzymes Ubc13 and UbcH5c [82]. Similarly, A20 also disrupts the binding of TRAF2 and cIAP1 with Ubc13. At later times (4-6 h) of LPS stimulation, A20 also triggers the proteasome-mediated degradation of the E2 enzymes Ubc13 and UbcH5c [82]. Ubc13 is also ubiquitinated and degraded in response to TNF- α stimulation (Figure 3). A20 disruption of E2-E3 enzymes is dependent on ZnF4 and Cys103 in the OTU domain consistent with previous studies [67, 82]. Therefore, it appears that A20 uses multiple complementary mechanisms to downregulate NF- κ B signaling.

A20 also negatively regulates signaling by nucleotide-binding oligomerization domain containing 2 (NOD2), a cytoplasmic pathogen recognition receptor that recognizes bacterial cell wall components, specifically mu-

ramyl dipeptide (MDP), and activates NF- κ B. RIP2 (also known as RICK) is a key downstream regulator of NOD2 that facilitates IKK activation upon K63-linked polyubiquitination and oligomerization [83]. A20-deficient macrophages display enhanced NOD2-mediated RIP2 ubiquitination, NF- κ B signaling and generation of proinflammatory cytokines [84]. *Tnfaip3*^{-/-} mice exhibit pronounced *in vivo* responses in response to MDP challenge as shown by greater amounts of serum IL-6 [84]. Mechanistically, A20 inhibits NOD2 signaling by deubiquitinating K63-linked polyubiquitin chains from RIP2. The E3 ligase Itch also negatively regulates MDP-induced NF- κ B activation, although Itch was shown to function as an E3 ligase for RIP2 [85].

In addition to regulating innate immunity, A20 also is important for adaptive immune responses. A20 is an important negative regulator of NF- κ B signaling in both T and B lymphocytes. Its regulation is unique in T lymphocytes because of the high basal levels compared to other cell types [65]. Nevertheless, overexpression of wild-type A20, but not an A20 DUB mutant, attenuates NF- κ B activation triggered by agonistic antibodies to CD3 and CD28 [86]. T-cell receptor ligation promotes the recruitment of A20 to the paracaspase MALT1 and the adaptor protein Bcl-10, where MALT1 cleaves A20 to impair its NF- κ B inhibitory function and fine-tune NF- κ B signaling [87]. A20 also removes MALT1 polyubiquitin chains to inhibit interactions between ubiquitinated MALT1 and the downstream IKK complex (Figure 2) [88]. Therefore, the balance between A20 cleavage by MALT1 and A20 deubiquitination of MALT1 determines NF- κ B activation in T lymphocytes. A similar cross-regulation between A20 and MALT1 is thought to exist in B lymphocytes, and is dysregulated in B-cell lymphomas which are characterized by uncontrolled NF- κ B activation. MALT1 paracaspase activity is constitutive in B-cell lymphomas, and inhibition of MALT1 catalytic activity preserves full-length A20, which then inhibits NF- κ B and promotes toxicity towards B-cell lymphoma cells [89].

A20 is also a potent negative regulator of dendritic cell (DC) maturation and antigen presentation. Silencing of A20 expression using siRNA in mouse myeloid DCs triggers spontaneous and enhanced expression of proinflammatory cytokines and costimulatory molecules [90]. A20-silenced DCs are particularly effective in anti-tumor responses by both inhibiting T regulatory cells and hyperactivating tumor-infiltrating cytotoxic T cells [90]. Due to the elevated expression of proinflammatory cytokines IL-6 and TNF- α in A20-silenced DCs, it is likely that constitutive NF- κ B activation is largely responsible for the marked activation and antigen presentation.

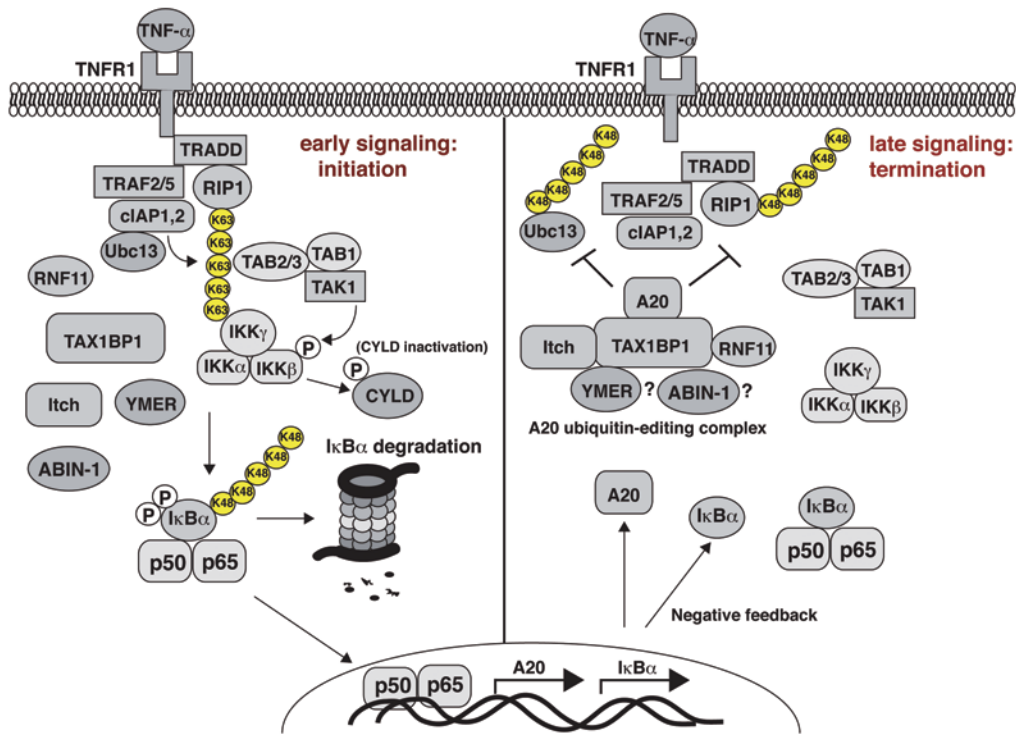


Figure 3 Temporal regulation of NF- κ B signaling downstream of TNFR1. During early stages of TNFR1 signaling (signal initiation—15–30 min after receptor engagement), RIP1 undergoes K63-linked ubiquitination, which activates IKK and NF- κ B. CYLD is inactivated by IKK-mediated phosphorylation. At later times (0.5–6 h), A20 and I κ B α expression is induced by NF- κ B as part of a negative feedback loop. A20 interacts with TAX1BP1, Itch, RNF11 and possibly ABIN-1 and YMER to form the A20 ubiquitin-editing complex. A20 removes K63-linked ubiquitin chains from RIP1 and catalyzes the formation of K48-linked chains to trigger RIP1 degradation. A20 also disrupts cIAP1 binding to Ubc13 and promotes Ubc13 ubiquitination and degradation.

Regulation of A20 by ubiquitin-binding proteins and adaptors

Given that A20 does not exhibit a strong preference for deubiquitinating K63-linked polyubiquitin chains *in vitro* [91], it is not surprising that A20 cooperates with several other proteins to inhibit NF- κ B signaling. The NF- κ B inhibitory function of A20 is dependent on several other proteins including Tax1 binding protein 1 (TAX1BP1), Itch, Ring finger protein (RNF) 11 and possibly others, including ABIN-1 and YMER, that together form a complex of proteins that we have termed the “A20 ubiquitin-editing complex” (Figure 3). TAX1BP1 was first identified in a yeast two-hybrid screen using the HTLV-I Tax oncoprotein as bait [92]. Shortly after, TAX1BP1 was also identified as a novel A20 binding protein that functions as an anti-apoptotic protein regulating the function of A20 [93].

Disruption of the *Tax1bp1* locus by gene trapping results in embryonic lethality of homozygous mutant mice

due to systemic hemorrhaging and cardiac defects at approximately E13.5 [94]. On the other hand, *Tax1bp1*^{-/-} mice, generated by conventional gene targeting methods, succumb to age-dependent inflammatory cardiac valvulitis [95]. Similar to *Tnfaip3*^{-/-} mice, *Tax1bp1*^{-/-} mice are hypersensitive to proinflammatory cytokines and succumb to sub-lethal doses of TNF- α and IL-1 β [95]. *Tnfaip3*^{-/-} and *Tax1bp1*^{-/-} MEFs display enhanced NF- κ B activation and a defect in the termination of NF- κ B signaling in response to TNF- α or IL-1 β stimulation [94, 95]. TAX1BP1 interacts with RIP1 downstream of TNFR and counteracts its ubiquitination since RIP1 ubiquitination is persistent upon TNF- α stimulation in *Tax1bp1*^{-/-} MEFs [94]. Together, these studies indicate that TAX1BP1 is a critical negative regulator of NF- κ B signaling.

TAX1BP1 contains an N-terminal SKIP (skeletal muscle and kidney enriched inositol phosphatase) carboxyl homology (SKICH) domain, originally described as a membrane targeting domain in the SKIP and PIPP phosphatases [96]. TAX1BP1 also contains three coiled-coil

domains and two C-terminal zinc finger domains. Due to the striking functional similarities between *Tnfaip3*^{-/-} and *Tax1bp1*^{-/-} MEFs, and the fact that TAX1BP1 lacks a DUB domain, it was hypothesized that TAX1BP1 inhibited NF- κ B through A20. Indeed, TAX1BP1 is required for A20 to interact with RIP1 upon TNF stimulation and also mediates the NF- κ B inhibitory function of A20 [94, 95]. Interestingly, one of the TAX1BP1 zinc finger domains functions as a novel UBD [95]. Therefore, TAX1BP1 likely serves as an adaptor by recruiting A20 to ubiquitinated substrates that are first recognized by the TAX1BP1 UBD.

The zinc finger domains of TAX1BP1 also harbor highly conserved 'PPXY' (where P=proline, X=any amino acid and Y=tyrosine) motifs that are precisely positioned in the center of both zinc finger domains [97]. PPXY motifs are well-established binding motifs for modular 'WW' (where W=tryptophan) domain-containing proteins [98]. Reconstitution of TAX1BP1-deficient MEFs with TAX1BP1 PPXY mutants fails to restore transient NF- κ B signaling, indicating the importance of these sites for TAX1BP1 function [97]. The HECT domain E3 ligase Itch contains multiple WW domains and interacts with TAX1BP1 via the PPXY motifs [99]. The TAX1BP1/Itch binding is inducible and occurs rapidly following TNF- α stimulation [97]. Itch also is essential for A20 recruitment to RIP1 upon TNF- α stimulation and the concomitant downregulation of RIP1 ubiquitination [97]. Consistent with these findings, *Itch*^{-/-} MEFs exhibit enhanced and prolonged NF- κ B signaling as observed in *Tnfaip3*^{-/-} and *Tax1bp1*^{-/-} MEFs. Despite the functional similarities observed between *Itch*^{-/-} and *Tnfaip3*^{-/-} MEFs, there are clear differences in the phenotypes of the knockout mice. Whereas A20-deficient mice exhibit spontaneous inflammation in multiple organs, the inflammation in Itch-deficient mice is mainly confined to the lungs and skin [100]. Furthermore, in *Tnfaip3*^{-/-} mice, the inflammation is independent of adaptive immunity, unlike *Itch*^{-/-} mice which are rescued from lethal inflammation by crossing with *Ragl*^{-/-} mice [97]. These *in vivo* differences may indicate a cell-type-specific functional interaction between A20 and Itch. Also, Itch clearly has functions in signaling and cell death that are independent of A20.

RNF11 is a RING-type E3 ligase overexpressed in breast, prostate and pancreatic cancers [101, 102]. RNF11 interacts with several regulators of the transforming growth factor beta (TGF- β) pathway such as Smurf2 and SMAD7 to potentiate TGF- β signaling [103, 104]. A high-throughput yeast two-hybrid screen identified over 70 putative binding proteins of RNF11 including A20, Itch, TAX1BP1, NEMO and ABIN-1 [105], suggesting

a possible link between RNF11 and NF- κ B signaling. Indeed, RNF11 inducibly interacts with TAX1BP1 and Itch in response to TNF- α or IL-1 stimulation [106]. Furthermore, siRNA knockdown of RNF11 enhances NF- κ B signaling, whereas overexpression of RNF11 downregulates NF- κ B, suggesting that RNF11 is a negative regulator of NF- κ B [106]. Knockdown of RNF11 is also associated with elevated levels of RIP1 and TRAF6 ubiquitination upon TNF- α or LPS stimulation, respectively [106]. A20 is also dependent on RNF11 to downregulate NF- κ B signaling, suggesting that RNF11 is a component of the multi-subunit A20 ubiquitin-editing protein complex together with A20, TAX1BP1 and Itch. Because the PPXY motif in RNF11 is essential for its NF- κ B regulatory function and is known to mediate binding to Itch [102], RNF11 may function as an adaptor for Itch or, alternatively, may regulate its catalytic activity. Determining the precise function of RNF11 *in vivo* will require the generation of gene-targeted mice.

ABIN-1 was initially identified in a yeast two-hybrid screen as an A20-interacting protein [70]. Overexpression of ABIN-1 inhibits NF- κ B signaling by multiple stimuli and mimics the NF- κ B inhibitory function of A20 [70, 107]. ABIN-1 is required for A20 to interact with and deubiquitinate NEMO, suggesting that ABIN-1 serves as an adaptor molecule that bridges A20 and NEMO [108]. Consistent with these findings, a recent study demonstrates that the Shigella effector protein IpaH9.8, which functions as an E3 ligase, hijacks ABIN-1 to interact with and ubiquitinate NEMO [109]. Furthermore, the ubiquitin-binding domain of ABIN-1 plays an important role in the adaptor function of ABIN-1 by promoting the interaction between IpaH9.8 and NEMO [109]. However, the exact role of NEMO ubiquitination in inflammatory signaling pathways remains unclear and ABIN-1-deficient MEFs do not exhibit major defects in NF- κ B regulation [110].

Another protein termed YMER (also known as CCDC50) was shown to interact with A20 in a yeast two-hybrid screen [111]. Overexpression of YMER downregulates NF- κ B signaling, whereas knockdown of YMER by siRNA enhances NF- κ B activation [111]. YMER contains a K63-linked polyubiquitin-binding motif that is essential for inhibiting NF- κ B [111]. Consistent with these findings, YMER was also identified in a screen for polyubiquitin-binding proteins [112]. Because YMER interacts with RIP1 and promotes an interaction of A20 with K63-linked polyubiquitin chains [111], YMER may also function as a ubiquitin-binding adaptor for A20. However, gene targeting studies of YMER are necessary to firmly establish its role as a regulator of A20 and NF- κ B.

Regulation of A20 deubiquitinase activity

The crystal structure of the OTU domain of A20 was determined by two groups, both suggesting a unique mechanism of catalytic function distinct from papain-like cysteine proteases [91, 113]. Although the overall structure of A20 differs from other cysteine proteases, the arrangements of catalytic histidine and cysteine residues are similar and conserved [113]. The catalytic cysteine residue (Cys103) in A20 resides within an alpha helix domain that is predicted to form a platform for ubiquitin binding [91]. Accordingly, Cys103 plays an important role in A20-mediated NF- κ B downregulation, suggesting a requirement for A20 DUB activity [67, 73, 94]. Conversely, others have reported that Cys103, and hence DUB activity, is dispensable for A20 to inhibit NF- κ B [66, 80]. Although the reasons for these discrepancies are unclear, they may be attributed to experimental factors such as varying amounts of transfected A20 and/or different genetic backgrounds of the cells (i.e. wild-type or A20-deficient). Nevertheless, it is clear that A20 inhibits antiviral signaling in a DUB-independent manner [114-116]. A20, together with TAX1BP1, inhibits antiviral signaling by disrupting a TRAF3-TBK1-IKKi protein complex via the C-terminal zinc finger domains [115].

Given that A20 opposes K63-linked polyubiquitination of RIP1 and TRAF6, it is surprising that A20 preferentially cleaves K48-linked polyubiquitin chains *in vitro* [91, 117]. It is likely that additional factors, possibly TAX1BP1, Itch and/or RNF11, determine A20 specificity *in vivo*. Another surprise from the A20 structural studies is that A20 cleaves polyubiquitin chains at the TRAF6/ubiquitin interface instead of disassembling ubiquitin chains in a step-wise manner [91]. Therefore, the specificity and catalytic activity of A20 are distinct from other OTU family cysteine proteases.

Although A20 is mainly regulated at the transcriptional level in a negative feedback loop, A20 is also subject to regulation by post-translational mechanisms. A20 is phosphorylated by IKK β on Ser381, leading to enhanced NF- κ B inhibition by A20 [118], although it is not clear if the DUB function of A20 is regulated by phosphorylation. A20 phosphorylation provides yet another layer of control by the NF- κ B pathway to terminate its activation in a negative feedback loop.

The role of A20 in autoimmune diseases and cancer

Dysregulation of A20 expression has been implicated in a number of autoimmune diseases and cancers. Polymorphisms within the A20 genomic locus increase susceptibility to a number of human autoimmune diseases,

including type 1 diabetes, psoriasis, rheumatoid arthritis, inflammatory bowel disease, celiac disease, coronary artery disease and SLE [119-124]. Presumably, the identified polymorphisms influence either the expression or function of A20. A recent study examined the functional effects of the African-derived A20 polymorphism A125V, which lies in the DUB domain [125]. The A20 A125V mutation impairs A20-mediated degradation, as well as deubiquitination, of TRAF2 [125]. The functional defects due to the mutation may result from a conformational change in A20 that alters protein-protein interactions.

A20 has also been implicated as a tumor suppressor in several subsets of B-cell lymphomas. Previous studies have demonstrated that chromosome 6q is frequently deleted in several types of non-Hodgkin lymphomas [126]. Inactivating point mutations and deletions are frequently found in the *A20* gene, encoded on chromosome 6q, in activated B-cell-like (ABC) diffuse large B-cell lymphoma (DLBCL), marginal zone lymphoma, MALT lymphoma, and Hodgkin lymphoma [127-130]. The *A20* promoter may also become methylated, leading to downregulation of A20 in MALT lymphoma [131]. Reintroduction of A20 into lymphoma cell lines with biallelic loss of A20 induces apoptosis, suggesting that A20 is indeed a tumor suppressor in B-cell lymphoma [128, 129].

Loss of A20 expression has also been demonstrated during oncogenic transformation of MEFs. Transformation of primary MEFs by E1A and Ras results in impaired TNF- α -induced expression of A20, despite upregulation of other NF- κ B-dependent genes [132]. Loss of A20 sensitizes transformed MEFs to TNF- α -mediated cell death despite enhanced NF- κ B activation [132]. The low A20 expression in transformed MEFs is attributed to impaired Bcl-3 binding to the *A20* promoter, suggesting yet another distinct mechanism of A20 downregulation in tumor cells.

Because A20 also functions as a potent prosurvival gene, A20 may even function as an oncogene in certain tumor types such as breast cancer and glioma. In breast cancer, A20 is an estrogen-regulated gene which confers resistance to the proapoptotic effects of tamoxifen [133]. A20 is overexpressed in estrogen receptor negative (ER-), progesterone receptor negative (PR-) and high-grade breast tumors suggesting that A20 may be a useful prognostic marker for aggressive breast carcinomas [133]. A20 is also overexpressed in glioma and is required for survival of glioma stem cells suggesting that the tumor stem cells may be "addicted" to A20 overexpression for survival [134]. Although A20 is commonly lost or mutated in B-cell lymphomas, recent large scale sequencing efforts of several tumor types (Catalogue of Somatic Mutations in Cancer (COSMIC): <http://www.sanger>.

ac.uk/genetics/CGP/cosmic/) suggest that A20 is not commonly mutated in other cancers. However, two novel A20 mutations V377M and P656L have been identified in lung carcinomas, although it is unclear if these mutations impact the function or stability of A20.

CYLD function and the regulation of NF- κ B

The cylindromatosis (CYLD) gene encodes a deubiquitinating enzyme of the USP family with a C-terminal catalytic domain, three N-terminal Cap-Gly domains and two proline-rich motifs (Figure 1) [135]. CYLD was originally identified as a tumor suppressor gene since mutations in the *Cyld* gene predispose individuals to tumors of skin appendages [136]. Three simultaneous publications demonstrated that CYLD negatively regulates NF- κ B signaling via DUB activity. Using an shRNA approach, Brummelkamp *et al.* showed that CYLD inhibits NF- κ B signaling by counteracting TRAF2 ubiquitination [137]. Two other groups used yeast two-hybrid screens to identify CYLD as a novel NEMO-interacting protein and negative regulator of NF- κ B activation [138, 139]. CYLD DUB activity is abolished by mutation of the conserved catalytic residue Cys601 [139], and CYLD DUB mutants are impaired in inhibiting NF- κ B signaling [139]. In addition to regulating NF- κ B, CYLD regulates a number of other pathways including c-Jun amino terminal kinase (JNK), p38 and retinoic acid-inducible gene I (RIG-I)-mediated innate antiviral signaling [140-143].

CYLD-deficient mice have been generated by multiple groups, revealing important roles for CYLD in immune cell development and homeostasis, as well as unexpected roles in osteoclastogenesis and spermatogenesis. *Cyld*^{-/-} mice exhibit reduced numbers of single positive CD4⁺ and CD8⁺ T cells in the thymus and periphery indicating an important role of CYLD in T-cell development [144]. Mechanistically, in thymocytes CYLD removes polyubiquitin chains from the Src family kinase Lck and promotes an interaction with its substrate Zap70 [144]. Therefore, CYLD is required for T-cell receptor (TCR) signaling in thymocytes. Conversely, peripheral T cells from *Cyld*^{-/-} mice are hyper-responsive to TCR stimulation and mediate spontaneous inflammation in the colon [145]. Thus, CYLD has developmentally-specific roles in regulating TCR signaling. In agreement with these results, thymocyte-restricted DUB-defective CYLD mutant mice lacking exon 9 exhibit severe defects in thymocyte development [146], underscoring the importance of CYLD DUB activity in thymocyte development. CYLD also plays essential roles in the development of natural killer T (NKT) cells by providing survival signals for immature NKT cells [147].

In mature T cells, CYLD targets TAK1 for inactivation to downregulate IKK and NF- κ B activation [145]. Specifically, CYLD removes K63-linked polyubiquitin chains from TAK1 that leads to its inactivation (Figure 2) [145]. CYLD also plays important roles in the development and activation of a variety of other immune cells. For example, B cells from *Cyld*^{-/-} mice display markers indicative of activation and are hyper-responsive to activation via the B-cell receptor (BCR) [148]. Although CYLD does not play a major role in B-cell development, there is a marked increase in marginal zone B cells in CYLD-deficient mice [148]. CYLD prevents the spontaneous activation of NF- κ B in B lymphocytes since *Cyld*^{-/-} B cells exhibit constitutive IKK and NF- κ B activation [148]. Taken together, these studies indicate that CYLD is essential to prevent spontaneous activation of NF- κ B in peripheral T and B lymphocytes and is important for the maintenance of immune homeostasis.

In addition to its crucial immune regulatory functions, CYLD also plays other important physiological roles including that in osteoclastogenesis and spermatogenesis. *Cyld*^{-/-} mice develop osteoporosis as a result of enhanced osteoclast differentiation [149]. CYLD-deficient osteoclast precursors are hypersensitive to RANKL signaling and produce more osteoclasts upon stimulation with RANKL [149]. CYLD inhibits RANK signaling by deubiquitinating TRAF6, and CYLD requires the adaptor molecule p62 to interact with TRAF6 [149].

CYLD is also an important regulator of spermatogenesis [150]. In the absence of CYLD, NF- κ B is turned on constitutively in testicular cells leading to the upregulation of antiapoptotic genes such as Bcl-2 and Bcl-xL and a block in the early wave of germ cell apoptosis [150]. *Cyld*^{-/-} testicular cells exhibit marked upregulation of RIP1 ubiquitination together with enhanced RIP1-IKK interactions [150]. Therefore, CYLD-mediated deubiquitination of RIP1 is an essential step in spermatogenesis.

A number of other *Cyld*^{-/-} or mutant mice have been generated further confirming the role of CYLD as an important negative regulator of NF- κ B and inflammation. B and T lymphocytes and macrophages from *Cyld*^{-/-} mice are hypersensitive to activating stimuli, resulting in enhanced NF- κ B and JNK signaling possibly due to increased TRAF2 and NEMO ubiquitination [151]. The *Cyld*^{-/-} mice have increased inflammation and tumor formation in a colitis-associated cancer model [151]. Another mouse strain was engineered to express a naturally occurring *Cyld* splice variant lacking exons 7 and 8 containing the TRAF2 and NEMO binding domains [152]. Mice expressing the *Cyld* splice variant have enlarged spleens and secondary lymphoid organs due to a dramatic accumulation of B lymphocytes as a result of

enhanced survival and expression of NF- κ B and TRAF proteins [152].

Because CYLD is an important tumor suppressor in the skin, CYLD-deficient mice are more prone to chemically induced skin tumors [153]. *Cyld*^{-/-} keratinocytes hyperproliferate and express higher levels of cyclin D1 in response to the mitogen 12-O-tetradecanoylphorbol-13 acetate (TPA) or ultraviolet (UV) light [153]. CYLD inducibly translocates to a perinuclear region where it binds to the I κ B family member Bcl-3 and removes K63-linked polyubiquitin chains, thus preventing nuclear translocation of Bcl-3-p50 and Bcl-3-p52 complexes [153]. Therefore, CYLD regulates Bcl-3 and cell proliferation in keratinocytes in a noncanonical pathway.

Since CYLD and A20 target many of the same substrates for deubiquitination, it is interesting to consider why no functional redundancy exists between these two proteins. It is likely that CYLD and A20 inhibit NF- κ B at different times during an inflammatory response. Whereas CYLD prevents spontaneous activation of NF- κ B, A20 is essential to terminate NF- κ B signaling in a negative feedback loop [135]. A20 is expressed at low levels in most cell types and is induced by NF- κ B activating stimuli. Therefore, A20 and CYLD serve precise and temporally distinct roles in inhibiting NF- κ B. It is also essential for NF- κ B to overcome repression by CYLD in response to activating stimuli like TNF- α or LPS. Indeed, CYLD is phosphorylated on multiple serine residues in a stimulus-dependent manner by IKK [154]. The phosphorylation is transient and is dependent on the NEMO subunit of IKK [154]. Furthermore, CYLD phosphorylation is required for TRAF2 ubiquitination and downstream activation of NF- κ B and JNK [154]. Therefore, IKK phosphorylates CYLD to inactivate its DUB function thus providing a window of NF- κ B activation prior to signal-induced termination by A20. This mechanism of CYLD inactivation has been subverted by the breast cancer oncogene IKK ϵ , which phosphorylates CYLD and inactivates its DUB function [155]. Presumably, inactivation of CYLD predisposes to enhanced NF- κ B activation and tumorigenesis.

Another key difference between A20 and CYLD is the specificity of CYLD for K63-linked polyubiquitin chains. *In vitro*, A20 preferentially cleaves K48-linked polyubiquitin chains whereas CYLD cleaves both K63-linked polyubiquitin chains as well as linear ubiquitin chains [91, 117, 156]. Therefore, A20 likely depends on ubiquitin-binding adaptor proteins such as TAX1BP1 and others to provide specificity. CYLD requires p62 as an adaptor molecule to interact with and deubiquitinate TRAF6 in RANK signaling in osteoclasts [149]. Whether CYLD uses distinct adaptors for other substrates such as

RIP1 is unknown.

The tumor suppressor role of CYLD

Cyld was originally identified as a gene mutated in familial cylindromatosis, an autosomal dominant condition characterized by the growth of benign tumors or cylindromas on skin appendages [136]. Familial cylindromatosis patients usually have germline mutations in *Cyld* and exhibit loss of heterozygosity of the remaining allele in the skin lesions [136]. The majority of the cylindromatosis mutations result in truncated CYLD proteins that lack DUB activity underscoring the importance of CYLD catalytic activity in its tumor suppressor function [157]. Emerging studies suggest that CYLD may play a broader role as a tumor suppressor for multiple cancers. For example, inactivating mutations have been identified in a number of NF- κ B regulatory proteins, including CYLD in multiple myeloma, a plasma cell malignancy exhibiting persistent canonical and noncanonical NF- κ B signaling [158, 159]. In T-cell acute lymphoblastic leukemia (T-ALL), CYLD expression is repressed by the Notch/Hes1 pathway to promote persistent IKK activation and cell survival [160]. CYLD expression is also downregulated in malignant melanoma by the transcription factor Snail [161]. As a result of CYLD downregulation, Bcl-3 displays persistent nuclear expression and upregulates cyclin D1 and N-cadherin, leading to enhanced proliferation and invasion of melanoma cells [161]. CYLD is also downregulated in other malignancies including colon and hepatocellular carcinomas [162].

It is likely that the tumor suppressor function of CYLD may not be entirely due to NF- κ B inhibition, since CYLD possesses a number of NF- κ B-independent functions. An shRNA screen for mitotic regulatory proteins identified CYLD; accordingly, knockdown of CYLD by siRNA delays entry of cells into mitosis [163]. This function of CYLD is independent of NF- κ B although it requires its catalytic activity [163]. A putative substrate of CYLD, Plk1, was also identified by mass spectrometry as a binding partner of CYLD [163]. Since loss of Plk1 or CYLD have a similar cell cycle phenotype, CYLD may regulate Plk1 activity by deubiquitinating K63-linked ubiquitin chains [163]. However, since CYLD regulation of the cell cycle may be considered pro-tumorigenic, it is not clear how this particular function of CYLD relates to cancer. Paradoxically, another study indicated that CYLD inhibits the mitotic kinase Aurora B by promoting its dephosphorylation via the PP2A phosphatase [164]. Additional studies are necessary to determine the complex roles of CYLD in cell cycle regulation.

CYLD contains three CAP-Gly domains commonly

found in microtubule-interacting proteins. Indeed, CYLD has been shown to interact with microtubules, predominantly via the first and second CAP-Gly domains [165]. CYLD promotes microtubule assembly by enhancing tubulin polymerization into microtubules [165]. CYLD also induces the acetylation of α -tubulin by inhibiting HDAC6 and thus counteracts the depolymerization of microtubules leading to an overall stabilization of the microtubule network [166]. As a result of its microtubule assembly function, CYLD also regulates angiogenesis by mediating the spread and migration of vascular endothelial cells [167]. The cell cycle regulatory function of CYLD may also be partly dependent on the modulation of microtubule stability by CYLD.

The Wnt signaling pathway is a key developmental pathway that is also linked to cancer. An siRNA screen identified CYLD as a negative regulator of Wnt signaling and β -catenin activation [168]. CYLD functions upstream of β -catenin by removing K63-linked polyubiquitin chains from the adaptor molecule Dishevelled (Dvl) [168]. Therefore, Wnt signaling is hyperactive in cylindromatosis skin tumors, suggesting a potential tumorigenic role in cylindromatosis. It will be interesting to examine Wnt/ β -catenin signaling in other tumor types exhibiting loss of CYLD.

Other DUBs targeting NF- κ B

Although A20 and CYLD clearly play vital and distinct roles in inhibiting NF- κ B, other DUBs may also regulate NF- κ B. For example, Cezanne is a member of the A20 family of cysteine protease DUBs [169]. Similar to A20, Cezanne is rapidly upregulated upon TNF- α stimulation and recruited to the TNFR, where it inhibits RIP1 ubiquitination and downstream IKK and NF- κ B signaling (Figure 2) [170]. Cezanne requires its DUB domain since a catalytically inactive mutant is impaired in inhibiting NF- κ B and RIP1 ubiquitination [170]. A recent study indicates that Cezanne preferentially hydrolyzes K11-linked polyubiquitin chains [171]. How this activity of Cezanne regulates NF- κ B signaling is unknown.

Ubiquitin-specific peptidase 21 (USP21) inhibits TNF-induced NF- κ B signaling by also promoting the deubiquitination of RIP1 (Figure 2) [172]. However, it is unclear why several DUBs including A20, Cezanne and USP21 are necessary to remove polyubiquitin chains from RIP1. One possibility is that these proteins may be working cooperatively in a large protein complex to inhibit RIP1 polyubiquitination. Regardless, gene targeting studies are essential to confirm the *in vivo* roles of Cezanne and USP21 in the regulation of NF- κ B signaling.

DUBs may also function downstream of RIP1 and

IKK in the NF- κ B signaling cascade. For example, the COP9 signalsome (CSN) inducibly interacts with I κ B α and regulates I κ B α stability and NF- κ B activation [173]. USP15 is a CSN-associated DUB that removes K48-linked polyubiquitin chains from I κ B α to enhance its stability and fine-tune NF- κ B activation [173].

Pathogens modulating NF- κ B activity via DUBs

Given the importance of A20 and CYLD in the negative regulation of NF- κ B signaling, it is not surprising that oncogenic viruses target these proteins for inactivation to promote persistent NF- κ B signaling. The human T-cell leukemia virus type I (HTLV-I) is etiologically linked to the development of a CD4+CD25+ malignancy termed adult T-cell leukemia (ATL). The HTLV-I genome encodes an oncogenic regulatory protein Tax that serves as a potent activator of NF- κ B [174]. Tax upregulates the expression of A20 via an NF- κ B binding site in the A20 promoter [175]. Tax also interacts with TAX1BP1, the ubiquitin-binding adaptor molecule for A20 [92, 176]. Indeed, it appears that Tax interacts with TAX1BP1 as a mechanism to disrupt its functions as an NF- κ B inhibitor and a coactivator for nuclear receptors [94, 176]. In the presence of Tax, TAX1BP1 binding to A20 and the E3 ligase Itch is impaired [94]. Furthermore, both A20 and Itch are impaired in the inducible binding to RIP1 when Tax is expressed in T cells [94]. Similarly, Tax also prevents the recruitment of A20 to TRAF6 in response to IL-1 stimulation [82]. Finally, Tax also prevents TNF- α -induced interaction of A20 with Ubc13 and the subsequent degradation of Ubc13 [82]. Taken together, it appears that Tax targets TAX1BP1, in part, to inhibit the function of A20 and promote persistent NF- κ B activation.

Other oncogenic viruses may also target A20 and its adaptor molecules. TAX1BP1 interacts with the human papillomavirus virus (HPV) E2 protein and enhances E2-dependent transcription and prevents its proteasomal degradation [177]. However, it is unclear if TAX1BP1 plays any role in NF- κ B activation in the context of HPV infection. LMP1, the Epstein Barr Virus (EBV)-encoded oncogenic protein, induces NF- κ B-dependent expression of A20 and also interacts with A20 [178, 179]. Although A20 displaces TRAF1 from the LMP1 complex [179], it is unclear if A20 retains NF- κ B inhibitory activity in the presence of LMP1 which promotes constitutive NF- κ B signaling. It is possible that LMP1 either inactivates A20 or turns on NF- κ B signaling in a manner that is insensitive to the inhibition by A20. A recent study has reported that A20 deubiquitinates IRF7 in EBV-infected Raji cells, thus providing evidence that A20 is indeed functional in

LMP1-expressing cells [180].

The HPV-encoded oncogenic protein E6 promotes hypoxia-induced NF- κ B activation by triggering the ubiquitination and proteasome-mediated degradation of CYLD [181]. Inactivation of CYLD leads to TRAF6 ubiquitination and IKK activation, thus promoting anchorage-independent growth of tumor cells [181]. Therefore, E6 targeting of CYLD appears to be an essential step in HPV-mediated tumorigenesis.

Conversely, pathogens such as viruses or bacteria may encode DUBs as a mechanism to inhibit NF- κ B and evade host immune responses. The Crimean Congo hemorrhagic fever virus (CCHFV) is a human nairovirus that causes hemorrhagic fever and lethality in 30% of infections [182]. The CCHFV-encoded L protein contains an OTU domain in its N-terminal region [183]. The OTU domain is also found in related nairoviruses Dugbe virus (DUGV) and Nairobi sheep disease virus. The L protein of CCHFV reduces levels of ubiquitinated and ISGylated proteins and downregulates TNF- α -mediated NF- κ B activation, presumably as a mechanism of immune evasion [183]. The bacterial pathogen *Yersinia*, the causative agent of plague, encodes a virulence factor YopJ that functions as a DUB for TRAF2, TRAF6 and I κ B α to inhibit NF- κ B and host proinflammatory responses [184]. YopJ does not appear to have specificity for K63-linked polyubiquitin chains since it also cleaves K48-linked polyubiquitin chains [184]. Collectively, certain pathogenic viruses and bacteria encode DUBs as a unique strategy of immune evasion by inhibiting NF- κ B.

Concluding remarks

Recent years have brought dramatic advances in our understanding of the mechanisms of NF- κ B regulation. DUBs such as A20 and CYLD have been shown to play prominent roles in inhibiting NF- κ B. However, many outstanding questions remain regarding the specific roles of DUBs in downregulating NF- κ B. For example, although it is clear that A20 relies on a number of adaptor and effector molecules to inhibit NF- κ B, what is the precise function of each of the molecules in the A20 ubiquitin-editing complex? Do these molecules play tissue-specific roles in NF- κ B regulation? Why are there several DUBs (i.e. A20, CYLD, Cezanne and USP21) that inhibit RIP1 ubiquitination downstream of TNFR signaling? What is the precise role of noncanonical polyubiquitin chains (i.e. K11-, K27-, K29-linked ubiquitin chains) in NF- κ B signaling and are these polyubiquitin chains counteracted by specific DUBs? Does CYLD require ubiquitin-binding adaptors to inhibit RIP1 ubiquitination? A more thorough understanding of how DUBs

regulate NF- κ B signaling may contribute to the design of novel therapies for inflammatory disorders and cancer.

Acknowledgments

We apologize for omitting any relevant references due to space limitations. The laboratory of EWH is supported by NIH grants PO1CA128115, RO1CA135362 and RO1GM083143.

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