

## Review Article

# Deubiquitinases in cell death and inflammation

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Apoptosis, pyroptosis, and necroptosis are distinct forms of programmed cell death that eliminate infected, damaged, or obsolete cells. Many proteins that regulate or are a part of the cell death machinery undergo ubiquitination, a post-translational modification made by ubiquitin ligases that modulates protein abundance, localization, and/or activity. For example, some ubiquitin chains target proteins for degradation, while others function as scaffolds for the assembly of signaling complexes. Deubiquitinases (DUBs) are the proteases that counteract ubiquitin ligases by cleaving ubiquitin from their protein substrates. Here, we review the DUBs that have been found to suppress or promote apoptosis, pyroptosis, or necroptosis.

## Introduction

Ubiquitination is the covalent, post-translational modification of a protein with the 8.4 kDa protein ubiquitin. An isopeptide bond is formed between the C-terminus of ubiquitin and a lysine side chain in the target protein, or less commonly, the C-terminal glycine is linked to either the N-terminus or a serine or threonine side chain of the protein [1–3]. Moreover, ubiquitin itself can be modified at its N-terminus or at one of its seven lysines allowing the assembly of polyubiquitin chains. Ubiquitination is mediated by the concerted action of ubiquitin-activating E1, ubiquitin-conjugating E2, and ubiquitin ligase E3 enzymes, resulting in altered protein stability, interactions, or localization. Deubiquitinating enzymes (DUBs) counter ubiquitin ligases by cleaving ubiquitin from their protein substrates (Figure 1). DUBs belong to the USP (ubiquitin-specific protease), UCH (ubiquitin C-terminal hydrolase), OTU (ovarian tumor), MINDY (motif-interacting with ubiquitin-containing novel DUB family), MJD (Machado–Josephin domain-containing), and JAMM (JAB1/MPN/Mov34) protease families [4]. Here we review our current understanding of DUBs that regulate the cell death programs of apoptosis, pyroptosis, and necroptosis.

## DUBs regulating caspase-8-dependent cell death and necroptosis

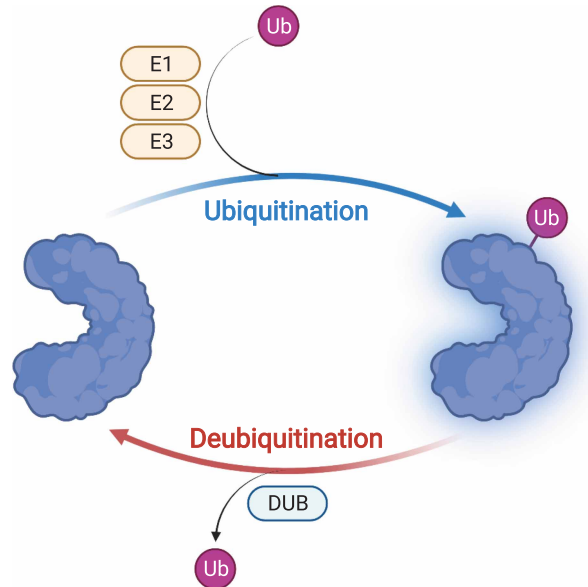
Tumor Necrosis Factor Receptor 1 (TNFR1), Toll-like Receptor 3 (TLR3), and TLR4 contribute to innate immune surveillance and defense against invading pathogens [5–7]. Endosomal TLR3 is activated by viral double-stranded RNA [6], whereas TLR4 on the plasma membrane responds to bacterial lipopolysaccharide [7]. TNFR1 responds to either TNF or lymphotoxin- $\alpha$  [8,9], the former produced by many cell types in response to infection. TNFR1, TLR3, and TLR4 each recruit ubiquitin ligases, including the linear ubiquitin chain assembly complex (LUBAC) [10–12], to build a ubiquitin scaffold for activating the protein kinases TAK1, IKK $\alpha/\beta$ , and IKK $\epsilon$ /TBK1 [13–17]. Activation of these kinases culminates in the transcription of proinflammatory genes, while formation of a secondary, death-inducing signaling complex is suppressed. Genetic [11,12,18–26], small molecule [17,20,27,28], or pathogen-induced perturbations [29] that compromise the assembly of the ubiquitin scaffold or activation of these kinases promotes the formation of the death-inducing complex. The nature of the perturbation governs whether the enzymatic activity of the kinase RIPK1 is required for assembly of the death-inducing complex (reviewed in [30]).

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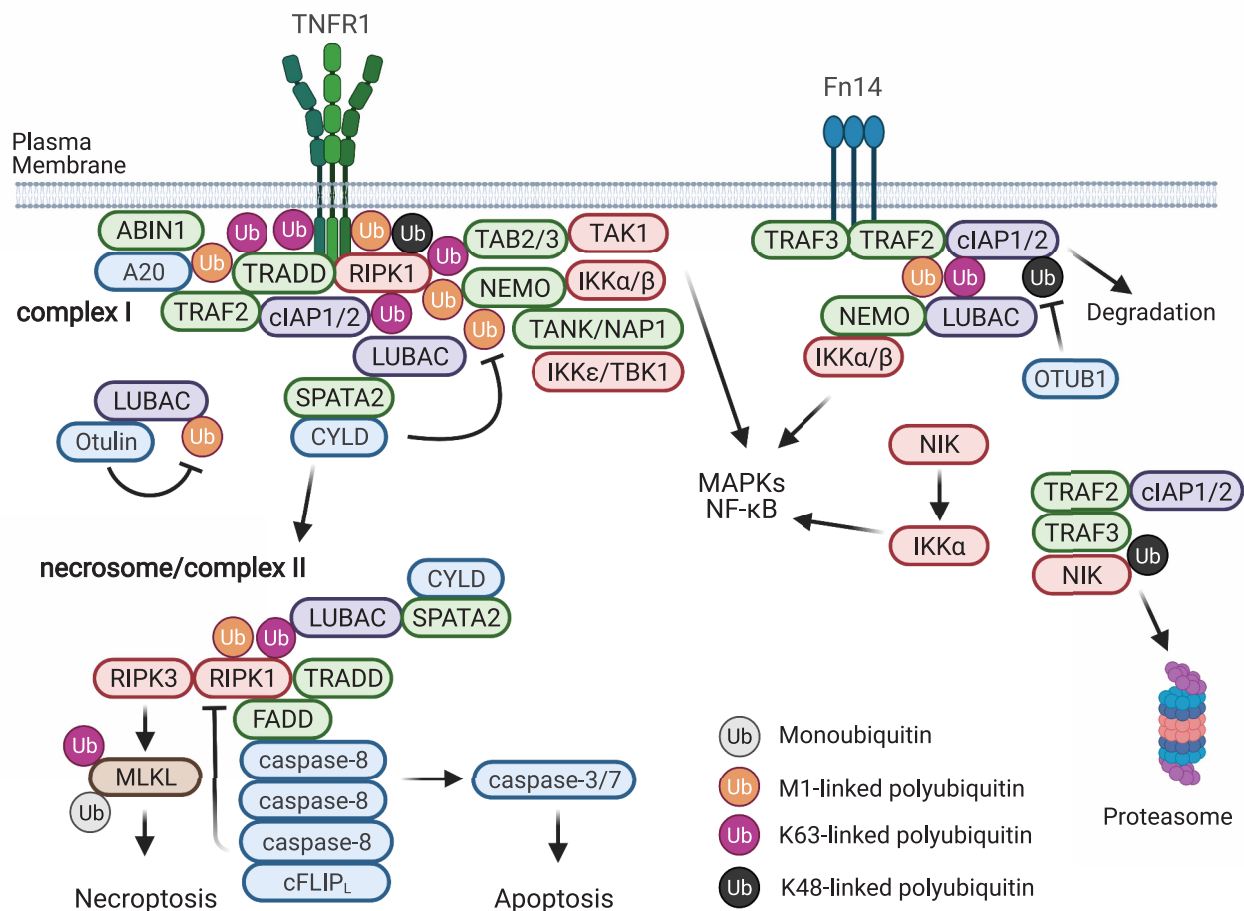
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**Figure 1. Deubiquitinases (DUBs) counteract protein modifications made by E3 ubiquitin ligase enzymes.**  
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In the case of the more extensively studied TNFR1, ligation of the receptor triggers the assembly of the TNFR1-associated signaling complex termed complex I [31] (Figure 2). The cytoplasmic death domain (DD) of TNFR1 recruits the DD-containing proteins TRADD and RIPK1 via homotypic interactions, with TRADD recruiting TRAF2, the adaptor for the E3 ubiquitin ligases cellular inhibitor of apoptosis protein 1 (cIAP1) and cIAP2 [32–37]. Subsequently, cIAP1/2 modify themselves and RIPK1 with lysine 63 (K63)-linked polyubiquitin and this contributes to the recruitment of TAB2 and TAB3, ubiquitin-binding adaptors for the kinase TAK1 [13,38]. K63-linked polyubiquitin within complex I also recruits LUBAC, composed of HOIP (also called RNF31), HOIL-1 (also called RBCK1), and SHARPIN, as both HOIP and SHARPIN bind to K63-linked polyubiquitin [10,15,39]. LUBAC then modifies several proteins in TNFR1 complex I with M1-linked polyubiquitin, including TNFR1 itself, TRADD, and RIPK1 [40–43]. Indeed, LUBAC can modify K63-linked polyubiquitin on RIPK1 with M1-linked ubiquitin to create hybrid polyubiquitin chains [16]. The M1-linked polyubiquitin in complex I recruits NEMO (also called IKK $\gamma$ ), the ubiquitin-binding regulatory subunit of the canonical I $\kappa$ B kinase (IKK) [14,44,45]. Hybrid polyubiquitin chains may position TAK1 and IKK to facilitate the activating phosphorylation of IKK by TAK1 [15,16,46]. Collectively, these molecular events stabilize complex I for productive signal transduction.

Many cells are not killed by TNF because the formation of a death-inducing complex II is transient and unproductive [31]. TRADD and RIPK1 move into the cytoplasm where they interact with FADD, caspase-8, and the long isoform of cFLIP (cFLIP<sub>L</sub>) [31], but cleavage of RIPK1 by the caspase-8/cFLIP<sub>L</sub> heterodimer then disrupts the complex [47–50]. Accordingly, heterozygous mutations altering the aspartic acid cleavage site in RIPK1 sensitize cells to TNF killing [48–50]. Notably, these mutations give rise to an autoinflammatory syndrome in humans [49,50]. Loss of the labile protein cFLIP, as in cells treated with the translational inhibitor cycloheximide, also sensitizes to TNF-induced cell death [31]. In this case, cells die because caspase-8 homodimers assemble within a stabilized complex II, autoprocess, and then cleave and activate caspases 3 and 7 to execute the apoptotic program [51–53]. If caspase-8 is eliminated or inactivated, however, cells may still die because RIPK1 in complex II can interact with RIPK3, if it is expressed, to form the necrosome [54–56]. Activation of RIPK3 within the necrosome leads to phosphorylation of the pseudokinase MLKL, which then mediates a lytic form of cell death termed necroptosis [57–61]. TLR3 and TLR4 utilize slightly different combinations of adaptor proteins and E3 ubiquitin ligases when compared with TNFR1, but they elicit similar death-inducing signaling complexes if LUBAC [11] or caspase-8 is compromised [62,63].



**Figure 2. Deubiquitinases OTULIN, CYLD, and OTUB1 modulate sensitivity to TNF-induced cell death.**

OTULIN suppresses cell death by removing M1-linked polyubiquitin from LUBAC and preserving its ligase activity. LUBAC modifies components of TNFR1 complex I with M1-linked polyubiquitin, which serves as a scaffold for the recruitment of kinases that activate gene expression via the MAPK and NF- $\kappa$ B pathways. Stabilization of complex I in this manner limits assembly of the death-inducing complex II/necrosome. CYLD promotes cell death by removing K63- and M1-linked polyubiquitin from components of TNFR1 complex I, which facilitates complex II/necrosome assembly. OTUB1 limits sensitization to TNF-induced cell death by TWEAK, which is the ligand for the receptor Fn14. cIAPs recruited to Fn14 modify the signaling complex with K48-linked polyubiquitin and target it for degradation. By removing this polyubiquitin, OTUB1 preserves the pool of cIAP1 available for recruitment into TNFR1 complex I. Within TNFR1 complex I, cIAP1 builds a K63-linked polyubiquitin scaffold that limits activation of the kinase RIPK1 and complex II/necrosome formation. Whether there are DUBs that limit ubiquitination of RIPK1 in the necrosome [216] or ubiquitination of MLKL [214,215] is unclear. Adaptor proteins are colored green, E3 ubiquitin ligases are purple, kinases are red, and proteases, including DUBs, are blue. Figure created with BioRender.com.

## A20

The deubiquitinase and ubiquitin-binding protein A20 (also called TNFAIP3) confers a degree of protection against TNF-induced apoptosis [64–66] or necroptosis [43,67,68]. Accordingly, loss of A20 in intestinal epithelial cells sensitizes mice to TNF toxicity that requires, in part, the kinase activity of RIPK1 [66,69,70]. It is worth noting, however, that enforced expression of A20 in intestinal epithelial cells also sensitizes mice to TNF toxicity driven by the kinase activity of RIPK1 [71]. Thus, the expression of *Tnfaip3*, which is an NF- $\kappa$ B-inducible gene [72], must be finely tuned for optimal signal transduction.

A20 is recruited to TNFR1 complex I by virtue of its ubiquitin-binding zinc finger 4 (ZnF4) and ZnF7 motifs [73–78]. ZnF4 binds to monoubiquitin or K63-linked polyubiquitin [79], while ZnF7 binds to

M1-linked polyubiquitin [73,74]. The ubiquitin-binding protein ABIN1 has also been implicated in the recruitment of A20 to TNFR1 complex I [80]. Ubiquitin binding by ZnF7, in particular, appears crucial for A20 to suppress TNF-induced NF- $\kappa$ B activation and cell death [66,73,74,77,78]. In mice, mutation of A20 ZnF7 results in TNF-dependent arthritis [78]. Mutation of A20 ZnF7 and ZnF4, however, results in lethal inflammation soon after birth [77,78] similar to A20 deficiency [65]. It is unclear if aberrant cell death is a major driver of lethality in either model. RIPK3 deficiency, but not MLKL deficiency, prolongs survival of A20-deficient mice [68,81], but the effect of eliminating both MLKL and caspase-8 to disable both caspase-8-dependent cell death and necroptosis has not been reported. When A20 deficiency is restricted to myeloid cells, however, mice develop arthritis that requires TLR4, RIPK3, and MLKL, but not TNFR1 [76,82]. These genetic data indicate that suppression of necroptosis is an important physiological function of A20 not only in the context of TNFR1 signaling. Consistent with A20 also suppressing caspase-8-dependent cell death, lethal inflammation in mice lacking both ABIN1 and A20 in intestinal epithelial cells is prevented by the combined loss of RIPK3 and caspase-8 [83].

Despite having an OTU domain that cleaves K48- or K63-linked polyubiquitin *in vitro* [84], the deubiquitinating activity of A20 appears largely dispensable for suppressing inflammation [43,75,85,86]. Mutation of the OTU catalytic cysteine in mice does not give an overt phenotype [75,85,86], although the mice are more sensitive to TNF toxicity [86]. Whether RIPK1 is a substrate of A20 in this context requires further study. Overall, available data indicate that A20 binding to polyubiquitin in TNFR1 complex I is more important than its DUB activity for suppressing complex II assembly. Recruitment of A20 to TNFR1 complex I may preserve the ubiquitin scaffold by protecting polyubiquitin from cleavage by the death promoting DUB CYLD (described in the next section) [43]. In keeping with this notion, M1-linked polyubiquitin in TNFR1 complex I is reduced by A20 deficiency or mutation of A20 ZnF7 [43,66,76,77].

## CYLD

CYLD (encoded by the cylindromatosis gene) promotes assembly of the TNFR1-induced necrosome [87–89]. Accordingly, knockdown, deletion, or inactivation of CYLD renders cells less sensitive to TNF-induced necroptosis [88–91]. CYLD also promotes, to varying degrees, caspase-8-dependent cell death induced by TNF plus cIAP antagonist [87], TNF plus cycloheximide [92], and TNF plus SHARPIN deficiency [93]. The role of CYLD in promoting cell death is evident in mice as well as cell culture. For example, inactivation of CYLD prevents RIPK3- and MLKL-dependent colitis in the FADD-deficient mouse intestine [91], and ameliorates RIPK3-dependent inflammation in the FADD-deficient mouse epidermis [94]. CYLD deficiency ameliorates TNFR1-, FADD- and RIPK1-dependent skin inflammation in SHARPIN-deficient mice [18,19,93,95].

CYLD is recruited to TNFR1 complex I via the adaptor protein SPATA2, which in turn binds to HOIP within LUBAC [96–99]. A PUB domain-interacting motif (PIM) in SPATA2 binds to the same HOIP PUB domain as the PIM in the DUB OTULIN (described in the next section). Consequently, OTULIN and CYLD exhibit mutually exclusive recruitment to LUBAC [43]. CYLD cleaves M1- or K63-linked ubiquitin chains [100], with phosphorylation of CYLD boosting its activity towards K63-linked polyubiquitin [101]. Contrary to expectations, however, ubiquitination of RIPK1 and TNFR1 in complex I is either unchanged or decreased, rather than increased in cells lacking either CYLD or SPATA2 [92,96,99,102]. Although one study reported that SPATA2 deficiency increased M1-linked polyubiquitin in TNFR1 complex I [97], others found that SPATA2 or CYLD deficiency decreased both M1- and K63-linked polyubiquitin in complex I [99,101]. Nonetheless, when whole cell lysates are analyzed, TNF-induced ubiquitination of RIPK1, TNFR1, and TRADD is increased by CYLD or SPATA2 deficiency [43,92,99,102]. M1-linked polyubiquitination, in particular, appears increased on RIPK1 [98]. These alterations, in the context of the necroptosis stimulus TNF plus zVAD, coincide with reduced activation of RIPK1 and reduced necrosome assembly [102]. Thus, the accumulation of polyubiquitin on TNFR1 complex I in cells lacking SPATA2 or CYLD may promote dissociation of complex I, thereby limiting dimerization and autophosphorylation of RIPK1, which in turn limits the ability of RIPK1 to engage RIPK3 [54,55,103,104]. SPATA2 and CYLD have also been observed in the TNF-induced necrosome [102], so deubiquitination of CYLD substrates may be important in both complexes. Whether hybrid polyubiquitin chains on complex I components influence deubiquitination by CYLD is unclear. In the context of interleukin-1 receptor signaling, however, modification of K63-linked ubiquitin chains with K48-linked chains is reported to protect K63-linked polyubiquitin from cleavage by CYLD [105].

Despite SPATA2- or CYLD-deficient cells being less sensitive than their wild-type counterparts to various forms of TNF-induced cell death, SPATA2-deficient mice are actually more sensitive than wild-type mice to

TNF toxicity. Moreover, this toxicity requires the kinase activity of RIPK1 [102]. How SPATA2 suppresses activation of RIPK1 in this context and whether CYLD-deficient mice are also more susceptible to TNF toxicity is unclear. Chronic NF- $\kappa$ B activation in intestinal epithelial cells sensitizes mice to TNF toxicity [106] and an early study identified CYLD as a negative regulator of TNF-induced NF- $\kappa$ B signaling [107]. However, it is unclear if there is aberrant NF- $\kappa$ B activation in SPATA2-deficient mouse intestines. Mouse macrophages, fibroblasts and keratinocytes lacking SPATA2 or CYLD exhibit, at best, a modest enhancement in TNF-induced activation of MAPKs or NF- $\kappa$ B [97,98,102,108–110].

Although SPATA2-deficient mice and several different strains of CYLD-deficient mice are viable [102,108–111], mice expressing inactive CYLD, owing to truncation of the C-terminal USP domain, die soon after birth [112]. Whether this lethality reflects a gain-of-function of the mutant CYLD scaffold remains unclear. In humans, germ-line mutations in *CYLD* are associated with a predisposition to tumors of skin appendages, with a majority of the disease-causing mutations predicted to C-terminally truncate CYLD [113]. Details of the pathway(s) perturbed in this setting by aberrant ubiquitination are unclear.

## OTULIN

OTULIN (OTU DUB with linear linkage specificity; also known as FAM105B or GUMBY) cleaves M1-linked polyubiquitin with exquisite specificity via substrate-assisted catalysis, a mechanism in which selective binding of OTULIN to M1-linked polyubiquitin activates its catalytic triad [41]. Strikingly, patients carrying biallelic loss-of-function *OTULIN* mutations develop a severe autoinflammatory syndrome termed OTULIN-related autoinflammatory syndrome (ORAS; also known as Otulipenia). These patients suffer from recurrent fevers, skin rashes, panniculitis, arthritis, and diarrhea, among other symptoms, and have been successfully treated with TNF-blocking therapeutics [114–118], highlighting the essential role of OTULIN in regulating TNF signaling.

In addition to its OTU domain, OTULIN possesses a PIM domain that interacts with the PUB domain in HOIP, and a PDZ-binding motif that interacts with the PDZ-containing protein SNX27 [41,119–121]. The physiological significance of the OTULIN-SNX27 interaction is unclear. Intriguingly, although OTULIN binds to HOIP, only HOIP is readily detected within TNFR1 complex I [43]. Why the CYLD-SPATA2-HOIP complex associates with complex I, but the OTULIN-HOIP complex does not remains unknown. One study that characterized complex I using mass spectrometry detected a small amount of OTULIN [96], raising the possibility that OTULIN is actively excluded from complex I. Cells lacking OTULIN or expressing catalytically inactive OTULIN contain more total M1-linked polyubiquitin than their wild-type counterparts, but have less M1-linked polyubiquitin in complex I [22,43,117]. The latter may stem from decreased expression of LUBAC components and/or reduced recruitment of LUBAC to complex I [22,115,117,122–124]. In some cell types, however, *OTULIN* mutations have less of an impact on LUBAC levels [117,118]. Thus, distinct *OTULIN* mutations and/or cell types may give rise to variable effects on LUBAC levels.

OTULIN bound to HOIP is thought to sustain LUBAC levels by cleaving M1-linked polyubiquitin attached to LUBAC itself [22,43]. Autoubiquitination of LUBAC is mediated by HOIL-1 monoubiquitinating itself, HOIP, and/or SHARPIN [2,125], and then HOIP modifying this monoubiquitin with M1-linked polyubiquitin [125]. The dynamic exchange of LUBAC components between OTULIN-containing complexes and the complexes assembled by receptors such as TNFR1 is poorly understood. Phosphorylation of tyrosine 56 within the OTULIN PIM limits HOIP binding [119,120], and appears to be increased in cells undergoing TNF-induced necroptosis [126], but whether this post-translational modification is crucial for OTULIN- and LUBAC-dependent functions *in vivo* remains to be shown.

By diminishing LUBAC activity, OTULIN deficiency destabilizes TNF-induced complex I and promotes the formation of complex II, leading to increased cell death [22,117,122–124,127]. Homozygous mutations compromising OTULIN DUB activity in mice cause embryonic lethality owing to excessive cell death, particularly among endothelial cells [22,128]. Embryonic lethality is prevented by the combined loss of RIPK3 and caspase-8, although the mice still die perinatally from RIPK1-dependent inflammation [22]. Systemic inactivation of OTULIN in adult mice [22], or *Otulin* deletion in keratinocytes [123,127], leads to severe inflammation that is ameliorated by *Tnfr1* deletion or the combined loss of RIPK3/MLKL-dependent necroptosis and FADD/caspase-8-dependent cell death. In contrast, while *Otulin* deletion in hepatocytes also produces a severe inflammatory phenotype, this is not ameliorated by *Tnf* or *Tnfr1* deletion [122,124], but is improved by *Fadd* deletion [124]. These data are consistent with OTULIN preventing autoinflammation by suppressing aberrant cell death, with intriguing differences observed in the TNF-dependence of these death programs in different cell types.

Similar phenotypes have been reported for mice deficient in HOIL-1 [21], further supporting the notion that LUBAC and OTULIN act in concert to favor signal transduction over cell death in TNF signaling.

In humans, loss of function mutations in LUBAC components cause a syndrome characterized variably by systemic autoinflammation, immunodeficiency, and amylopectinosis [129–131]. Thus, while these conditions partially overlap with those of ORAS, they differ in key features as well. Overall, these findings point to a model in which OTULIN and LUBAC act cooperatively in a linear pathway downstream of TNFR1 that favors complex I-mediated signaling over complex II-driven cell death. Functions of OTULIN and LUBAC that may be independent of one another, and their importance in certain cell types, remain an important area of study. The mechanism by which autoubiquitination destabilizes LUBAC and/or alters its activity also awaits elucidation.

## OTUB1

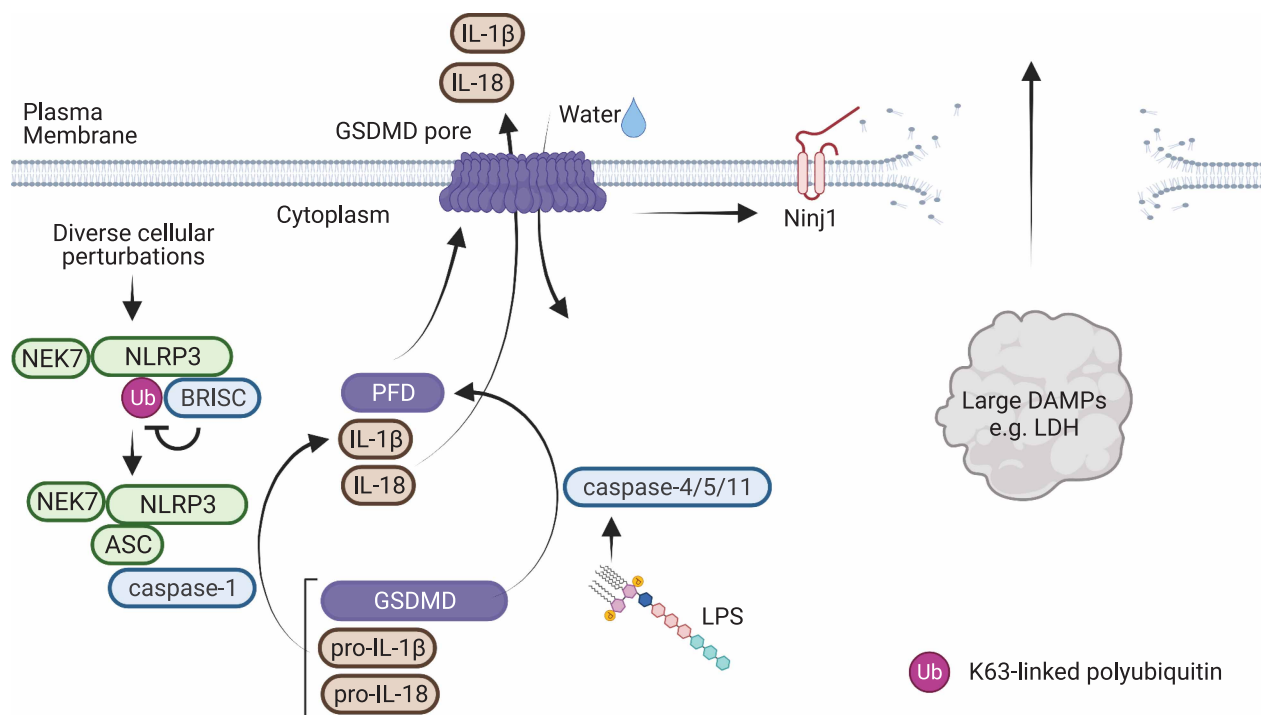
OTU deubiquitinase, ubiquitin aldehyde binding 1 (OTUB1) suppresses TNF-induced cell death in a manner that is distinct from A20 and OTULIN [132]. It functions in the signaling complexes of TNF receptor family members, such as Fn14, that recruit cIAP1/2, TRAF2, and TRAF3 (Figure 2). These receptors activate MAPKs and NF- $\kappa$ B, leading to increased expression of many genes, including *Tnf*. Both the canonical and non-canonical NF- $\kappa$ B pathways are stimulated. The non-canonical pathway is mediated by the kinase NIK, which is freed from constitutive cIAP-dependent ubiquitination and degradation when cIAP1/2, TRAF2, and TRAF3 are sequestered by the ligated receptor [27,28,133,134]. cIAP-dependent ubiquitination of the receptor complex eventually culminates in the degradation of cIAP1/2, which sensitizes cells to TNF-induced apoptosis [135,136]. Cell death is tempered by OTUB1 removing the K48-linked polyubiquitin on cIAP1 that marks it for degradation [132]. Accordingly, loss of OTUB1 in certain cell lines exacerbates apoptosis induced by TWEAK, the ligand for Fn14.

Interestingly, loss of OTUB1 in hepatocytes sensitizes mice to intravenous infection with *Listeria monocytogenes* in an MLKL-dependent manner. Thus, exacerbated pathology is due to aberrant necroptosis rather than apoptosis [137]. However, the use of full body *Mlkl* knockout mice makes it unclear if MLKL acts in Kupffer cells and/or hepatocytes of the infected liver. The role of RIPK3, which is difficult to detect in healthy hepatocytes [138], was not assessed genetically. Restricting deletion of both *Otub1* and *Mlkl* (or *Otub1* and *Ripk3*) to hepatocytes would be informative. Another study suggested that MLKL inhibits *Listeria* replication in epithelial cells without inducing necroptosis [139]. Thus, the mechanisms underlying MLKL-dependent pathology in *Listeria*-infected *Otub1* hepatocyte-specific knockout mice warrant further study.

## DUBs regulating pyroptosis

Pyroptosis is a lytic form of cell death mediated by members of the gasdermin family [140]. Gasdermins are intracellular proteins expressed in latent form that promote cell death after their pore-forming domain (PFD) is liberated by proteolytic cleavage. For example, gasdermin D (GSDMD) induces pyroptosis after it is cleaved by human caspases 1, 4, and 5 (mouse caspases 1 and 11) (Figure 3). These caspases get activated when cells are exposed to pathogen-derived molecules (examples include toxins, cytoplasmic lipopolysaccharide (LPS), and cytoplasmic DNA) or sterile insults (examples include uric acid and cholesterol crystals, which are associated with gout and atherosclerosis, respectively) [141,142]. The N-terminal PFD of GSDMD, having been released from its C-terminal inhibitory domain, assembles oligomeric pores in the plasma membrane that disrupt the electrochemical gradient and release small proteins such as IL-1 $\alpha$ , IL-1 $\beta$ , and IL-18 [143–147]. Subsequent rupture of the plasma membrane through the ill-defined activity of membrane protein NINJ1 then allows larger intracellular components, including lactate dehydrogenase (LDH), to escape the dying cell [148]. In other contexts, caspase-8, neutrophil elastase or cathepsin G may cleave GSDMD to unleash pyroptosis [29,149,150].

Caspase 1 is activated within canonical inflammasome complexes whose makeup is governed by the nature of the cellular insult. The NLRP3-ASC inflammasome activates caspase-1-dependent pyroptosis in response to diverse cellular perturbations, including extracellular ATP, bacterial toxin nigericin, non-canonical caspase-11-dependent pyroptosis, and RIPK3-dependent cell death [141,151–153]. In many cell types, including mouse macrophages and hepatocytes, optimal activation of the NLRP3 inflammasome relies on transcriptional up-regulation of *Nlrp3* gene expression by NF- $\kappa$ B [154,155]. This priming step is satisfied in culture by treatment with TLR agonists, including LPS. Loss of WDR48 (also called UAF1), a cofactor that stimulates the



**Figure 3. Deubiquitinase BRISIC promotes pyroptosis induced by the NLRP3 inflammasome.**

Assembly of the NLRP3 inflammasome is triggered by a range of cellular insults. BRISIC interacts with NLRP3 in complex with NEK7 and removes K63-linked polyubiquitin from NLRP3. Deubiquitination of NLRP3 is required for NLRP3 to interact with ASC, the adaptor for caspase-1. Activation of caspase-1 within the NLRP3-nucleated complex results in cleavage of its substrates pro-IL-1 $\beta$  and pro-IL-18, yielding the biologically active cytokines. Caspase-1 also cleaves GSDMD, producing an N-terminal fragment that assembles oligomeric pores in the plasma membrane capable of releasing IL-1 $\beta$  and IL-18. GSDMD pores also disrupt the electrochemical gradient and kill the cell. Pyroptosis culminates in large scale rupture of the plasma membrane, which is mediated by the membrane protein NINJ1. Precisely how NINJ1 triggers membrane breakdown and the release larger intracellular proteins such as lactate dehydrogenase (LDH) is unclear. Other proteases that can elicit the pore-forming fragment of GSDMD include human caspase-4 (mouse counterpart caspase-11) and caspase-5, which are activated by cytosolic lipopolysaccharide (LPS). Figure created with BioRender.com.

DUB activity of USP1, USP12, and USP46 [156–158], impairs LPS-induced up-regulation of NLRP3 in mouse macrophages [159]. Therefore, WDR48-associated DUBs may facilitate pyroptosis indirectly.

## BRISIC

The K63 linkage-specific DUB BRCC3 (also called BRCC36) [160] promotes activation of the NLRP3 inflammasome by deubiquitinating NLRP3 [161,162] (Figure 3). Belonging to the JAMM DUB family, BRCC3 may cleave K63-linked polyubiquitin that is conjugated to NLRP3 by the ubiquitin ligase RNF125 [163]. BRCC3 is part of the BRCC3 isopeptidase complex (BRISIC), wherein the activity of BRCC3 is dependent on interactions with the pseudoDUB ABRAXAS2 (also called KIAA0157 and ABRO1) [164]. Accordingly, ABRAXAS2-deficient mouse macrophages phenocopy BRCC3-deficient macrophages and exhibit impaired NLRP3-dependent processing of caspase-1, despite evidence of normal priming [162]. Aberrant ubiquitination of NLRP3 in ABRAXAS2-deficient cells appears to limit interactions between NLRP3 and ASC, rather than target NLRP3 for degradation.

Biochemical experiments suggest that ABRAXAS2 and BRCC3 associate with NLRP3 after priming. This interaction requires phosphorylation of NLRP3 serine 194 and the NLRP3-interactor NEK7. However, ABRAXAS2- and BRCC3-dependent deubiquitination of NLRP3 also requires an NLRP3 activation stimulus [162,165]. Thus, priming is proposed to recruit BRISIC to NLRP3 so that it is poised to deubiquitinate NLRP3 upon receipt of an activation stimulus [162]. Activating stimuli may induce conformational changes in NLRP3 that facilitate its deubiquitination by BRISIC. The autoactivating mutant NLRP3 A350V (equivalent to human

Muckle–Wells syndrome mutant NLRP3 A352V) is deubiquitinated in macrophages after priming alone [162], consistent with this mutation destabilizing the inactive conformation of NLRP3 [166].

Interestingly, BRISC is bound and inhibited by the inactive form of the metabolic enzyme serine hydroxymethyltransferase 2 (SHMT2) [167,168], suggesting a connection between metabolism and deubiquitination of BRISC substrates. Contrary to what might be expected, however, interactions between SHMT2 and BRISC are required for the latter to deubiquitinate type I interferon (IFN) receptor IFNAR1 at the cell surface [167,169]. Deubiquitination of IFNAR1 limits internalization and lysosomal degradation of the receptor, and thereby promotes IFN signaling. It was suggested that SHMT2 involvement in substrate targeting combined with its reversible inhibition of BRISC, perhaps through its displacement by K63-linked polyubiquitin on BRISC substrates, might prevent non-specific BRISC DUB activity [167]. Deubiquitination of the HIV-1 Tat protein by BRISC has also been shown to require SHMT1 or SHMT2 [170]. Whether deubiquitination of NLRP3 by BRISC is dependent on SHMT enzymes has not been investigated.

Small molecule inhibitors of JAMM DUBs, thiolutin and holomycin, limit pyroptosis and inflammation induced by both wild-type and autoactivating NLRP3 mutants, in large part by inhibiting BRCC3 [171]. Therefore, inhibition of BRISC with more specific inhibitors may represent an alternative strategy to NLRP3 inhibitors for the treatment of NLRP3-driven diseases. However, effects on other BRISC substrates, including IFNAR1 [169] and JAK2 [172] must be considered. Further complicating matters, BRCC3 has been implicated in oligodendrocyte differentiation [173], plus BRCC3 and some of the other BRISC components also function in the nuclear BRCA1-A complex involved in DNA repair [168].

## Other DUBs

In contrast with BRCC3, CYLD and its binding partner SPATA2 suppress activation of the NLRP3 inflammasome [174]. Mechanistically, it was suggested that CYLD deubiquitinates centrosomal PLK4, leading to interactions between NEK7 and PLK4 at the centrosome that interfere with inflammasome assembly by preventing interactions between NEK7 and NLRP3. NEK7 serves as a scaffold bridging adjacent NLRP3 subunits [166]. A20 also suppresses activation of the NLRP3 inflammasome [175]. Eliminating A20 from mouse macrophages causes arthritis that involves NLRP3, ASC, IL-1R, RIPK3, and MLKL [76,176]. RIPK3-dependent cell death can activate the NLRP3 inflammasome [152,153]. Therefore, aberrant necroptosis of A20-deficient macrophages is thought to activate the NLRP3-ASC-caspase-1 inflammasome to process pro-IL-1 $\beta$  into biologically active IL-1 $\beta$  [76]. Cleavage of GSDMD by caspase-1 and subsequent pyroptosis are probably dispensable because MLKL-dependent necroptosis suffices to release proinflammatory IL-1 $\alpha$  and IL-1 $\beta$  [76].

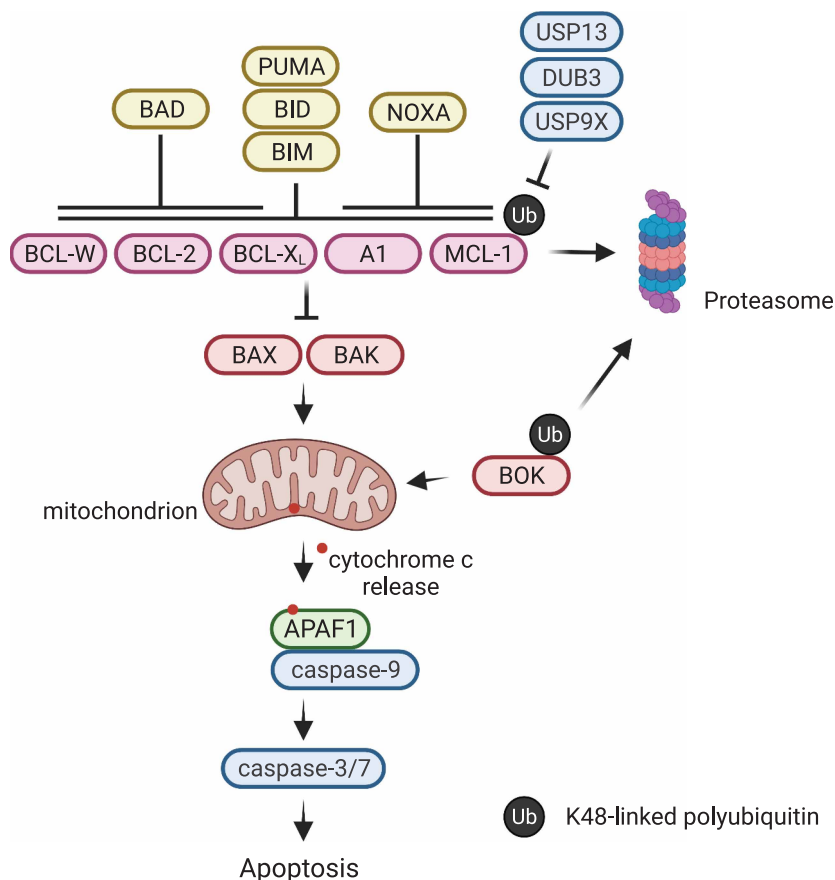
DUBs regulating activation of the other inflammasomes have been described, but the details are still emerging. For example, USP21 was shown to deubiquitinate and stabilize AIM2 that is activated by cytosolic double-stranded DNA [177], whereas CYLD may remove K63-linked polyubiquitin from NLRP6 to suppress inflammasome activation in mice infected with *Citrobacter rodentium* [178].

## DUBs regulating intrinsic apoptosis

The intrinsic apoptosis pathway is triggered by diverse cellular insults, including DNA damage, oncogene activation, and survival factor withdrawal. The pathway is regulated by members of the BCL-2 protein family, which feature up to four BCL-2 homology domains (BH1-4). Death is unleashed when the BH3-only proteins (BAD, BID, BIK, BIM, BMF, HRK, NOXA, or PUMA) are up-regulated. BH3-only proteins bind to a selection of their pro-survival relatives (BCL-2, BCL-2 related protein A1, BCL-W, BCL-X<sub>L</sub>, or MCL-1) and prevent them from sequestering the pro-apoptotic effectors BAK and BAX (Figure 4). Certain BH3-only proteins may also activate BAK and BAX directly. Oligomerization of BAX and BAK leads to permeabilization of the outer mitochondrial membrane and cytochrome c is released into the cytoplasm. Interactions between cytochrome c and cytoplasmic APAF1 lead to assembly of the apoptosome complex that activates caspase-9, the apical caspase in a proteolytic cascade that dismantles the cell (reviewed by [179,180]). Another pro-apoptotic effector, BOK, appears to disrupt mitochondria and trigger apoptosis only when it escapes ubiquitin-dependent proteasomal degradation mediated by the gp78 ubiquitin ligase complex [181]. Whether there is a DUB that can reverse the constitutive ubiquitination of BOK is unclear.

MCL-1 is the most labile of the pro-survival proteins, being modified with K48-linked polyubiquitin and targeted for proteasomal degradation by several ubiquitin ligases, including HUWE1 (also called MULE) [182], SCF<sup>FBW7</sup> [183,184], and MARCH5 [185]. Different ligases appear to act in different contexts. For example, MARCH5 drives degradation of MCL-1 that is bound to NOXA [186,187], HUWE1 promotes degradation of





**Figure 4. Deubiquitinases USP9X, DUB3, and USP13 promote cell survival by stabilizing MCL-1.**

Activation of the intrinsic apoptosis pathway is regulated by members of the BCL2 protein family. The BH3-only pro-apoptotic proteins (colored yellow) bind to and neutralize their pro-survival relatives (colored pink), thereby unleashing the pro-apoptotic effectors BAX and BAK (colored red) to permeabilize the mitochondrial outer membrane. Some BH3-only proteins may also engage BAX and BAK directly. Mitochondrial cytochrome c is released into the cytoplasm, triggering assembly of an APAF1 complex that activates caspase-9. The executioner caspases 3 and 7 are cleaved and activated by caspase-9 resulting in the highly orchestrated proteolytic events that dismantle the cell. Deubiquitinases, including USP9X, DUB3, and USP13, promote cell survival by cleaving K48-linked polyubiquitin from MCL-1 that would otherwise target it for proteasomal degradation. The pro-apoptotic effector BOK is unstable owing to its constitutive ubiquitination. A DUB that removes ubiquitin from BOK has not been described. Figure created with BioRender.com.

MCL-1 in response to DNA damage [182], and SCF<sup>FBW7</sup> instigates degradation of MCL-1 by antitubulin chemotherapeutics [184]. DUBs shown to enhance cell survival by deubiquitinating MCL-1 and limiting its turnover include USP9X, DUB3 (also called USP17L2), and USP13 [188–190]. Elevated expression of these DUBs correlates with elevated expression of MCL-1 protein in certain patient tumors [188–190]. Thus, aberrantly high expression of DUBs that can stabilize MCL-1 may contribute to tumor development and resistance to chemotherapy. Interactions between MCL-1 and its DUBs may be regulated. For example, phosphorylation of MCL-1 following DNA damage was shown to limit interactions between MCL-1 and USP9X [188].

USP9X is essential for mouse embryogenesis [191–194], but whether MCL-1 instability and activation of the intrinsic apoptosis pathway contributes to lethality in USP9X-deficient embryos is unclear. USP9X has other substrates besides MCL-1 that are essential for normal embryogenesis, including PEG10 and polycomb repressive complex 2 (PRC2) components SUZ12 and EZH2 [194,195]. DUB3 and USP13 also have other substrates that regulate diverse cellular processes [196–198].

BAP1 is a UCH family DUB that suppresses intrinsic apoptosis in some mouse cell types by promoting the expression of *Mcl1* and *Bcl2* [199]. In complex with ASXL1, ASXL2, or ASXL3, BAP1 removes monoubiquitin

from histone H2A lysine 119 and thereby counters transcriptional repression mediated by ubiquitin ligases RNF2 and RING1 of the polycomb repressive complex 1 (PRC1) [199–203]. There is considerable interest in understanding gene regulation by BAP1 because it is a potent tumor suppressor. Humans with an inactivating germline mutation in *BAP1* are predisposed to cancer, especially uveal melanoma and mesothelioma (reviewed by [204]). The transcriptional changes that promote tumor development upon loss or inactivation of the other *BAP1* allele remain unclear.

USP7 (also called HAUSP) is another DUB that suppresses intrinsic apoptosis indirectly. In unstressed cells, USP7 deubiquitinates the ubiquitin ligase HDM2 (or its mouse counterpart MDM2) to limit proteasomal degradation of HDM2 and its binding partner HDMX (MDMX in mice) (reviewed by [205]). Stabilization of the HDM2-HDMX ligase promotes ubiquitination and proteasomal degradation of the tumor suppressor and transcription factor p53, whose target genes include *BBC3* (encoding PUMA), *PMAIP1* (encoding NOXA), and *BAX* [206]. Thus, one role of USP7 in healthy cells is to limit p53-dependent expression of pro-apoptotic BCL-2 family members. After DNA damage, however, phosphorylation of the HDM2-HDMX ligase disrupts interactions between HDM2 and USP7, leading to degradation of the ligase and activation of p53-dependent transcription [205]. This regulatory mechanism is reminiscent of how USP9X activity towards MCL-1 is disrupted after DNA damage. Disabling the pro-survival roles of DUBs in stressed cells makes sense because it favors activation of the intrinsic apoptosis pathway if the cells are damaged beyond repair. USP7, like most of the DUBs reviewed here, can deubiquitinate several substrates. For example, it can also deubiquitinate histone H2B [207] and N-MYC [208]. Accordingly, USP7 deficiency in mice has both p53-dependent and p53-independent consequences [209,210].

In contrast with USP7, OTUB1 can stabilize p53 in cells, but this activity does not require its catalytic activity [211]. OTUB1 may instead interfere with ubiquitination of p53 by inhibiting the E2 enzyme UbcH5. Other DUBs implicated in the deubiquitination and stabilization of p53 include USP10 [212] and the MJD DUB Ataxin-3 [213].

## Conclusions and future directions

DUBs cleaving either monoubiquitin or polyubiquitin can modulate the cell death machinery directly (for example, USP9X and BRISC) or indirectly (for example, OTULIN and BAP1) by controlling the abundance, conformation, and/or interactions of key cell death proteins. Although this review has discussed some of the DUBs regulating cell death signaling, there are ubiquitination events in these pathways where the ubiquitin ligases and DUBs have yet to be identified. For example, the enzymes controlling ubiquitination of MLKL to either limit [214] or promote necroptosis [215] remain unknown. The mechanisms regulating DUB-substrate interactions are also an area of interest. Relatively little is known about the regulation of DUBs such as OTULIN and BAP1. Finally, most of the DUBs reviewed here do not target a single protein or cell death signaling alone, but have multiple substrates involved in diverse biological processes. Thus, much remains to be uncovered in exploring the therapeutic potential of targeting DUBs to manipulate cell death signaling.

### Competing Interests

KN is an employee of Genentech. ADG is a visiting scientist at Genentech.

### Abbreviations

BH1-4, four BCL-2 homology domains; BRISC, BRCC3 isopeptidase complex; DD, death domain; DUBs, Deubiquitinases; GSDMD, gasdermin D; IFN, interferon; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; LUBAC, linear ubiquitin chain assembly complex; MJD, Machado–Josephin domain; ORAS, OTULIN-related autoinflammatory syndrome; OTUB1, OTU deubiquitinase, ubiquitin aldehyde binding 1; PFD, pore-forming domain; PIM, PUB domain-interacting motif; SHMT2, serine hydroxymethyltransferase 2; TLR3, Toll-like Receptor 3; TNFR1, Tumor Necrosis Factor Receptor 1; UCH, ubiquitin C-terminal hydrolase; USP, ubiquitin-specific protease.

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