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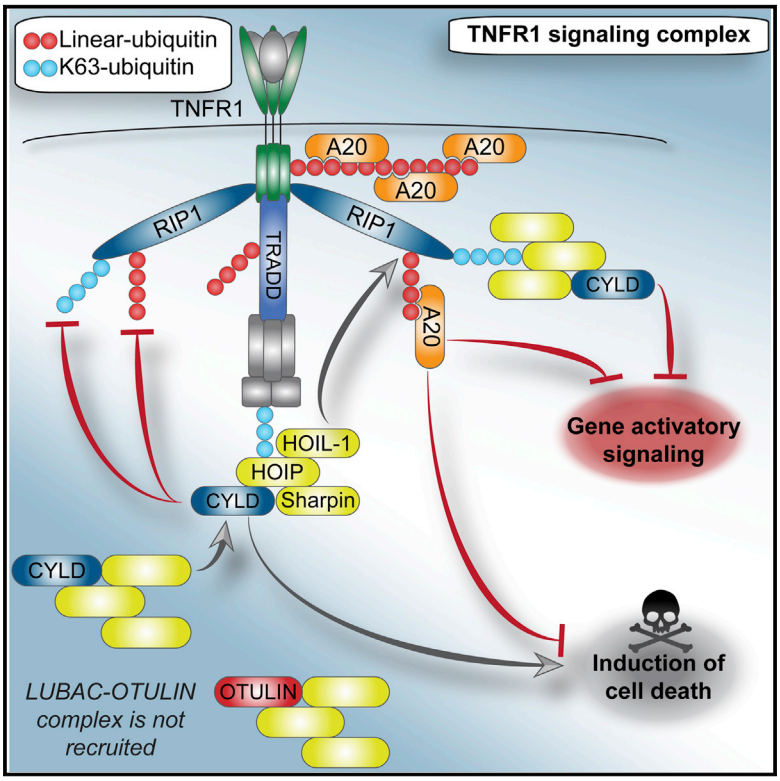
LUBAC-Recruited CYLD and A20 Regulate Gene Activation and Cell Death by Exerting Opposing Effects on Linear Ubiquitin in Signaling Complexes

Graphical Abstract



Highlights

- LUBAC directly recruits CYLD to the TNFR1 complex where it antagonizes M1 linkages
- M1-ubiquitin chains recruit A20, which, in turn, protects them from degradation
- CYLD and A20 inhibit gene activation but oppose each other in regulating cell death
- OTULIN controls LUBAC activity prior to stimulation but not in signaling complexes

Authors

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In Brief

Linear ubiquitin is an important regulator of immune signaling. Draber et al. show that the deubiquitinase CYLD antagonizes linear ubiquitin in signaling complexes. A20 is recruited to complexes via linear ubiquitin and, in turn, protects linear ubiquitin from cleavage. A20 and CYLD cooperatively inhibit gene activation but oppose each other to regulate TNF-induced cell death via their respective activities on linear ubiquitin.

Draber et al., 2015, Cell Reports *13*, 2258–2272
December 15, 2015
http://dx.doi.org/10.1016/j.celrep.2015.11.009

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LUBAC-Recruited CYLD and A20 Regulate Gene Activation and Cell Death by Exerting Opposing Effects on Linear Ubiquitin in Signaling Complexes

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http://dx.doi.org/10.1016/j.celrep.2015.11.009

SUMMARY

Ubiquitination and deubiquitination are crucial for assembly and disassembly of signaling complexes. LUBAC-generated linear (M1) ubiquitin is important for signaling via various immune receptors. We show here that the deubiquitinases CYLD and A20, but not OTULIN, are recruited to the TNFR1- and NOD2-associated signaling complexes (TNF-RSC and NOD2-SC), at which they cooperate to limit gene activation. Whereas CYLD recruitment depends on its interaction with LUBAC, but not on LUBAC's M1-chain-forming capacity, A20 recruitment requires this activity. Intriguingly, CYLD and A20 exert opposing effects on M1 chain stability in the TNF-RSC and NOD2-SC. While CYLD cleaves M1 chains, and thereby sensitizes cells to TNF-induced death, A20 binding to them prevents their removal and, consequently, inhibits cell death. Thus, CYLD and A20 cooperatively restrict gene activation and regulate cell death via their respective activities on M1 chains. Hence, the interplay between LUBAC, M1-ubiquitin, CYLD, and A20 is central for physiological signaling through innate immune receptors.

INTRODUCTION

Ubiquitin is an evolutionarily highly conserved small protein of 76 amino acids (8.6 kDa). Ubiquitination is a post-translational protein modification, carried out by three classes of enzymes, namely the ubiquitin-activating- (E1), ubiquitin-conjugating- (E2), and ubiquitin-ligating-enzymes (E3). The consecutive activity of these enzymes leads to the attachment of ubiquitin via its C terminus to a target protein (Hershko and Ciechanover, 1998). Ubiquitin itself can be ubiquitinated by attachment of the incoming ubiquitin to either of seven different lysine (K) residues (K6, K11, K27, K29, K33, K48, K63) or the N-terminal methionine (M1). Thus, depending on the linkage type(s) target proteins can be decorated with ubiquitin chains that are diverse in their compositions and exhibit different three-dimensional conformations (Kulathu and Komander, 2012).

Whereas K48-ubiquitin linkages serve to signal for protein degradation by the proteasome (Hershko and Ciechanover, 1998), non-degradative ubiquitin chains have emerged as important regulators of signals emanating from diverse immune receptors including TNFR1, NOD2, CD40, TLR2, TLR4, and IL-1R. Upon stimulation by their respective ligands, components within the primary receptor-associated signaling complexes (SCs) are modified by addition of K63- and M1-linked and, in certain cases, also other types of ubiquitin chains (Fiil and Gyrd-Hansen, 2014; Iwai et al., 2014; Shimizu et al., 2015; Zinngrebe et al., 2014). Formation of K63 chains is mediated by various E3 ubiquitin ligases specific for individual SCs. The linear ubiquitin chain assembly complex (LUBAC), consisting of HOIL-1, SHARPIN, and the catalytically active subunit HOIP, is the only currently known E3 capable of forming M1 chains de novo (Gerlach et al., 2011; Haas et al., 2009; Ikeda et al., 2011; Kirisako et al., 2006; Tokunaga et al., 2011). In all of the above signaling pathways, LUBAC has been determined to be responsible for M1 chain formation (Damgaard et al., 2012; Emmerich et al., 2013; Gerlach et al., 2011; Rodgers et al., 2014). K63 chains are recognized by the ubiquitin binding domains of TAB2 or TAB3 (Kanayama et al., 2004; Wang et al., 2001), resulting in recruitment of the TAK/TAB complex as well as LUBAC (Haas et al., 2009; Wang et al., 2001). LUBAC then enables efficient recruitment of NEMO and, consequently, of the NEMO/IKKα/IKKβ (NEMO/IKK) complex (Haas et al., 2009). These two functional units then cooperatively trigger activation of the NF-κB and MAPK signaling pathways (Walczak et al., 2012). Absence of LUBAC therefore attenuates gene induction by the above receptors and causes early embryonic lethality in mice due to aberrant TNFR1-induced endothelial cell death. Importantly, this cell death is due to increased formation of complex II of TNFR1 and not

caused by attenuated gene activation from the TNF-RSC (Peltzer et al., 2014).

To signal at the physiological level in response to a given stimulus, it is not only required that the corresponding SC forms, but it also has to disassemble with the appropriate kinetics. Regulated assembly and disassembly of ubiquitin chains within SCs are essential to achieve this. The enzymes responsible for removing ubiquitin moieties from target proteins and cleaving polyubiquitin chains are deubiquitinases (DUBs). DUBs implicated in the regulation of signaling by TNFR1 and other immune receptors are CYLD, A20 (Harhaj and Dixit, 2012), and the M1-specific DUB OTULIN, which was recently proposed to specifically antagonize LUBAC at SCs, including in the context of the TNF-RSC and the NOD2-SC (Fiil et al., 2013; Keusekotten et al., 2013; Rivkin et al., 2013). While CYLD antagonizes K63 linkages in SCs (Trompouki et al., 2003; Wright et al., 2007), it cleaves various linkages in vitro, albeit with preference for K63 and M1 linkages (Komander et al., 2008; Ritorto et al., 2014). A20 is induced by NF-κB upon stimulation of various immune receptors and hydrolyzes K11, K63, and K48 but not M1 linkages (Mevissen et al., 2013; Ritorto et al., 2014; Wertz et al., 2004). A20 binds to both K63 and M1 linkages via its Zinc finger (ZnF) domains 4 and 7, respectively (Bosanac et al., 2010; Tokunaga et al., 2012; Verhelst et al., 2012). Deficiency in these DUBs results in distinct phenotypes. In mice, OTULIN deficiency is embryonically lethal due to vascular defects (Rivkin et al., 2013). CYLD deficiency causes cylindromatosis in humans, a disease characterized by formation of benign tumors in the skin of affected individuals (Bignell et al., 2000; Blake and Toro, 2009; Zhang et al., 2004). Deficiency in A20, but interestingly not inactivation of its DUB activity, causes early death in mice due to severe inflammation, implying that A20 likely exerts major functions independently from its DUB activity (Lee et al., 2000; Lu et al., 2013). Recently, HOIP was found to directly associate with both CYLD and OTULIN in non-stimulated cells (Elliott et al., 2014; Fiil et al., 2013; Schaeffer et al., 2014; Takiuchi et al., 2014).

Here, we analyzed the interplay between LUBAC, M1-ubiquitin, and the various before-mentioned DUBs in assembly and disassembly of immune SCs and the functional impact this interplay has on the regulation of their signaling output.

RESULTS

CYLD, but Not OTULIN, Forms Part of the Native TNF-RSC

As linear ubiquitination is crucial for various immune signaling pathways (Gerlach et al., 2011; Haas et al., 2009), we aimed to understand how it is regulated in SCs. To do so, we created cell lines that are genetically deficient in HOIP, the M1-chain-forming component of LUBAC, and re-expressed tandem affinity purification (TAP)-tagged (2 × Strep-tag II followed by a PreScission cleavage site and 1 × Flag) HOIP in these cells (Figure S1A). We first analyzed the unstimulated LUBAC obtained from these cells by mass spectrometry following TAP. In line with recent reports (Elliott et al., 2014; Fu et al., 2014; Rivkin et al., 2013; Schaeffer et al., 2014; Takiuchi et al., 2014), our data showed that prior to stimulation, both CYLD and OTULIN interacted with HOIP (Figures S1B–S1D). We therefore deemed it likely that LUBAC would mediate recruitment of both of these DUBs to SCs. Unexpectedly, however, a kinetic analysis following tumor necrosis factor (TNF) stimulation revealed that OTULIN was not recruited to the TNF-RSC following TNF stimulation, despite being present in lysates, whereas recruitment of CYLD was evident, interestingly with kinetics reminiscent of LUBAC recruitment (Figure 1A). Thus, CYLD forms part of the TNF-RSC and OTULIN does not.

It is currently unclear how CYLD is recruited to SCs, including the TNF-RSC, although Optineurin was previously suggested to be involved (Nagabhushana et al., 2011). As CYLD interacts with LUBAC prior to stimulation, and because it is recruited to the TNF-RSC with similar kinetics as LUBAC, we wondered whether recruitment of CYLD may require HOIP. Analyzing the native TNF-RSC in HOIP-proficient versus -deficient cells revealed that while CYLD was present in the TNF-RSC of HOIP-proficient A549 (Figure 1B) and HaCaT (Figure S1E) cells, it was absent from it in their HOIP-deficient counterparts (Figures 1B and S1E). Thus, HOIP is required for recruitment of CYLD to the TNF-RSC.

CYLD Recruitment to the TNF-RSC Requires LUBAC, but Not M1-Ubiquitin

To determine whether absence of M1 chains from the TNF-RSC affected CYLD presence and OTULIN absence, we reconstituted HOIP-deficient cells with either TAP-tagged wild-type (WT) or enzymatically inactive HOIP-C885S (Smit et al., 2012; Stieglitz et al., 2012). When examining the effects of absence of LUBAC activity on the constitutive interaction of HOIP with CYLD and OTULIN, we found that neither of these interactions required HOIP activity (Figure 1C). Regarding the TNF-RSC, in line with our previous results, this complex is less stable in the absence of HOIP or its activity (Haas et al., 2009). Consequently, all proteins were retained less efficiently in the complex (Figure 1D). Importantly, however, cells expressing HOIP-C885S maintained the capacity to recruit CYLD to the TNF-RSC, yet OTULIN remained absent from this complex (Figure 1D). We therefore conclude that the enzymatic activity of HOIP is not required for recruitment of CYLD to the TNF-RSC.

CYLD, but Not OTULIN, Forms Part of the NOD2-SC and Is Recruited via HOIP

As mentioned above, the mechanism of CYLD recruitment to SCs is currently unclear. As CYLD interaction with LUBAC was responsible for TNF-RSC recruitment, we wondered whether LUBAC might also be responsible for recruitment of CYLD to other SCs. To test this hypothesis, we analyzed the receptor-associated complex that forms upon stimulation of NOD2 because, despite of being composed quite differently from the TNF-RSC, signaling via NOD2 also involves LUBAC (Damgaard et al., 2012; Fiil et al., 2013). NOD2 is an intracellular pattern-recognition receptor (PRR) recognizing components of the bacterial cell wall, and it plays a critical role in gastrointestinal host defense (Chen et al., 2009). As previous studies employed a system triggered by overexpression of NOD2 that does not require ligand-induced stimulation and to avoid

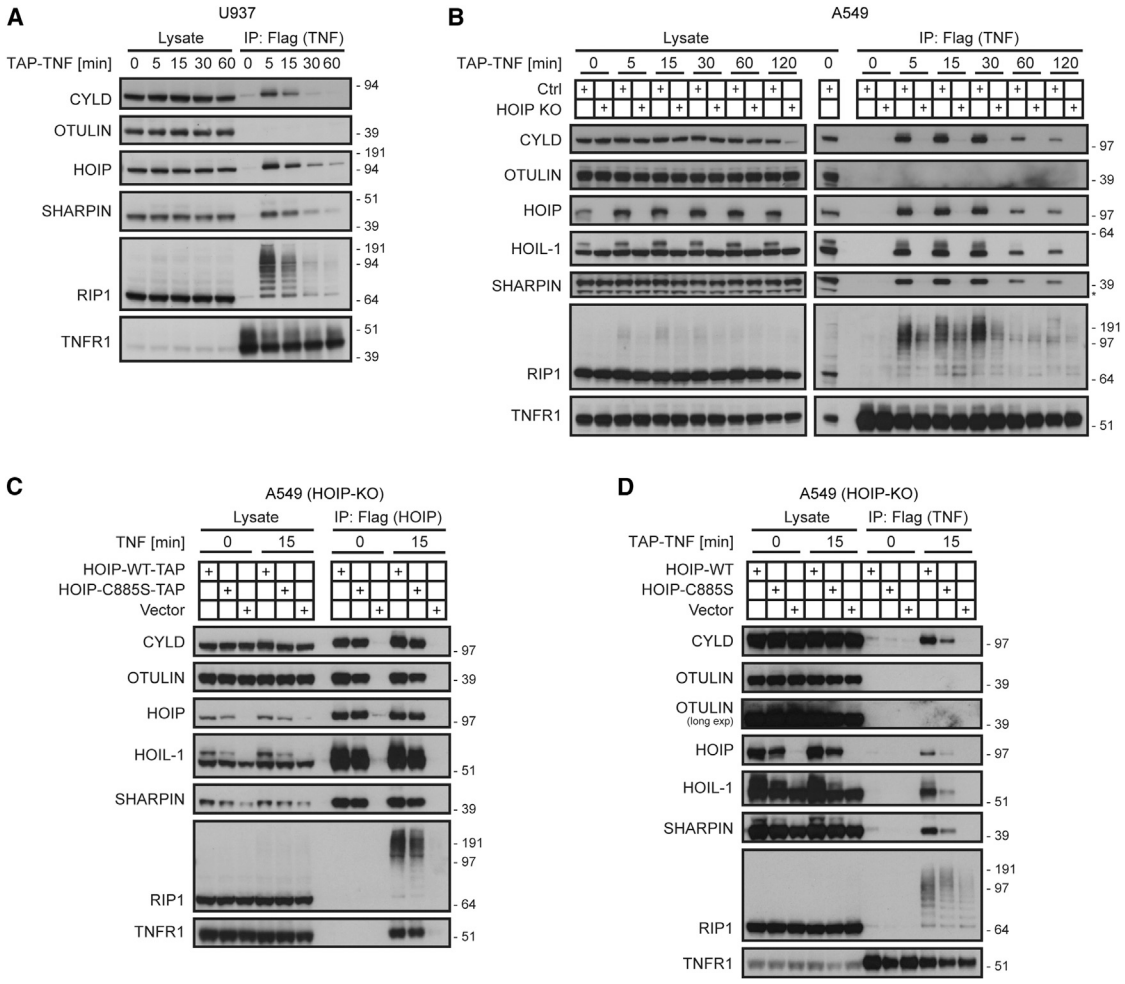


Figure 1. HOIP Is Required for Recruitment of CYLD to the TNF-RSC whereas OTULIN Is Not Recruited

(A) U937 cells were stimulated with TAP-TNF (1 μg/ml) for the indicated times. The TNF-RSC was immunoprecipitated via α-Flag beads and analyzed by western blot.

(B) WT and HOIP-deficient A549 cells were stimulated with TAP-TNF (500 ng/ml) and subjected to immunoprecipitation as in (A).

(C) HOIP-deficient A549 cells were reconstituted with either HOIP-WT-TAP or HOIP-C885S-TAP or vector control. Cells were subsequently stimulated with TNF (500 ng/ml) for 15 min or left untreated prior to LUBAC immunoprecipitation via α-Flag beads and analysis by western blot.

(D) The TNF-RSC isolated from HOIP-deficient A549 cells reconstituted with vector control, HOIP-WT, or catalytically dead HOIP-C885S was analyzed by western blot.

possible non-physiological events resulting from ligand-independent signaling by NOD2 overexpression, we generated stable clones expressing TAP-tagged NOD2 at intermediate levels. In these cells, NOD2-SC formation requires ligand-induced stimulation, which more closely resembles the physiological situation. Stimulation of these cells with synthetic muramyl dipeptide (MDP) L18-MDP, a known ligand for NOD2 (Grimes et al., 2012), induced strong NF-κB activation in HOIP-proficient, but not in HOIP-deficient cells (Figures 2A and S2A). In line with reports on HOIP's requirement for Erk activation upon CD40 and TNFR1 stimulation (Peltzer et al., 2014; Sasaki et al., 2013), we found that activation of Erk was also substantially inhibited in HOIP-deficient NOD2-stimulated cells (Figure S2A). Thus, NOD2-induced phosphorylation of ERK and IκB largely depends on LUBAC.

We next analyzed the NOD2-SC and found that CYLD also forms part of this complex (Figures 2B and S2B). As with the TNF-RSC, HOIP is essential for recruitment of CYLD to the NOD2-SC (Figures 2B and S2B), while OTULIN also does not form part of this complex (Figure 2B). CYLD was previously described as an inhibitor of TNF signaling (Brummelkamp et al., 2003; Kovalenko et al., 2003). To evaluate the role of CYLD in NOD2-mediated signaling, we isolated bone-marrow derived macrophages (BMDMs) from WT and CYLD-deficient mice and stimulated them with L18-MDP, as these cells constitutively express NOD2. This revealed that in the absence of CYLD, NOD2-induced gene activation via NF-κB and MAPKs (Erk, p38, and JNK) is enhanced (Figure 2C). Thus, CYLD limits gene activation induced by the NOD2-SC.

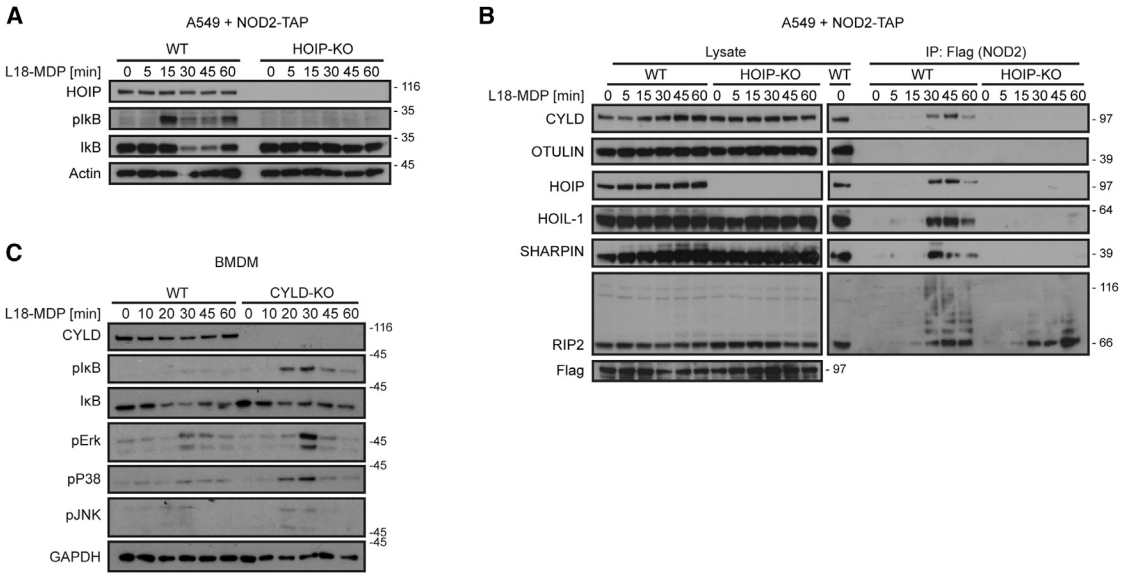


Figure 2. HOIP Recruits CYLD to the NOD2-SC

(A) A549 cells pro- or deficient in HOIP expression were stably transfected with NOD2-TAP, stimulated with L18-MDP (200 ng/ml) for the indicated times, and analyzed by western blot.

(B) A549 cells pro- or deficient in HOIP were virally transfected with NOD2-TAP and stimulated with L18-MDP (200 ng/ml) for the indicated times. The NOD2-SC was isolated by Flag-tag immunoprecipitation.

(C) Bone-marrow-derived macrophages (BMDMs) isolated from mice pro- or deficient in CYLD were stimulated with L18-MDP (200 ng/ml) for the indicated times and analyzed by western blot.

Mutually Exclusive Binding of CYLD and OTULIN to HOIP Causes CYLD-Selective Recruitment to SCs

As it was unclear why OTULIN could be absent from LUBAC-containing SCs despite interacting with LUBAC in the cytosol prior to stimulation, we next aimed to find a biochemical explanation for this unexpected phenomenon. The crystal structure of the HOIP PUB domain bound to the OTULIN PIM peptide (aa 49–67) shows that Tyr56 in OTULIN and Asn102 in HOIP are crucial for their interaction (Elliott et al., 2014; Schaeffer et al., 2014). Accordingly, phosphorylation of an OTULIN-derived peptide on the residue corresponding to Tyr56 prevented its association with HOIP's PUB domain (Elliott et al., 2014). Therefore, one possible explanation of OTULIN's absence from SCs could be its release from LUBAC as a result of Tyr56 phosphorylation. This event would have to be postulated to occur at, or shortly following, recruitment. TNF stimulation, however, did not abolish the interaction between HOIP and OTULIN (Figures 1C and S3A). Furthermore, induction of global tyrosine phosphorylation by pervanadate treatment, which induces irreversible inhibition of phosphatases, did not prevent this association either (Figure 3A). Conversely, reduction of overall tyrosine phosphorylation by phosphatase treatment did not increase it (Figure 3A). Thus, stimulation-associated tyrosine phosphorylation of OTULIN, including at Tyr56, cannot be responsible for OTULIN's absence from SCs.

Intriguingly, HOIP's relatively small PUB domain mediates the interaction with both CYLD and OTULIN and even short deletions in this domain abolished interaction with both factors (Figures S3B–S3D). This suggested that steric hindrance may prevent simultaneous interaction of HOIP with CYLD and OTULIN. If that was the case, they would compete for binding to HOIP so that CYLD-interacting LUBAC would be devoid of OTULIN and vice versa. To test this possibility, we precipitated TAP-tagged OTULIN and checked for binding of LUBAC components and CYLD. While LUBAC components were co-precipitated, CYLD was not (Figure 3B). Thus, CYLD and TAP-tagged OTULIN do not form part of the same individual LUBAC complexes, implying that they indeed cannot simultaneously interact with an individual HOIP protein. To address whether this also holds true for endogenous OTULIN, we next quantitatively immunoprecipitated OTULIN from WT cells, which would result in removal of the fraction of LUBAC bound to endogenous OTULIN, yet without co-precipitating CYLD. In accord with this hypothesis, CYLD did not form part of endogenous OTULIN-associated LUBAC complexes. Subsequent CYLD immunoprecipitation from OTULIN-depleted samples revealed that a second fraction of LUBAC is bound to CYLD (Figure 3C). Hence, there are two distinct fractions of LUBAC in the cell, one associated with CYLD, and another one bound to OTULIN.

The HOIP-OTULIN interaction can be disrupted by mutation of a critical residue (N102) in HOIP's PUB domain (Elliott et al., 2014). To test whether the CYLD-HOIP interaction would also be affected by this mutation, we reconstituted HOIP-deficient cells with HOIP-N102A and checked for association with CYLD. This revealed that HOIP-N102A was unable to bind to CYLD (Figure 3D). Together, these results show that CYLD and OTULIN interact with HOIP via the same or an overlapping site and that HOIP's interactions with CYLD and OTULIN are mutually exclusive. Based on these results, in combination with our findings regarding the composition of the TNF-RSC (Figure 1)

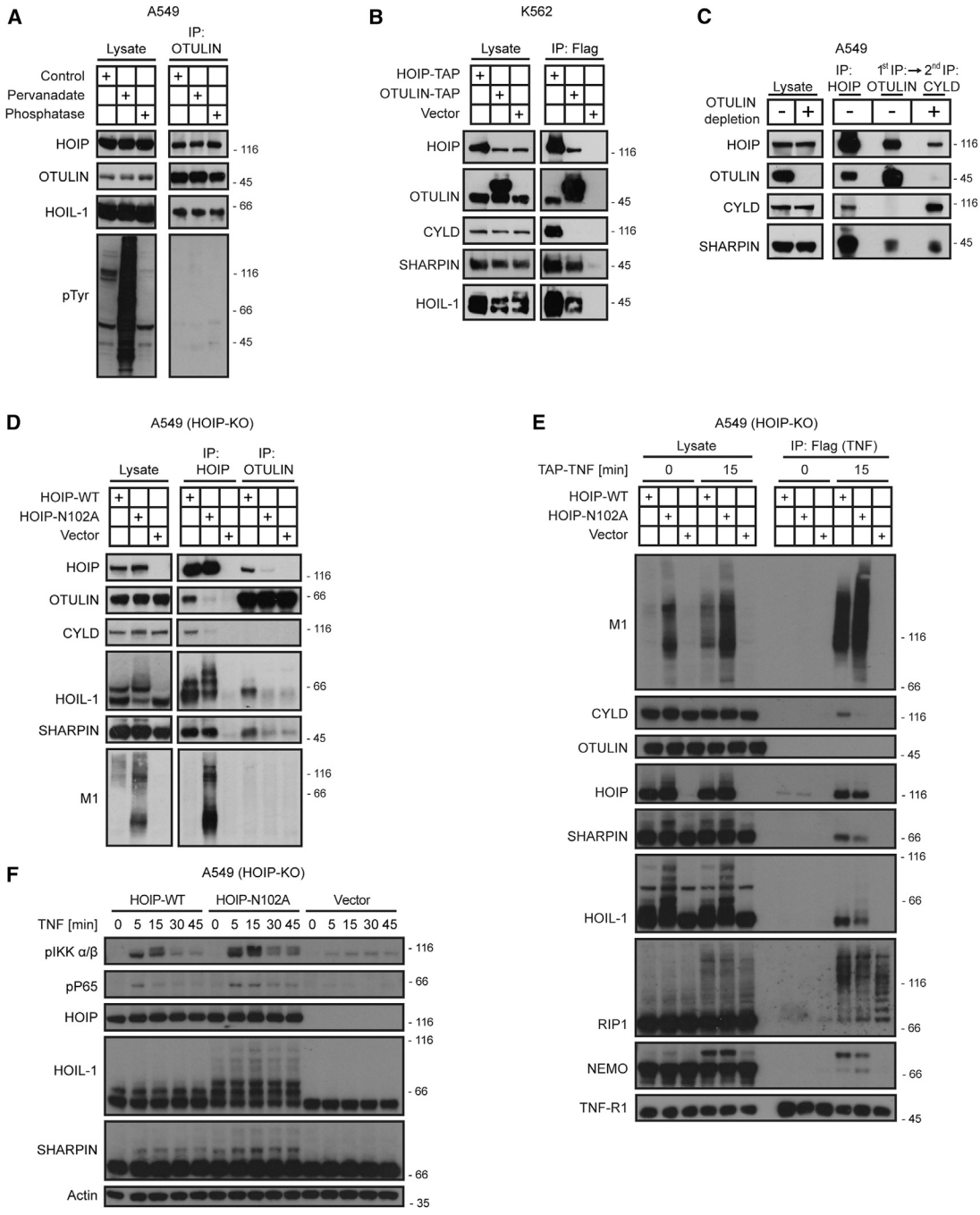


Figure 3. Mutually Exclusive Binding of CYLD and OTULIN to HOIP Causes CYLD-Selective Recruitment to SCs

(A) A549 cells were incubated with pervanadate prior to lysis or left untreated. Indicated lysates were subjected to phosphatase treatment prior to OTULIN immunoprecipitation.

(B) K562 cells expressing either HOIP-TAP, OTULIN-TAP, or vector control were subjected to α-Flag immunoprecipitation and analyzed by western blot.

(C) Lysate from A549 cells was subjected to immunoprecipitation for HOIP (IP: HOIP) or OTULIN (first IP: OTULIN). OTULIN-immuno-depleted lysate was subsequently subjected to CYLD immunoprecipitation (second IP: CYLD).

(D) A549 cells deficient in HOIP and reconstituted with HOIP-WT, HOIP-N102A, or vector control were subjected to immunoprecipitation for HOIP or OTULIN and subsequently analyzed by western blot.

(E) A549 cells deficient in HOIP and reconstituted with HOIP-WT, HOIP-N102A, or vector control were stimulated with TAP-TNF (500 ng/ml) for 15 min or left untreated. The TNF-RSC was immunoprecipitated via α-Flag beads and analyzed by western blot.

(F) A549 cells deficient in HOIP and reconstituted with HOIP-WT, HOIP-N102A, or vector control were stimulated with TNF (500 ng/ml) for indicated times, and lysates were analyzed by western blot.

and the NOD2-SC (Figure 2), it can be concluded that CYLD-associated LUBAC is recruited to SCs, whereas LUBAC associated with OTULIN is not.

Concomitant Loss of OTULIN and CYLD Interaction with HOIP Increases M1 Ubiquitination at the TNF-RSC and Enhances TNF-Induced Gene Activation

TNF stimulation induces expression of various response genes, which help counteract invading pathogens but also promote autoinflammation (Walczak, 2011). We therefore analyzed the TNF-RSC in cells expressing HOIP N102A to assess functional consequences of the loss of DUB interaction with HOIP. The recruitment of CYLD was abrogated in cells expressing this HOIP variant (Figure 3E), confirming that the interaction with LUBAC is required for CYLD recruitment. Following TNF stimulation, these cells showed increased M1 ubiquitination at the TNF-RSC (Figure 3E) and enhanced activation of NF-κB (Figure 3F). Similar results were obtained with cells expressing HOIP devoid of the PUB domain (Figure S3E). Furthermore, the lack of HOIP's PUB domain resulted in increased TNF-induced expression of *IRF1*, *ICAM1*, *TNF*, and *IκBα* (Figure S3F). NEMO was previously shown to be linearly ubiquitinated (Gerlach et al., 2011; Tokunaga et al., 2009), and this polyubiquitination event, albeit weak, could be observed in cells expressing HOIP-WT but not in cells lacking HOIP or expressing inactive HOIP. In cells expressing HOIP-N102A, however, the polyubiquitination of NEMO was enhanced (Figure S3G). Thus, abolishing HOIP's capacity to interact with both CYLD and OTULIN enhances M1 ubiquitination in the TNF-RSC and, consequently, TNF-induced gene activation.

OTULIN Deficiency Leads to Accumulation of M1 Chains in the Cytosol but Not at the TNF-RSC or NOD2-SC

As HOIP-associated OTULIN was not recruited to SCs, we next studied whether OTULIN deficiency may increase levels of linear ubiquitin found in the cytosol. Using CRISPR-Cas9, we generated OTULIN-deficient cells. Already prior to stimulation, these cells contained significantly more M1-linked ubiquitin chains in their cytosol than control cells (Figures 4A and S4A). These aberrant M1 chains were not free chains but conjugated to substrates (Figure S4B). The accumulation of linear ubiquitin linkages was found to be due to absence of OTULIN's enzymatic activity (Figure S4C). However, in accordance with our observation that OTULIN is not present at the TNF-RSC, linear ubiquitination within the TNF-RSC was not increased in OTULIN-KO as compared to WT cells (Figures 4A and S4A). This was also the case in the NOD2-SC (Figures 4B and S4D).

These circumstances led us to suspect that, instead of antagonizing M1-ubiquitin at SCs, OTULIN might regulate LUBAC components themselves. To identify which proteins show increased linear ubiquitination in lysates of OTULIN-deficient cells, we devised a new strategy to enrich for M1-ubiquitinated proteins. This method, which we will refer to as M1-affinity purification (M1-AP), employs a bead-resin-immobilized, enzymatically inactive portion of OTULIN (aa 58–352, C129A) that binds to, but does not cleave, M1 linkages with high affinity and specificity (Keusekotten et al., 2013) (Figures S4E and S4F). Prior to M1-AP, proteins were completely denatured in 1% SDS and subsequently renatured so that only proteins directly modified by ubiquitin chains containing M1 linkages, and not proteins that are only non-covalently associated with such chains, could be detected in these assays. Notably, M1-AP showed that M1 ubiquitination of HOIL-1 and SHARPIN was substantially increased in OTULIN-deficient cells (Figure 4C). For technical reasons, we could not determine M1 ubiquitination of HOIP. In accord with the results of the TNF immunoprecipitation, analysis of the components of the TNF-RSC by M1-AP showed that M1 ubiquitination of RIP1, a known LUBAC target in the TNF-RSC (Gerlach et al., 2011), was not increased in OTULIN-deficient cells (Figure 4D). Hence, rather than serving as a negative regulator of LUBAC activity at SCs, OTULIN keeps components of LUBAC free of aberrant M1 linkages prior to stimulation.

CYLD Antagonizes M1 Ubiquitination of TNF-RSC Components

Since its association with LUBAC was the recruiting principle for CYLD and because expression of HOIP-N102A, unable to recruit CYLD to the TNF-RSC, increased the amount of M1 linkages in this SC, we next sought to determine whether CYLD could be responsible for antagonizing LUBAC activity in SCs. We employed CRISPR-Cas9 to create CYLD-deficient A549 cells and stimulated them with TNF before subjecting them to M1-AP or K63-AP, the latter being based on isolation of K63-ubiquitin linkages with tUIM (Sims et al., 2012) (Figure S5A). The absence of CYLD increased overall TNF-induced M1 and K63 ubiquitination. When determining which proteins were modified by these linkages, we found that both M1 and K63 ubiquitination of RIP1, TNFR1, and TRADD were increased in the absence of CYLD (Figures 5A and S5B). This suggested that each one of these proteins carries M1 and K63 chains when present in the TNF-RSC, and that CYLD antagonizes both of them on all of these proteins.

To determine whether TNFR1 and TRADD are bona fide LUBAC targets, subsequent to TNF stimulation and isolation by M1-AP we treated M1-ubiquitinated proteins with two different recombinant DUBs (Figure S5C). Treatment with OTULIN resulted in removal of all M1-linked chains from these proteins (Figure 5B). Crucially, this treatment significantly reduced the high-molecular-weight species of TNFR1, TRADD, and RIP1 (Figure 5B). Thus, prior to OTULIN treatment M1-linked ubiquitin chains had been present on these proteins. It should be noted that complete removal of ubiquitin chains by OTULIN would be expected only for exclusively linearly ubiquitinated targets. However, in line with our previous results regarding RIP1 (Gerlach et al., 2011), TRADD and TNFR1 also carry other chain types, and the degree of reduction in their overall ubiquitination by OTULIN treatment is indicative of the ratio between M1 linkages and other linkages present on these proteins. In contrast, the DUB vOTU is capable of cleaving all ubiquitin linkages except for the M1 linkage (Akutsu et al., 2011). Treatment with vOTU resulted in removal of all linkages from M1-affinity purified TNFR1, TRADD, and RIP1 (Figures 5B and S5D). As this includes the ubiquitin moieties through which M1 chains are linked to these proteins, a ladder of linear ubiquitin chains that are shed

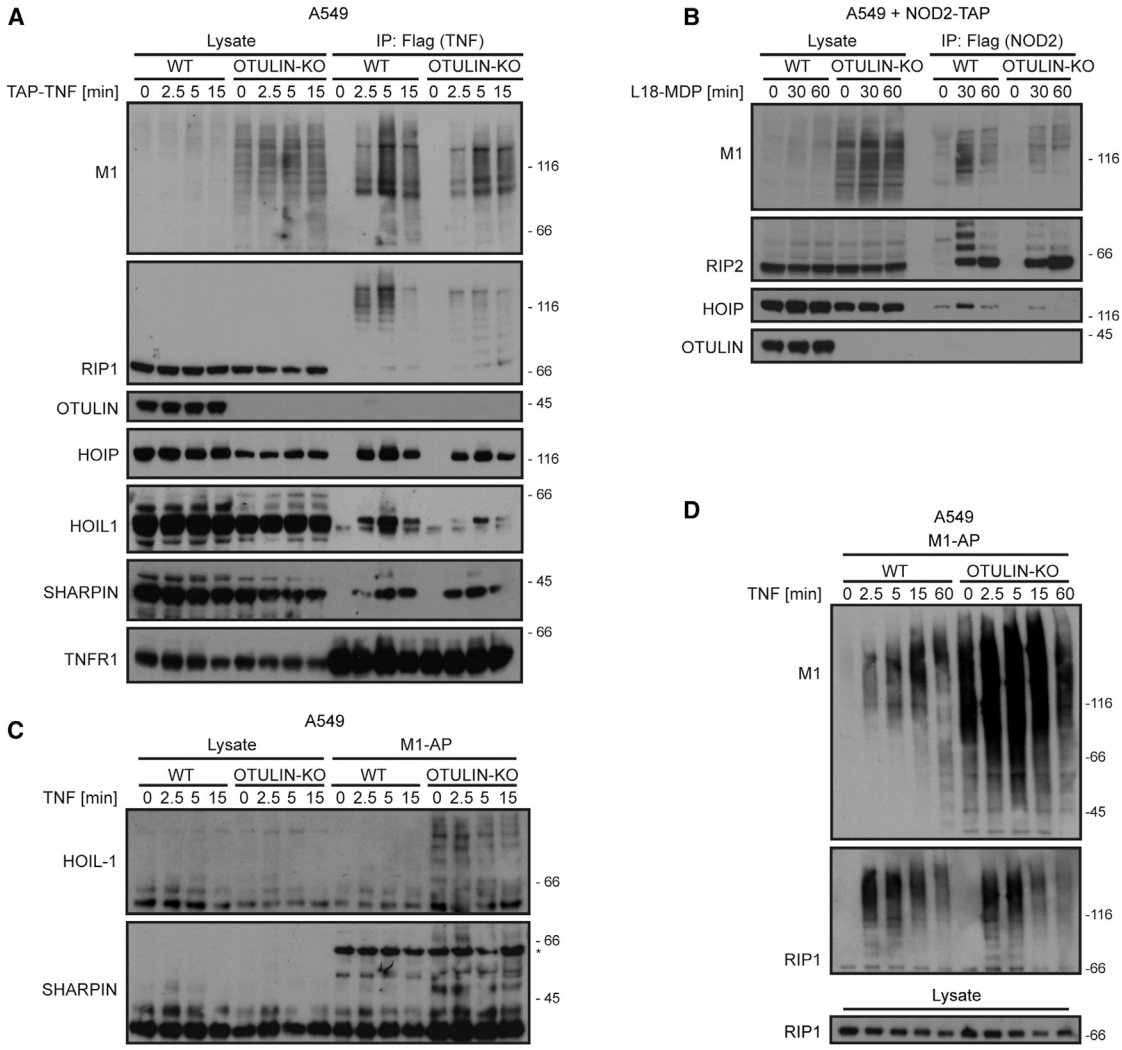


Figure 4. OTULIN Constitutively Removes M1-Ubiquitin from LUBAC in Non-stimulated Cells

(A) The TNF-RSC was isolated from either WT or OTULIN-KO A549 cells stimulated with TAP-TNF (500 ng/ml) for the indicated times and subjected to western blot.

(B) A549 cells pro- or deficient in OTULIN and virally transduced to express NOD2-TAP were stimulated with L18-MDP (200 ng/ml) for the indicated times. The NOD2-SC was isolated by α-Flag immunoprecipitation.

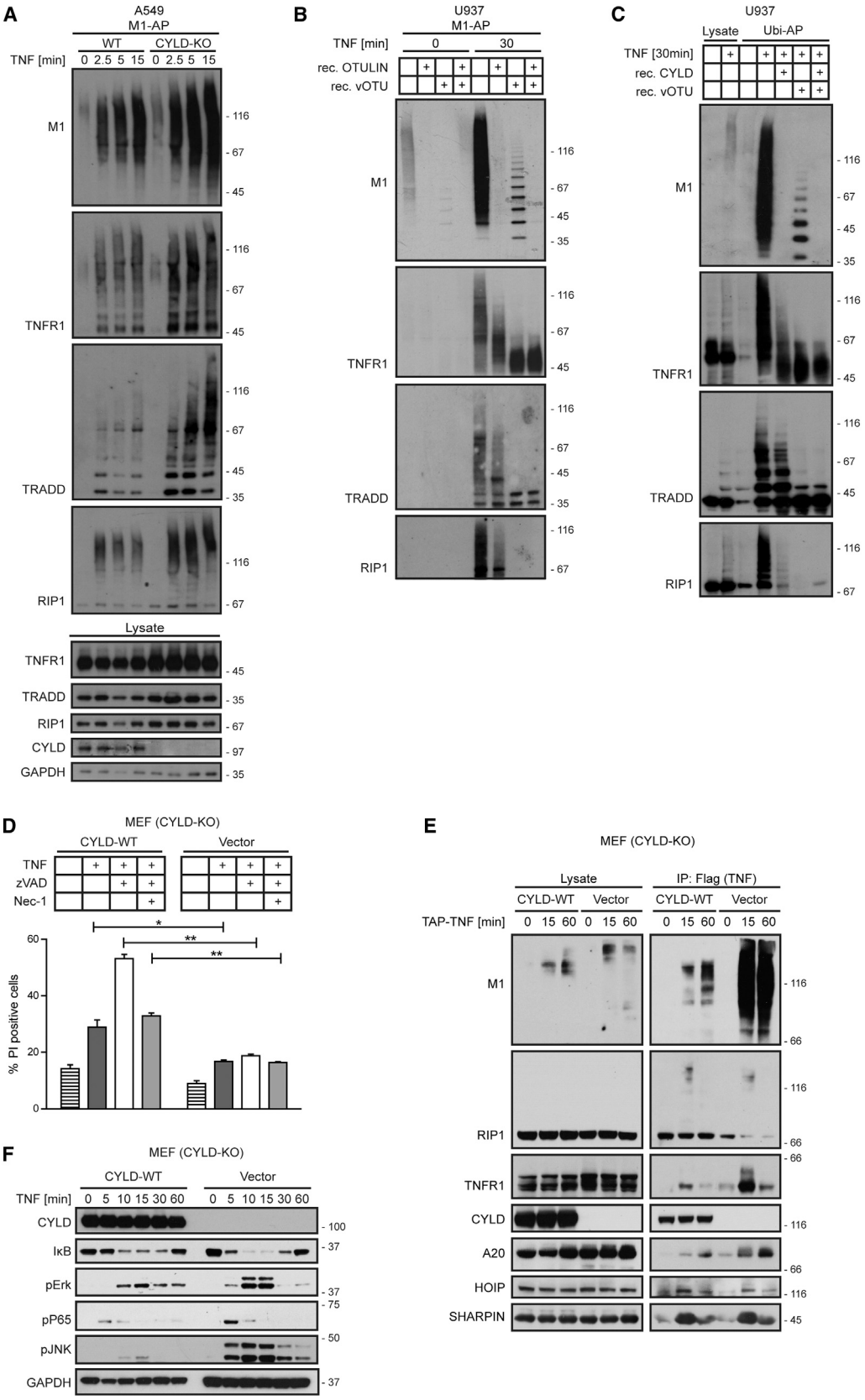
(C and D) WT or OTULIN-deficient A549 cells were stimulated with TNF (200 ng/ml) for the indicated times. M1-ubiquitin-specific affinity purification (M1-AP) was performed and samples were examined by western blot.

from these proteins by vOTU treatment becomes apparent. As they are completely hydrolyzed when OTULIN is added together with vOTU, it can be concluded that these chains are pure M1-linked chains (Figure 5B). Importantly, no linear ubiquitination of RIP1, TNFR1, or TRADD was observed in cells lacking HOIP (Figure S5D). These results identify TNFR1 and TRADD as previously unrecognized, additional bona fide targets of LUBAC in the TNF-RSC. In addition, they show that the linear chains present on these targets are of considerable length.

As absence of CYLD enhanced M1 ubiquitination of TNF-RSC components, we assessed whether the recombinant USP domain (aa 583–956) of CYLD (CYLD-USP), encompassing its catalytic DUB activity, could be capable of removing ubiquitin chains from them. Isolation of ubiquitinated proteins following TNF stimulation and subsequent treatment with recombinant CYLD-USP or vOTU showed that CYLD's DUB domain is capable of removing the majority of overall ubiquitination from TNFR1, TRADD, and RIP1 (Figure 5C). The fact that treatment with CYLD, in contrast to treatment with vOTU, did not release M1 chains demonstrates that CYLD indeed hydrolyzes M1 linkages present on these target proteins (Figure 5C). Thus, CYLD acts as a DUB that antagonizes both linear and K63 ubiquitin linkages in the TNF-RSC.

CYLD Limits Gene Activation and Enhances Cell Death in Response to TNF

As CYLD was previously described to be a positive regulator of TNF-induced necroptosis (O'Donnell et al., 2011) and because



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we previously showed that linear ubiquitination in the TNF-RSC protects from TNF-induced cell death (Peltzer et al., 2014), we next wanted to assess the impact of CYLD's activity on M1 chains in the TNF-RSC in relation to TNF-induced cell death. In line with previous studies, we found that CYLD-reconstituted but not -deficient MEFs were prone to TNF- and TNF/zVAD-induced cell death (Figure 5D). Importantly, this coincided with a decrease in both linear ubiquitination of TNF-RSC components and TNF-induced gene activation in CYLD-reconstituted MEFs (Figures 5E and 5F). Thus, CYLD-mediated removal of ubiquitin chains, including of linear chains, from components of the TNF-RSC results in diminished TNF-induced gene activation and, at the same time, enhanced cell death.

Recruitment of A20 to the TNF-RSC Requires LUBAC and M1-Ubiquitin

Another major DUB involved in TNF signaling is A20. It is, however, debated how it is recruited to signaling complexes. The ZnF4 domain of A20 selectively recognizes K63-linked ubiquitin, and mutations in this domain were reported to impair A20 recruitment to the TNF-RSC (Bosanac et al., 2010; Lu et al., 2013). Interestingly, the ZnF7 domain of A20 is involved in A20's ability to suppress NF-κB and cell death (Tokunaga et al., 2012; Verhelst et al., 2012). In addition, this domain binds with high affinity to linear ubiquitin chains, and TNF-RSC recruitment of A20 devoid of ZnF7 is reduced (Tokunaga et al., 2012). This prompted us to study the biochemical and functional interplay between LUBAC, linear ubiquitination, and A20 in TNFR1 and NOD2 signaling.

A20 is induced by various stimuli in an NF-κB-dependent manner (Catrysse et al., 2014). In all cell lines tested, we observed, however, that A20 was already present before stimulation. Consequently, it formed part of the TNF-RSC already 5 min after TNF stimulation, yet interestingly only in WT but not HOIP-deficient cells (Figures 6A, S6A, and S6B). Prolonged stimulation of up to 3 hr resulted in increased A20 expression and recruitment to the TNF-RSC, again only in control and not in HOIP-deficient cells (Figure S6C). A20 functions as a negative regulator of NOD2 signaling (Hitotsumatsu et al., 2008). It was unknown, however, whether it is recruited to the NOD2-SC. We therefore next analyzed the NOD2-SC for A20 presence and, if there, what the role of LUBAC would be in its recruitment. This analysis revealed that A20 forms part of the NOD2-SC and that HOIP is required for this (Figure 6B).

As LUBAC deficiency reduces gene activation, we next addressed the role of HOIP's enzymatic activity in induction of A20. As expected, HOIP-deficient cells showed substantially decreased activation of NF-κB and, consequently, reduced production of CCL2 and IL-8 as well as a comparably weak upregulation of A20 upon TNF stimulation (Figures 6C–6E). In cells expressing catalytically inactive HOIP-C885S the TNF-induced stimulation of gene-activatory signaling pathways and cytokine production was also decreased, yet not abolished (Figures 6C and 6D). Interestingly, these cells had increased basal expression of A20 before stimulation, but 3 hr after stimulation the A20 levels were almost identical to the ones observed in HOIP-WT cells (Figure 6E). To evaluate whether the enzymatic activity of HOIP is required for A20 recruitment to the TNF-RSC, we therefore compared the TNF-RSC that forms after 3 hr in cells expressing HOIP-WT and HOIP-C885S. This showed that HOIP-C885S expressing cells are almost completely defective in A20 recruitment to the TNF-RSC (Figure 6F). Together, this identifies LUBAC-generated M1 chains as required for recruitment of A20 to the TNF-RSC.

A20 Presence Stabilizes M1 and K63 Linkages in TNFR1 and NOD2 Complexes

Even though A20 is unable to cleave M1 linkages, we hypothesized that it could affect M1 chains in SCs indirectly through cleavage of other linkages. We therefore next assessed the impact of A20 absence on the presence of linear ubiquitin chains in the TNF-RSC. To do so, we again employed CRISPR-Cas9, this time to generate A20-deficient A549 cells. Unexpectedly, rather than being increased, M1 ubiquitination was markedly decreased in the TNF-RSC of A20-deficient as compared to control cells, a finding that also applied to TNFR1 (Figure 7A). This was confirmed using M1-AP (Figure S7A). A20 deficiency also reduced the amount of M1-linked ubiquitin present in the NOD2-SC (Figure 7B) and on RIP2 in this complex (Figure S7B). Finally, also MEFs deficient in A20 showed a marked reduction in linear ubiquitination at the TNF-RSC (Figure S7C). Together, these results indicate that A20 presence stabilizes M1 linkages in SCs.

Even though this result made it unlikely that A20's DUB activity was responsible for this effect, we could not formally exclude it based on the experiments performed so far. Independently thereof, we reasoned that because A20 is recruited to SCs via linear ubiquitin chains, this interaction could be responsible for their A20-endowed stabilization. Since A20's ZnF7 was known to bind M1 chains and to be required for TNF-RSC recruitment (Tokunaga et al., 2012), we tested whether ZnF7 could be responsible for A20-mediated stabilization of linear ubiquitin chains at the TNF-RSC. To do so, we used CRISPR-Cas9 to prepare cells lacking only the ZnF7 domain of A20 (Figure S7D). In

Figure 5. CYLD Removes M1- and K63-Ubiquitin from TNFR1, TRADD, and RIP1

(A) WT or CYLD-deficient A549 cells were treated with TNF (200 ng/ml). M1-affinity purification (AP) was performed, and the samples were subsequently analyzed by western blot.

(B) U937 cells were stimulated with TNF (200 ng/ml). Samples were denatured and subjected to M1-AP with subsequent treatment with recombinant OTULIN, vOTU, or both.

(C) Cells were treated as in (B), and samples were subjected to total ubiquitin-AP followed by treatment with recombinant CYLD (aa 583–956), vOTU, or both.

(D–F) MEFs deficient in CYLD were reconstituted with CYLD-WT or vector control. Cells were stimulated with TNF (200 ng/ml) for 24 hr in the presence or absence of zVAD (20 μM), Nec-1 (10 μM), or both, and cell death was evaluated as percentage of propidium iodide positive cells (data are presented as mean ± SEM [n = 3], $^{*}p < 0.05$, $^{**}p < 0.0005$, statistics were performed using t test) (D). Cells were stimulated with TAP-TNF (500 ng/ml) for the indicated times, and the TNF-RSC was isolated by α-Flag immunoprecipitation (E). Cells were stimulated with TNF (200 ng/ml) for the indicated times, and activation of gene-activatory signaling pathways was analyzed by western blot (F).

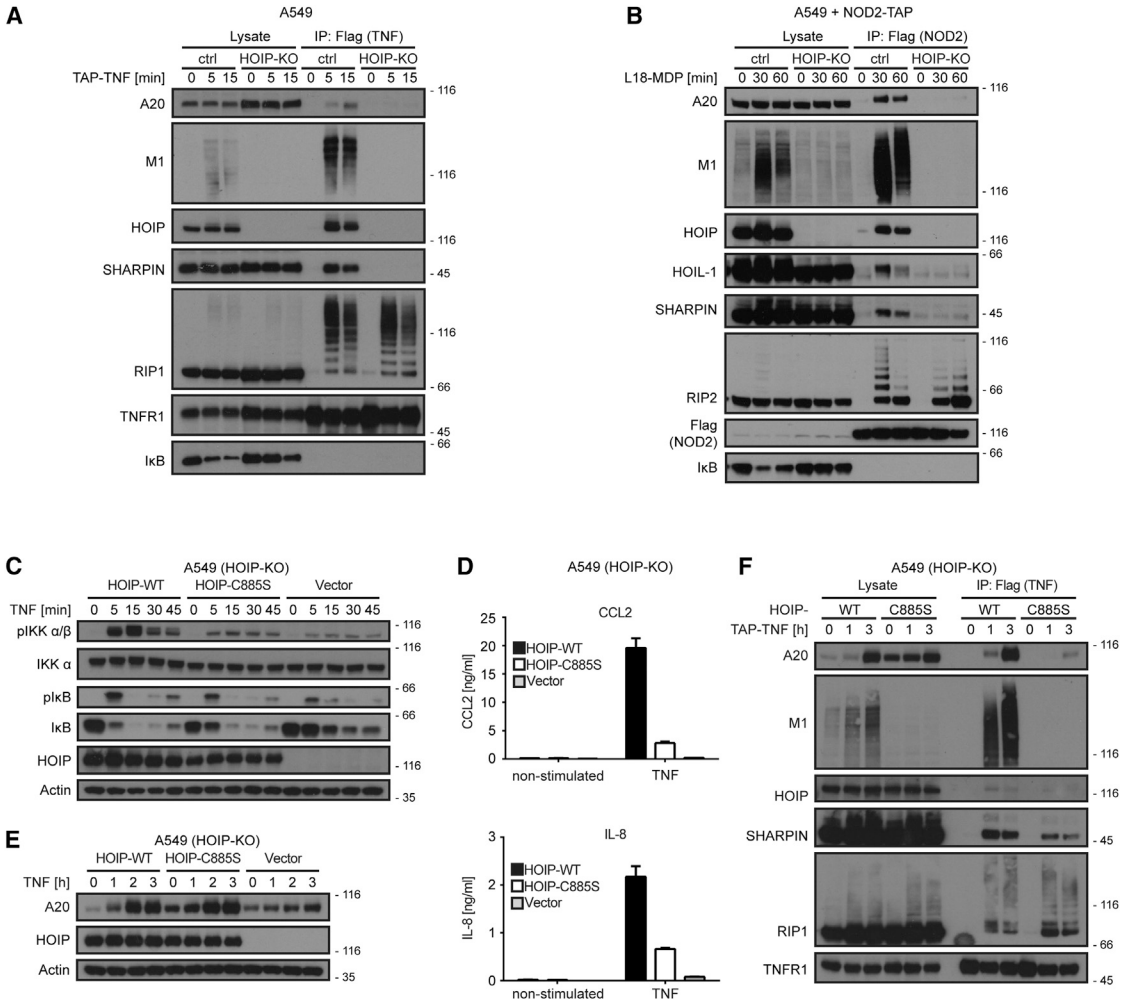

Figure 6. Recruitment of A20 to the TNF-RSC Requires Linear Ubiquitination

(A) HOIP-pro- or deficient A549 cells were stimulated with TAP-TNF (500 ng/ml) for the indicated times and subjected to TNF-RSC isolation and western blot analysis.

(B) A549 cells pro- or deficient in HOIP were transfected with NOD2-TAP and subsequently stimulated with L18-MDP (200 ng/ml). The NOD2-SC was immunoprecipitated via α-Flag beads and then analyzed by western blot.

(C–E) A549 cells deficient in HOIP were reconstituted with HOIP-WT, enzymatically inactive HOIP-C885S, or vector control. Cells were stimulated with TNF (200 ng/ml) for the indicated times before analysis by western blot (C and E). Additionally, production of CCL2 and IL-8 was measured by ELISA after stimulation with TNF (50 ng/ml) for 24 hr (data are presented as mean ± SEM [n = 3]) (D).

(F) HOIP-deficient A549 cells were reconstituted with HOIP-WT or enzymatically inactive HOIP-C885S. Samples were analyzed as in (A).

line with previous results (Tokunaga et al., 2012), we found in both A549 and HaCaT cells that absence of ZnF7 severely compromised recruitment of A20 to the TNF-RSC and accumulation of ubiquitin chains therein (Figures 7C and S7E). To assess whether the DUB activity of A20 or its M1-binding function was responsible for its capacity to stabilize M1 linkages in the TNF-RSC, we reconstituted A20-deficient MEFs with A20-WT, DUB-inactive A20 (A20-C103S), or an A20 ZnF7 point mutant C779A/C782A (A20-ZnF7mut), which is unable to interact with M1 chains (Tokunaga et al., 2012). Both A20-WT and A20-C103S, but not A20-ZnF7mut, were recruited to the TNF-RSC and stabilized linear ubiquitin linkages within this complex (Figures 7D and S7F). Thus, the linear-ubiquitin-binding activity of A20, but not its DUB activity, is required for stabilization of linear chains. In summary, these results show that direct binding of A20 to linear ubiquitin chains enables recruitment of A20 to the TNF-RSC and that this, in turn, results in stabilization of M1-ubiquitin chains in this complex.

The observation that ZnF7 is necessary for recruitment of A20 to the TNF-RSC fits with the finding that A20-WT and A20-C103S, but not A20-ZnF7mut, inhibit TNF-induced gene activation (Figures 7E and S7G) and protect cells from TNF/zVAD-induced necroptosis (Figure 7F) (Yamaguchi and Yamaguchi, 2015). Hence, the activity of A20 as a binder and stabilizer of linear ubiquitin linkages appears to be functionally more significant than its activity as a DUB.

DISCUSSION

OTULIN Antagonizes Basal LUBAC Activity, but Not M1-Ubiquitin in SCs

Our analysis of LUBAC obtained from non-stimulated cells confirmed previous reports that CYLD and OTULIN bind to the PUB domain of HOIP (Takiuchi et al., 2014). To our surprise, we found that, unlike CYLD, OTULIN formed part of neither the native TNF-RSC nor the NOD2-SC. This contrasts with previous reports addressing OTULIN recruitment to SCs. Fiil et al. (2013) reported that transient overexpression of Flag-tagged NOD2 resulted in its interaction with OTULIN. However, this system does not require stimulation by a NOD2 ligand, and it is therefore possible that the detected interaction could be a non-physiological event related to NOD2 overexpression. In addition, Schaeffer et al. (2014) proposed OTULIN to be recruited to the TNF-RSC. Even though we were able to detect CYLD in two different SCs, including the TNF-RSC, in all cell lines studied, we never found OTULIN to be recruited. These results implied that SC recruited HOIP was not associated with OTULIN, despite the fact that OTULIN and HOIP interacted prior to stimulation. Indeed, we found that OTULIN antagonizes LUBAC-mediated linear ubiquitination in the cytoplasm but not at SCs. The function of OTULIN is therefore likely to prevent accumulation of M1-ubiquitin linkages outside of SCs (Figure 7G).

Aiming to find a mechanistic explanation for the unexpected absence of OTULIN from SCs, we first turned our attention to phosphorylation, as phosphorylation of OTULIN Tyr56 was previously reported to be capable of disrupting the interaction between HOIP and OTULIN (Elliott et al., 2014). Our data, however, show that phosphorylation is not responsible for OTULIN absence from SCs. The explanation was provided by our discovery that HOIP cannot simultaneously bind OTULIN and CYLD as both require HOIP-Asn102 for binding. We next demonstrated the existence of two separate pools of cytoplasmic LUBAC: one associated with OTULIN, the other one with CYLD. Importantly, whereas CYLD-bound LUBAC is recruited to the TNF-RSC and the NOD2-SC, OTULIN-associated LUBAC is not. It will be interesting to determine how OTULIN's interaction with HOIP prevents its recruitment to SCs, e.g., whether OTULIN binding could interfere with HOIP's ability to bind ubiquitin chains, a requirement for LUBAC recruitment to SCs (Gerlach et al., 2011; Haas et al., 2009).

CYLD Antagonizes LUBAC Activity in SCs

We show here that CYLD is recruited to SCs due to its interaction with HOIP, independently of LUBAC's enzymatic activity. Furthermore, in cells expressing HOIP-N102A, which can interact with neither CYLD nor OTULIN, CYLD is not recruited to the TNF-RSC, demonstrating that CYLD's interaction with HOIP is essential for CYLD recruitment.

CYLD is a DUB with broad specificity, yet the enzymatically active USP domain of CYLD, when produced recombinantly, was previously shown to most efficiently hydrolyze M1- and K63-linked tetra-ubiquitin in vitro (Komander et al., 2009). It has been unclear to date, however, whether endogenous CYLD would be able to regulate M1-ubiquitin, in addition to its previously demonstrated role as a DUB that cleaves K63 linkages on targets within the TNF-RSC (Brummelkamp et al., 2003; Kovalenko et al., 2003; Trompouki et al., 2003; Wright et al., 2007). Employing newly devised protocols for K63-AP and M1-AP, we demonstrate here that, as a consequence of CYLD deficiency, both K63- and M1-ubiquitin are increased on several components of the TNF-RSC, including TNFR1, TRADD, and RIP1. Furthermore, the recombinant USP domain of CYLD completely removes M1 linkages, and indeed the majority of other linkage types, from components of the TNF-RSC. Importantly, M1 linkages are fully hydrolyzed and not merely released from complex components, as no free M1 chains appear following treatment with CYLD. Collectively, these results identify CYLD as an antagonist of linear ubiquitination in SCs, in addition to its previously described role as an antagonist of K63-linked ubiquitination (Figure 7H).

Removal of M1-Ubiquitin from the TNF-RSC Accounts for CYLD's Pro-cell Death Role

We show that reconstitution of CYLD-deficient MEFs with CYLD, but not with vector control, decreases M1-ubiquitin in the TNF-RSC. This is accompanied by decreased gene activation, yet interestingly, also with enhanced TNF-induced cell death. This is in line with the fact that CYLD has previously been described as being both an inhibitor of gene activation and promoter of cell death, the latter by enhancing complex II formation upon TNF stimulation (Hitomi et al., 2008; O'Donnell et al., 2011). We previously showed that lack of linear ubiquitination in the TNF-RSC enhances formation of complex II of TNF signaling (Peltzer et al., 2014). Crucially, these experiments were performed with HOIP-deficient cells. Based on the results presented here, CYLD requires HOIP for TNF-RSC recruitment. Hence, in cells lacking HOIP, CYLD does not form part of the TNF-RSC. Consequently in these cells, CYLD cannot mediate the transition from complex I of TNFR1 signaling (i.e., the TNF-RSC) to complex II. Decisively, however, in HOIP-deficient cells this event does not require CYLD activity; it readily occurs without it (Peltzer et al., 2014). This identifies the lack of M1 chains in complex I as decisive to render this complex unstable so that complex II can readily form. Importantly, in this situation CYLD is not required to enable this transition. We thus conclude that the M1-chain-antagonizing activity of CYLD in complex I of TNFR1 signaling is responsible for its pro-cell death role. Whether this is due to direct cleavage of M1 chains and/or their indirect removal through the previously demonstrated cleavage of K63 linkages that are extended by linear chains remains to be determined. Equally, whether removal of a specific M1 chain from a particular target enables complex II formation, or multiple such events together account for it, remains to be resolved.

A20 Binding to M1 Chains Inhibits Gene Activation

With respect to A20, we show that in absence of HOIP or M1-ubiquitin A20 recruitment to SCs is almost completely prevented and that genomic deletion of A20's ZnF7, in turn, drastically impairs recruitment. Thus, LUBAC, by placing M1 chains on SC components, recruits A20 to these SCs via its ZnF7 (Figure 7H). These results are in line with the previous finding that A20 can bind to M1-ubiquitin via its ZnF7 domain (Tokunaga et al., 2012; Verhelst et al., 2012). Additionally, they provide the

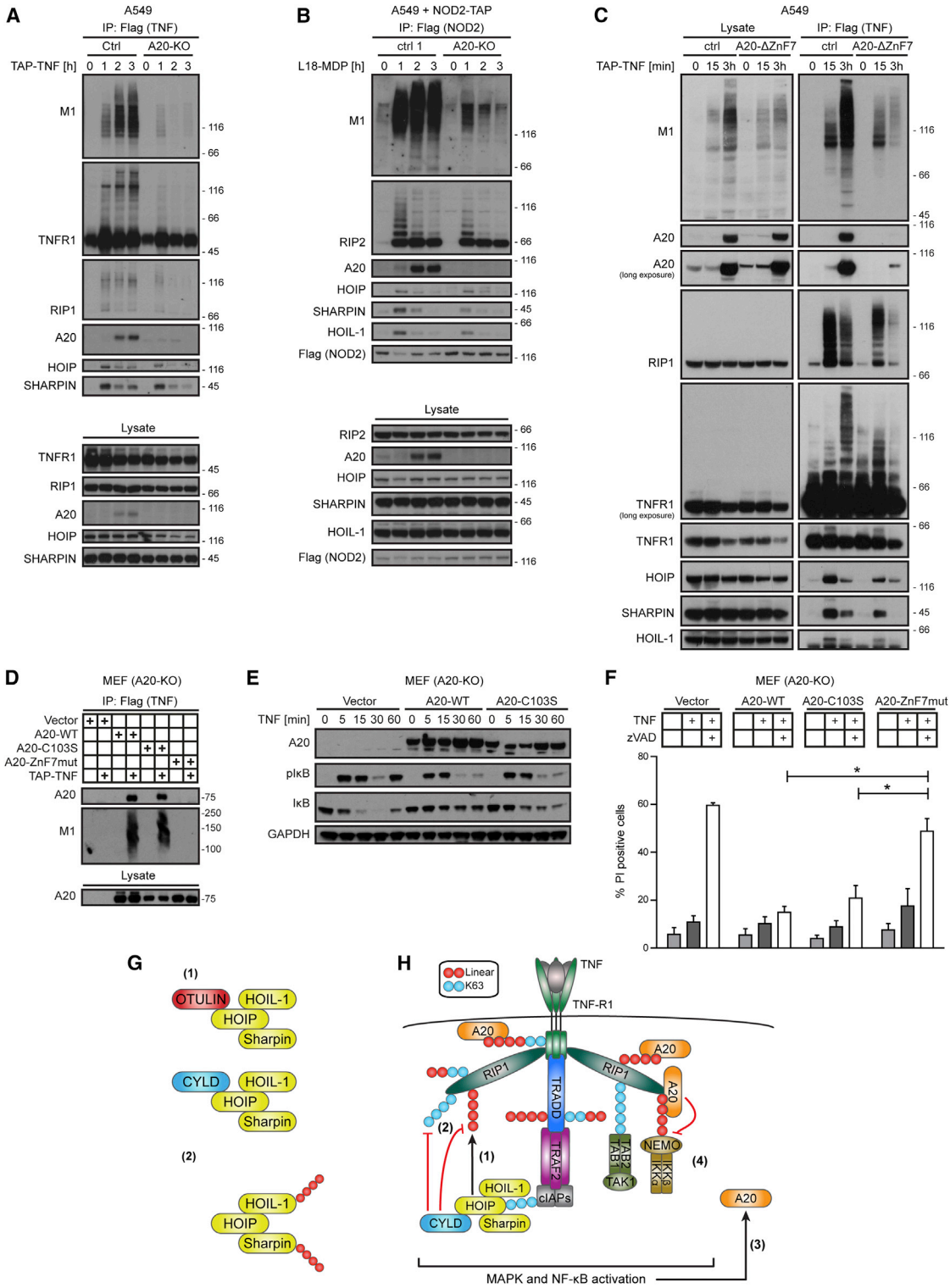

Figure 7. A20 Stabilizes Linear Ubiquitination at the TNF-RSC and NOD2-SC

(A) A549 control or A20-KO cells were stimulated with TAP-TNF (500 ng/ml) for the indicated times, subjected to TNF-RSC purification and analyzed by western blot.

(B) A549 cells pro- or deficient in A20 were virally transfected with NOD2-TAP and stimulated with L18-MDP (200 ng/ml) for the indicated times. The NOD2-SC was isolated by α-Flag immunoprecipitation.

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biochemical explanation for the observation that TNF-RSC recruitment of A20 lacking ZnF7 was significantly reduced (Tokunaga et al., 2012).

Even though A20 was shown to be incapable of cleaving M1 chains (Mevissen et al., 2013), we were surprised to find that A20 significantly stabilized M1-ubiquitin in SCs. Given that M1 chains are required for full gene-activatory signaling (Fiil and Gyrd-Hansen, 2014; Iwai et al., 2014; Walczak et al., 2012) and that A20 is a DUB previously described to inhibit NF-κB signaling (Bosanac et al., 2010; Tokunaga et al., 2012; Wertz et al., 2004), this finding seemed counterintuitive, at first. Indeed, results obtained in reconstitution experiments showed that it is ZnF7 and not A20's enzymatic DUB activity or ZnF4 that is required for A20-mediated restriction of gene activation (Skaug et al., 2011). Combined with our observation that ZnF7 is required for A20 recruitment to SCs due to its interaction with LUBAC-generated M1 chains, which are, in turn, stabilized by it, these findings now offer a possible alternative explanation for A20's NF-κB-inhibitory activity. A20 binding to M1 chains in SCs could compete with the binding of other gene-activatory factors, e.g., the NEMO/IKK complex, to them. Rising levels of A20 protein, induced as a consequence of NF-κB activation, would render M1 chains less available for NEMO/IKK retention over time so that the negative feedback loop would be completed.

A20 and CYLD Regulate Cell Death via Their Opposing Activities on Linear Ubiquitin

A20 has also been implicated as a negative regulator of TNF-induced cell death (Lee et al., 2000; Yamaguchi and Yamaguchi, 2015), whereas CYLD was shown to promote it (Hitomi et al., 2008; O'Donnell et al., 2011). Thus, while they cooperatively restrict gene activation from various SCs, they act in opposing ways on TNF-induced cell death. Linear ubiquitination in the TNF-RSC prevents TNF-induced cell death by restricting complex II formation (Gerlach et al., 2011; Ikeda et al., 2011; Peltzer et al., 2014). We show here that A20 binding to M1 chains in the TNF-RSC stabilizes them, whereas CYLD antagonizes M1 chains in this complex. Together this implies that the ability of A20 to bind M1 chains protects them from cleavage by DUBs that are capable of cleaving linear ubiquitin chains in signaling complexes. Having identified herein CYLD as a DUB with precisely this activity at the TNF-RSC, we therefore propose a model according to which A20 protects M1 chains in the TNF-RSC from CYLD-mediated cleavage, thereby providing the sought after explanation for the opposing roles played by A20 and CYLD with regards to TNF-induced cell death. Intriguingly, both roles depend on LUBAC-generated linear ubiquitin in the TNF-RSC. Hence, an intricate interplay between LUBAC, linear ubiquitination, A20, and CYLD is crucial for assembly and disassembly of SCs to enable efficient, yet properly controlled, immune signaling.

EXPERIMENTAL PROCEDURES

For description of cell lines, antibodies, and plasmids, see Supplemental Experimental Procedures.

Recombinant Proteins

TAP-TNF, untagged TNF, recombinant deubiquitinases, and proteins used for M1-AP, K63-AP, and Ubi-AP were produced in *E. coli*.

Generation of Knockout Cell Lines

HOIP- and HOIL-1-deficient K562 and HOIP-deficient A549 and HeLa cell lines were prepared by transfecting mRNA encoding gene-specific zinc finger nucleases (Sigma). CYLD-, OTULIN-, A20-deficient, and A20ΔZnF7-expressing cells were prepared by lentiviral transduction with LentiCRISPR v2 vectors (Sanjana et al., 2014) provided by Feng Zhang (Addgene plasmid #52961). Single cell clones with protein knockout or control clones were verified by western blotting.

Retroviral Transduction of Cells

Coding sequences of HOIP-WT, HOIP-C885S, HOIP-N102A, deletion mutants of human HOIP fused or not at the C terminus to the TAP-tag and TAP-tagged NOD2 (aa 28–1040) were inserted into the retroviral MSCV vector containing GFP as selection marker. Upon infection, cells were sorted using MoFlo FACS (Beckman Coulter).

Isolation of Ubiquitin Conjugates from Cell Lysates and Deubiquitination Assay

Cells were lysed and proteins denatured in AP-lysis buffer containing 1% SDS. Samples were subsequently diluted to 0.1% SDS before M1-, K63-, or total ubiquitin-specific recombinant affinity protein coupled to HALO beads was added for overnight incubation at 4°C. Beads were washed, and samples were subjected to treatment with 1 μM recombinant deubiquitinase for 1 hr at 37°C or eluted with reducing sample buffer.

Cell Stimulation and Immunoprecipitation

To analyze the native TNF-RSC, cells were treated with TAP-TNF as indicated. Cells stably expressing NOD2-TAP were stimulated with L18-MDP. Cells expressing TAP-tagged proteins were stimulated with untagged TNF. After cell lysis, samples were subjected to anti-Flag immunoprecipitation using M2 beads (Sigma) or incubated with protein A/G-Agarose beads (Santa Cruz Biotechnology) coupled to indicated antibodies. For pervanadate treatment, cells were incubated with 1 mM pervanadate for 20 min before lysis. Phosphatase treatment was performed on lysates using 50U of FastAP (Thermo Scientific) per mg of protein in absence of phosphatase inhibitors.

(C) A549 control cells or A549 cells lacking zinc finger 7 of A20 (A20-ΔZnF7) were analyzed as in (A).
(D–F) A20-deficient MEFs were reconstituted with A20-WT, A20-C103S, A20-ZnF7mut, or empty vector. Cells were stimulated with TAP-TNF (500 ng/ml) and subjected to TNF-RSC purification (D). Cells were stimulated with TNF (200 ng/ml) for the indicated times before analysis by western blot (E). Cells were stimulated with TNF (200 ng/ml) in presence or absence of zVAD (20 μM) as indicated for 24 hr, and cell death was evaluated as percentage of propidium iodide positive cells (data are presented as mean ± SEM [n = 3], *$p < 0.05$, statistics were performed using t test) (F).
(G) Model of LUBAC regulation: (1) LUBAC is associated with OTULIN or CYLD in a mutually exclusive manner; (2) loss of LUBAC interaction with CYLD and OTULIN leads to unregulated LUBAC activity, ultimately resulting in enhanced linear ubiquitination of LUBAC components themselves.
(H) Model of CYLD and A20 recruitment to, and activity at, the TNF-RSC: (1) CYLD-associated LUBAC is recruited to the SC, thereby enabling CYLD recruitment in a HOIP-dependent manner. (2) At the SCs, CYLD antagonizes M1- and K63-linked ubiquitination, thereby limiting gene activation and rendering cells more prone to TNF-induced cell death. (3) A20 is recruited to the SC by its ZnF7 domain interacting with LUBAC-generated M1 linkages placed on SC components. (4) A20 binding to M1 chains prevents their removal and restricts gene-activatory signaling, likely by competing with factors required for gene activation and, in case of the TNF-RSC, renders cells more resistant to cell death induction from this SC.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.11.009.

AUTHOR CONTRIBUTIONS

H.W. conceived the project. P.D., S.K., M.R., and H.W. designed the research and wrote the manuscript. P.D., S.K., M.R., H.D., E.L., D.M., L.S., and T.H. performed experiments; S.S. performed mass spectrometric analysis of TAP-purified LUBAC; L.T. and E.R. helped establish knockout cell lines; K.R. and L.M. prepared the recombinant USP domain of CYLD.

ACKNOWLEDGMENTS

We thank Geert van Loo for providing A20-deficient MEFs, Reinhard Fässler for providing CYLD-deficient mice, and the UCL Cancer Institute's "Cancer Genome Engineering Facility," supported by the BRC, the Welton Foundation and the Cancer Research UK-UCL Centre, for providing technical support in CRISPR engineering. We thank Anne Stinn and Marcel Hahn for excellent technical assistance. This work was supported by a Wellcome Trust Senior Investigator Award (096831/Z/11/Z) and an ERC Advanced Grant (294880) awarded to H.W., a BBSRC CASE studentship (BB/J013129/1) awarded to M.R., and MRC funding for L.M. and K.R.

Received: October 12, 2015
Revised: October 12, 2015
Accepted: October 29, 2015
Published: December 3, 2015

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