Cellular IAP proteins and LUBAC differentially regulate necroosome-associated RIP1 ubiquitination

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Necroptosis is a caspase-independent regulated type of cell death that relies on receptor-interacting protein kinases RIP1 (receptor-interacting protein kinases 1) and RIP3. Tumor necrosis factor-α (TNF-α)-stimulated assembly of the TNFRI (TNF receptor 1)-associated signaling complex leads to the recruitment of RIP1, whose ubiquitination is mediated by the cellular inhibitors of apoptosis (c-IAPs). Translocation of RIP1 to the cytoplasm and association of RIP1 with the necroosome is believed to correlate with deubiquitination of RIP1. However, we found that RIP1 ubiquitination was dispensable for necroptosis. In this study, we found that necrosome-associated RIP1 ubiquitination serves as a platform for the recruitment of other components of the necroosome, and RIP1 ubiquitination in the necroosome coincides with RIP3 phosphorylation. Both cellular IAPs and LUBAC (linear ubiquitin chain assembly complex) modulate RIP1 ubiquitination in IAP antagonist-treated necrotic cells, but they use different mechanisms. c-IAP regulates RIP1 recruitment to the necroosome without directly affecting RIP1 ubiquitination, whereas HOIP and HOIL1 mediate linear ubiquitination of RIP1 in the necroosome, but are not essential for necroptosis formation. Knockdown of the E3 ligase c-IAP1 decreased RIP1 ubiquitination, necrosome assembly and necroptosis induced by TNF-α, BV6 and zVAD-fmk. c-IAP deficiency likely decreases necrotic cell death through the activation of the noncanonical NF-κB pathway and consequent c-IAP2 upregulation. The ability to upregulate c-IAP2 could determine whether c-IAP1 absence will have a positive or negative impact on TNF-α-induced necrotic cell death and necroptosis formation. Collectively, these results reveal unexpected complexity of the roles of IAP proteins, IAP antagonists and LUBAC in the regulation of necroptosis.

Cell Death and Disease (2015) 6, e1800; doi:10.1038/cddis.2015.158; published online 25 June 2015

Proper cell death regulation is critical for tissue homeostasis with impaired or excessive cell death contributing to numerous pathologies.¹,² The best understood form of regulated cell death is apoptosis, which activates cysteine proteases called caspases.³ Recent advances have defined another type of cell death called necroptosis, a highly regulated process that occurs when caspases are inhibited. Necroptosis involves activation of receptor-interacting protein kinases 1 (RIP1, aka RIPK1) and RIP3 and has distinct cellular features, which include early loss of plasma membrane integrity, organelle swelling and inflammation.⁴–⁶

Necroptosis can be induced by TNFα (tumor necrosis factor α), TLRs (toll-like receptors) or by viral infection.⁷ Binding of TNFα to TNFRI (TNF receptor 1) triggers the recruitment of adaptor proteins TRADD and TRAF2, ubiquitin E3 ligases cellular inhibitor of apoptosis (c-IAP) proteins and RIP1 to form the receptor-associated signaling complex-I.⁷ Within complex-I, c-IAPs promote ubiquitination of RIP1 with K11- and K63-linked polyubiquitin chains.⁸–¹¹ RIP1 ubiquitination serves as a platform for the recruitment of other components of the NF-κB and mitogen-activated protein kinase signaling pathways including LUBAC (linear ubiquitin chain assembly complex).¹²,¹³ LUBAC is an ubiquitin E3 ligase complex that attaches linear polyubiquitin chains on RIP1 and consists of the catalytic subunit HOIP and the auxiliary proteins HOIL1 and sharpin.¹⁴–¹⁷ Diverse ubiquitination chains assembled on RIP1 are proposed to keep RIP1 within complex-I,¹⁰,¹³ c-IAP deficiency, which can be achieved by gene targeting or by using IAP antagonists that stimulate c-IAP proteosomal degradation, permits non-ubiquitinated RIP1 to leave the TNFR1-associated complex and join FADD, caspase-8/10 and FLIP in cytoplasmic complex-II.¹⁰,¹₈–²¹ If caspase activation is inhibited, RIP1 engages RIP3 to form the necrosome signaling complex and switches cell death from apoptosis to necroptosis.²² The kinase activities of RIP1 and RIP3 are essential for necroptosis and the RIP1 kinase inhibitor, necrostatin-1, blocks necrotic cell death.²³–²⁵ Following autophosphorylation of RIP1 and RIP3, phosphorylated RIP3 recruits its substrate, the pseudokinase MLKL (mixed lineage kinase domain-like). Phosphorylated MLKL oligomerizes and translocates to membranes resulting in cell rupture.²⁶–³⁰

RIP1 is a key factor in determining both whether a cell lives or dies, and the mode of death.³¹ Ubiquitination of RIP1 is essential for survival signaling,³² but it may also be integral to necroptosis. In this study, we found that necrosome-associated RIP1 was ubiquitinated, at least in part, by linear polyubiquitin chains assembled by HOIP and HOIL1. Interestingly, this linear ubiquitination on RIP1 was dispensable for necroptosis. In contrast, c-IAPs were dispensable for ubiquitination of RIP1 within the necroosome. Loss of c-IAP1 resulted in the

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Abbreviations: RIP1, receptor-interacting protein 1; RIP3, receptor-interacting protein 3; TNF-α, Tumor necrosis factor-α; TNFR1, TNF receptor 1; IAP, inhibitor of apoptosis; c-IAP, cellular inhibitor of apoptosis; XIAP, X chromosome-linked IAP; LUBAC, linear ubiquitin chain assembly complex; MLKL, mixed lineage kinase domain-like; MEF, mouse embryonic fibroblasts; IP, immunoprecipitation

Received 30.1.15; revised 03.4.15; accepted 04.5.15. Edited by B Turk
upregulation of c-IAP2 and noncanonical NF-κB signaling in some cells with a concomitant decrease in TNFα plus IAP antagonist-induced necroinflammation and necroptosis. These results reveal unexpected complexity in the roles of c-IAP1 and c-IAP2 in regulating necroinflammation.

Results

RIP1 is ubiquitinated in the necroinflammation. RIP1 is ubiquitinated within the TNFR1-associated signaling complex-I, and RIP1 deubiquitination is reported to be necessary for the assembly of cytoplasmic complex-II. To investigate the ubiquitination status of RIP1 during necroinflammation, human colon carcinoma HT29 cells were induced to undergo necroinflammation with TNFα, IAP antagonist BV6 and the pancaspase inhibitor zVAD-fmk (combination hereafter referred to as TBZ). Surprisingly, immunoblotting of total RIP1 revealed slower migrating forms of RIP1 at 3 h after TBZ but not after the apoptotic stimulus of TNFα and BV6 or the individual stimuli (Figure 1a and Supplementary Figure S1A). This modified form of RIP1 coincided with a slower migrating form of RIP3 (Figure 1a), which was sensitive to phosphatase treatment and therefore represented phosphorylated RIP3 (Supplementary Figure S1B). Phosphorylated RIP3 and what appeared to be ubiquitinated RIP1 in cells treated with TBZ was markedly reduced by the RIP1 kinase inhibitor necrostatin-1 (Nec-1) (Figure 1a). Consistent with previous reports, Nec-1 protected HT29 cells from killing by TBZ (Figure 1b). Similar modification of RIP1 was observed in another cell line commonly used to study necroinflammation, the mouse cell line L929 (Supplementary Figure S1C).

We confirmed that RIP1 was indeed ubiquitinated during necroinflammation by treating HT29 and mouse embryonic fibroblasts (MEFs) with TNFα+BV6+zVAD and immunoblotting for RIP1 after immunoprecipitating ubiquitinated proteins (Figures 1c and d). To determine if RIP1 within the necroinflammation was ubiquitinated, we treated HT29 cells with Flag-tagged TNF, BV6 and zVAD-fmk and then isolated TNFR1-associated complex-I (Figure 1e, middle panels). Antibodies to caspase-8 were then used to capture the necroinflammation complex-II from complex-I-depleted lysates (Figure 1e, right panels). Following early ubiquitination of TNFR1-associated RIP1 (5 min after stimulation), RIP1 ubiquitination decreases and RIP1 leaves the TNFR1 complex to bind caspase-8 where it is ubiquitinated again (Figure 1e). Similar TBZ-induced and caspase-8-associated RIP1 ubiquitination was observed in several additional cell lines (Supplementary Figure S1D). To confirm that the modifications observed in caspase-8-bound RIP1 upon TBZ treatment were ubiquitination, a first immunoprecipitation with caspase-8 was performed, followed by disruption of the complex and a second immunoprecipitation with ubiquitin antibody (Figure 1f). Collectively, these data demonstrate that ubiquitinated RIP1 is part of the necroinflammation complex together with caspase-8, FADD and phosphorylated RIP3.

Necroinflammation stimulates K63 and linear chain-linked polyubiquitination of RIP1. To investigate the nature of necroinflammation, we used ubiquitin chain-specific antibodies to determine the ubiquitination status of several proteins associated with necroinflammation (Figure 2 and Supplementary Figures S2 and 3). We treated HT29 cells with TBZ to induce necroinflammation, lysed them in denaturing urea buffer and immunoprecipitated with ubiquitin chain-specific antibodies. Our experiment revealed strong, necroinflammation stimulus-dependent RIP1 ubiquitination with K63 and linear polyubiquitin chains (Figure 2a). We also observed modest modification of FLIP and caspase-8 (Figure 2a). In addition, we also noticed minimal linear ubiquitination of RIP3, and K63-linked ubiquitination of c-IAP1 protein that was not completely degraded (Figure 2a). In addition to HT29 cells, TBZ-induced necroinflammation stimulated K63-linked and linear RIP1 polyubiquitination in other human cell lines as well (Supplementary Figure S2A). To verify if this ubiquitination pattern is selective to necroinflammation stimulus, we treated HT29 cells with TNFα and BV6 to activate apoptosis (Supplementary Figure S2B). TB produced minimal RIP1 ubiquitination at 3.5 h when compared with TBZ (Figure 2a and Supplementary Figures S2A and B).

Next, we examined if this pattern of necroinflammation ubiquitination could be observed in murine cells. To that end, we treated MEFs with TBZ and L929 cells with TAZ and investigated ubiquitination using ubiquitin chain-specific antibodies. Again, we observed significant K63 and linear polyubiquitination of RIP1 but not of RIP3 or caspase-8 (Figures 2b and c and Supplementary Figure S2C). We further expanded our analyses of necroinflammation ubiquitination by investigating LPS-induced cell death in L929 cells (Supplementary Figure S3A). Treatment of L929 cells with LPS, BV6 and zVAD (LBZ) prompted K63-linked and linear RIP1 ubiquitination, but not as prominently as TAZ treatment (Supplementary Figures S3B and C). On the other hand, LPS alone or together with zVAD did not stimulate RIP1 ubiquitination, although IRAK1, a critical component of LPS-induced signaling, was modified by K63 and linear ubiquitin linkages (Supplementary Figures S3B and C). Taken together, these results indicate that RIP1 is the primary target of necroinflammation ubiquitination, predominantly with K63 and linear chain linkages.

c-IAPs do not mediate ubiquitination of RIP1 during necroinflammation. Given that c-IAP proteins are E3 ligases for RIP1, we investigated their role in RIP1 ubiquitination during necroinflammation. Knockdown of c-IAP1 caused a noticeable decrease in RIP1 ubiquitination at 3 h after TBZ (Figure 3a). c-IAP1 downregulation also reduced phosphorylation of MLKL and improved cell viability in response to TBZ (Figures 3b and c and Supplementary Figures S4A and B). c-IAP2 knockdown did not affect cell viability in the same way as c-IAP1 knockdown (Figures 3b and c). However, double knockdown of c-IAP1 and c-IAP2 restored necroinflammation levels (Figure 3c and Supplementary Figure S4B).

To assess if c-IAP1 was the E3 ligase for RIP1 during necroinflammation, cells were pretreated with the proteasome inhibitor MG132 to limit proteasomal degradation of c-IAP1 triggered by BV6. The expectation was that we would see more RIP1 ubiquitination. Contrary to expectations, pretreatment of cells (3 h time point) with MG132 decreased RIP1 ubiquitination, although c-IAP1 was (at least partly) stabilized (Figure 3d). This was accompanied by the stabilization of procaspase-8 and FLIP, and a decrease in RIP3.
phosphorylation. Administration of MG132 for just the last hour of TBZ treatment (1 L time point) did not affect c-IAP1 degradation or RIP1 ubiquitination (Figure 3d). Looking specifically at RIP1 in complex-I and the necrosome/complex-II, MG132 pretreatment appeared to stabilize modified RIP1 at complex I and prevent assembly of the necrosome/complex-II (Figure 3e). Therefore, MG132 pretreatment and c-IAP1 stabilization inhibit RIP1 translocation to caspase-8-associated necrosome complex, and consequently necrosome-associated RIP1 ubiquitination.

BV6 treatment caused transient elimination of c-IAP2 in HT29 cells (Figure 3f). Interestingly, the return of TBZ-induced c-IAP2 coincided with the appearance of RIP1 ubiquitination (Figure 3f, compare lanes 3 and 4 with 8 and 9). This increase in c-IAP2 protein abundance at 4 h after TBZ followed increased expression of c-IAP2 messenger RNA (mRNA), raising the possibility that c-IAP2 could promote RIP1 ubiquitination in necroptotic signaling (Supplementary Figure S4C). To determine definitively if c-IAP2 and/or c-IAP1 were responsible for RIP1 ubiquitination in necroptosis, we compared RIP1 modifications in a WT, c-IAP1−/− and c-IAP1−/− c-IAP2−/− MEFs. TBZ-induced ubiquitination of RIP1 was comparable in the different MEF lines (Figure 3g), indicating that c-IAP1 and c-IAP2 are dispensable for ubiquitination of RIP1 induced by TBZ. In addition, in the absence of c-IAP1 and c-IAP2, TNFα plus zVAD (TZ)
treatment was sufficient to trigger RIP1 ubiquitination (Figure 3g). siRNA knockdown of TRAF2, which is the adaptor protein that bridges c-IAPs and RIP1 within complex-I, did not affect TBZ-induced ubiquitination of RIP1 or necroptosis in HT29 cells either (Supplementary Figures S4D and E). Collectively, these data indicate that ubiquitination of RIP1 during necroptosis can occur independently of c-IAPs.

Upregulation of c-IAP2 in the absence of c-IAP1 decreases necroptosis. We were intrigued that knockdown of c-IAP1 in HT29 cells decreased TBZ-induced ubiquitination of RIP1 and necrotic cell death (Figures 3a and c and Supplementary Figures S4A and B). Analysis of complex-I and the necrosome/complex-II revealed that c-IAP1 knockdown in HT29 cells caused a slight increase in the amount of RIP1 in TBZ-induced complex-I, whereas less RIP1 was incorporated into the caspase-8-containing necrosome/complex-II (Figure 4a). The association of caspase-8 with RIP3 and FADD was also reduced (Figure 4a).

Interestingly, knockdown of c-IAP1 increased the expression of c-IAP2 protein (Figure 4b) and mRNA (Figure 4c), and the increased c-IAP2 protein could not be quickly depleted by BV6 in the absence of c-IAP1 (Figure 4b). Upregulation of c-IAP2 was most likely the result of noncanonical NF-κB activation, as demonstrated by processing of the NF-κB2/p100 transcription factor to p52 (Figure 4d) and accumulation of the p52 subunit in the nucleus (Figure 4e). Indeed, combined knockdown of c-IAP1 and p100 abrogated c-IAP2 upregulation (Figure 4f). Similarly, in KatoIII and Colo201 cells, c-IAP1 knockdown also upregulated c-IAP2 levels and reduced TBZ-induced

**Figure 2.** RIP1 undergoes K63 and linear chain-linked polyubiquitination during necroptosis. (a) HT29 cells were treated with TNFα 20 ng/ml (T), BV6 2 μM (B) and zVAD (20 μM) for 3.5 hours. Cells were lysed in 6 M urea buffer and immunoprecipitated using linkage-specific anti-ubiquitin antibodies or control antibody. Immunoprecipitated proteins were detected using indicated antibodies. (b) and (c) MEF (b) or L929 (c) cells were not treated or treated with TNFα (100 ng/ml), BV6 (2 μM) and zVAD (20 μM) for 3 h (b) or with TNFα (10 ng/ml) and zVAD (20 μM) for 2 or 3 h (c). Cells were lysed and immunoprecipitated as in (a) and immunoprecipitated proteins were detected using indicated antibodies.
necroptosis and RIP1 ubiquitination (Supplementary Figures S5A and E). To assess more precisely differences in necrosome formation, HT29 cells were treated with TBZ for 1 h (Figure 4g and Supplementary Figures S6A and B). Although downregulation of c-IAP1 decreased the association of RIP1 with caspase-8, c-IAP2 knockdown had no effect (Supplementary Figure S6A). Elevated c-IAP2 appeared to contribute to the effects of c-IAP1 knockdown on TBZ-induced assembly of the necrosome/complex-II because combined knockdown of c-IAP1 and c-IAP2 resulted in stronger RIP1
necrosome association in comparison with c-IAP1 knockdown (Figure 4g).

Triple knockdown of c-IAP1, c-IAP2 and XIAP triggered a noticeable increase in necrosome formation in agreement with reports that XIAP is a negative regulator of necroptosis (Supplementary Figure S6B). To assess which combination of IAP knockdowns would mimic BV6 activity in necroptosis, we treated cells with TNFα and zVAD, and interestingly, we found that triple knockdown of c-IAP1, c-IAP2 and XIAP had the same effect as BV6 (Supplementary Figure S6C). However, individual downregulation of XIAP did not alter RIP1 necrosome recruitment or TBZ-induced cell death, suggesting that the absence of XIAP exerts strong effects on TBZ-stimulated necrosome formation mostly in the context of

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**Figure 4**  c-IAP1 knockdown inhibits necrosome formation and cell death due to c-IAP2 upregulation. (a–h) HT29 cells were transfected with the indicated siRNAs or treated with BV6 for 72 or 48 h. (a) Cells were treated with Flag-TNFα 1 μg/ml (T), BV6 2 μM (B) and zVAD 20 μM (Z) for the indicated periods of time. Cell lysates were first immunoprecipitated with Flag-beads, and the supernatants underwent a second immunoprecipitation with caspase-8 antibody. The pull-downs and lysates were analyzed by western blotting with the indicated antibodies. (b) Cells were treated with TNFα 20 ng/ml (T), BV6 2 μM (B) and zVAD 20 μM (Z) for the indicated periods of time and cellular lysates were analyzed by western blotting. (c) Total mRNA was extracted and c-IAP2 and c-IAP1 mRNA levels were analyzed by quantitative RT-PCR real time. (d) Lysates of siRNA-transfected and BV6-treated cells were analyzed by western blotting with the indicated antibodies. (e) Nuclear and cytoplasmic extracts of siRNA-transfected cells were analyzed by western blotting with the indicated antibodies. (f) Lysates of siRNA-transfected cells were analyzed by western blotting with the indicated antibodies. (g) Cells were treated with TNFα 20 ng/ml (T), BV6 2 μM (B) and zVAD 20 μM (Z) for the indicated periods of time. Cell lysates were immunoprecipitated with caspase-8 antibody. The pull-downs and lysates were analyzed by western blotting with the indicated antibodies. (h) Total mRNA was extracted and c-IAP2 mRNA levels were analyzed as in (c)
c-IAP1/2 loss (Supplementary Figures S6D and E). These results point to an interesting interplay of c-IAPs and IAP antagonists in necroptosis. IAP antagonists eliminate c-IAP proteins to induce necroptosis in HT29 cells, but at the same time, IAP antagonists or c-IAP1 knockdown induce c-IAP2 upregulation owing to noncanonical NF-xB activation. However, the loss of c-IAP1 by knockdown allows the accumulation of c-IAP2, whereas BV6 treatment causes degradation of newly synthesized c-IAP2 (Figures 4d, e and h).

Next, we investigated the regulation of c-IAP2 levels in MEts during necroptotic signaling. Both c-IAP1−/− knockout (KO) and c-IAP1/2−/− DKO MEts were more sensitive to necroptotic cell death compared with wild-type (WT) MEts (Figure 5a and 3g). Interestingly, c-IAP1−/− KO MEts did not have elevated levels of c-IAP2 in basal conditions, and c-IAP2 upregulation after necrotic stimulation was lower compared with that in WT MEts (Figure 5b). Additionally, transient knockdown of c-IAP1 in MEts did not significantly affect c-IAP2 mRNA levels (Figure 5c). Contrary to HT29 cells, p100 is constitutively processed to p52 in MEts and c-IAP1 downregulation caused no significant differences in p100 processing and p52 nuclear translocation (Figures 5d and e). Collectively, these data suggest that the ability to upregulate c-IAP2 determines whether c-IAP1 absence will have positive or negative impact on TNFα-induced necroptotic cell death.

RIP1 undergoes linear ubiquitination in necroptosis. LUBAC (consisting of HOIP, HOIL1 and sharpin) has been shown to promote linear ubiquitination of RIP1 within the TNFR1 signaling complex.13,16 We examined whether LUBAC mediates RIP1 linear ubiquitination in the necrosome by pretreating cells with BV6 to eliminate c-IAP proteins and thus prevent TNF-mediated recruitment of LUBAC to complex-I.12,13 HT29 cells were treated with TBZ and the necrosome was immunoprecipitated using anticaspase-8 antibody. Necrosome was then disrupted in urea buffer and immunoprecipitated with the linear polyubiquitin chain-specific antibody. This demonstrated that necrosome-associated RIP1 is modified with linear polyubiquitin linkages (Figure 6a). To examine the role of LUBAC in necrosome-associated linear polyubiquitination of RIP1, we knocked down HOIP and HOIL1. Downregulation of HOIP and HOIL1 reduced RIP1 ubiquitination in the necrosome (Figure 6b) and necroptosis-specific linear ubiquitination (Figure 6c). HOIP knockdown reduced the levels of caspases-8-bound ubiquitinated RIP1, suggesting that HOIP absence affected RIP1 ubiquitination similarly to c-IAP1 knockdown (RIP1 (dark exposure); Figure 6d). However, quantification of RIP1 levels revealed that c-IAP1 downregulation decreased RIP1 ubiquitination because of the inhibition of necroptosome formation (changes in A numbers—no changes in B/A ratio) but no significant changes in B/A ratio—ubiquitinated over unmodified RIP1) (Figure 6d and Supplementary Figure S7A). On the other hand, HOIP knockdown did not affect necroptosome formation (no changes in A numbers) but reduced ubiquitination of RIP1 (lower B/A ratio) (Figure 6d and Supplementary Figure S7B). Knockdown of HOIP did not affect TBZ-stimulated necroptotic cell death either.
Therefore, c-IAPs and LUBAC regulate RIP1 ubiquitination and necrosome formation through different mechanisms.

We also explored necroptotic signaling in cpdm MEFs, which have deficient sharpin expression.\textsuperscript{36} Investigation of linear polyubiquitination of RIP1 during necroptosis revealed no differences between cpdm and WT MEFs (Supplementary Figure S8A). As expected, K63-linked RIP1 polyubiquitination was not affected. We verified this result with sharpin knockdown in HT29 cells, and again observed that RIP1 can undergo linear ubiquitination in necroptosis without sharpin (Supplementary Figure S8B). Sharpin knockdown did not

(Supplementary Figures S7C and D). Therefore, c-IAPs and LUBAC regulate RIP1 ubiquitination and necrosome formation through different mechanisms.

**Figure 6** RIP1 undergoes linear ubiquitination upon induction of necroptosis. (a–c) HT29 cells were pretreated with BV6 2 μM (B) and zVAD 20 μM (Z), and 20 min latter TNFα 20 ng/ml (T) was added for another 2 h. (a) Cell lysates were first immunoprecipitated with caspase-8 antibody, and the pull-downs were disrupted in 6 M urea and underwent a second immunoprecipitation with linear ubiquitin or control antibody. (b–d) HT29 cells were transfected with the indicated siRNAs for 72 h. (b) Cell lysates were immunoprecipitated with caspase-8 antibody. (c) Cell lysates were immunoprecipitated in 6 M urea with linear ubiquitin or control antibody. (d) HT29 cells were pretreated with BV6 2 μM (B) and zVAD 20 μM (Z), and 20 min latter with Flag-TNFα 1 μg/ml (T) for the indicated periods of time. Cell lysates were first immunoprecipitated with Flag beads, and the supernatants underwent a second immunoprecipitation with caspase-8 antibody. The pull-downs and lysates were analyzed by western blotting with the indicated antibodies. The amount of RIP1 immunoprecipitated by caspase-8 was quantified and indicated below each lane. RIP1 quantification in the upper panel (A values) corresponds to the levels of unmodified RIP1 in each lane in comparison with RIP1 levels in GFP 1 h. RIP1 quantification in the lower panel (B/A values) corresponds to the ratio between the intensity of RIP1 ubiquitination (B values) and the levels of co-immunoprecipitated unmodified RIP1 in the upper panel (A values).
affect necroosome formation, even though RIP1 ubiquitination in the TNFR1-associated signaling complex was reduced in the absence of sharpin (Supplementary Figure S8C). In addition, sharpin or HOIP downregulation did not influence each other’s protein levels in any of the examined cell lines (Supplementary Figures S8D and F). Therefore, unlike HOIP and HOIL1, sharpin does not seem to be crucial for necroosome-associated RIP1 linear ubiquitination during IAP antagonist-stimulated necrotic signaling.

**Discussion**

Ubiquitination of RIP1 by c-IAP proteins within TNFR1-associated complex-I inhibits RIP1 dissociation from complex-I. Deubiquitination allows RIP1 to move from the TNFR1-associated complex to the cell death-promoting complex-II or necroosome. Thus, it is believed that the necroosome contains non-ubiquitinated RIP1. However, contrary to previous reports, our study shows that RIP1 is ubiquitinated within IAP antagonist and TNFα, as well as IAP antagonist and LPS, stimulated necroptosis predominantly with K63 and linear polyubiquitin chains, and that ubiquitinated RIP1 is part of the necroosome complex where it associates with phosphorylated RIP3. Our data show that following approaches, it is conceivable that the results from two studies might not completely overlap. Nevertheless, the data from our study and Lawlor et al. shed light on the importance of ubiquitination in necrotic signaling, which should trigger more investigations of the role of this posttranslational modification in inflammatory cell death pathways.

While our manuscript was in review, another study showed that c-IAPs promote RIP1 ubiquitination within the TNFR1 signaling complex. We postulated that they regulate necroptosis-associated RIP1 ubiquitination. Further supporting that hypothesis, we observed a decrease in RIP1 ubiquitination after c-IAP1 knockdown and a correlation between c-IAP2 reappearance and RIP1 ubiquitination. However, given that prospeosome inhibitor treatment to stabilize c-IAP1 eliminated RIP1 ubiquitination and complete absence of c-IAPs in double KO MEFs did not affect RIP1 ubiquitination, we are fairly certain that c-IAPs are not RIP1 E3 ligases in the necroosome.

Nevertheless, c-IAP1 downregulation inhibits necrotic cell death, whereas IAP antagonists promote necroptosis. The possible key for understanding this conundrum is c-IAP2, whose levels increase upon c-IAP1 loss because of the activation of noncanonical NF-κB signaling. Thus, c-IAP2 protein levels may explain the interesting differences between IAP antagonist treatment and c-IAP1 downregulation: specific c-IAP1 loss boosts c-IAP2 levels, whereas BV6 also promotes c-IAP2 upregulation, but ultimately causes its degradation. However, c-IAP2 upregulation is not a global event as in some cell types, such as MEFs, c-IAP1 loss does not activate noncanonical NF-κB signaling nor upregulates c-IAP2 levels.

As a consequence, the absence of c-IAP1 does not provide any survival benefit. Therefore, the ability to activate noncanonical NF-κB signaling and induce c-IAP2 upregulation can determine responsiveness to IAP antagonist-mediated necrotic cell death. Interestingly, only the triple knockdown of c-IAP1, c-IAP2 and XIAP reproduces BV6-like effects on necroptosis and necrotic cell death. Recent studies suggest that XIAP could potentially ubiquitinate necroosome components; however, more studies are needed to decipher the exact role of XIAP in necroptosis.

LUBAC also promotes RIP1 ubiquitination within the TNFR1 signaling complex, but through linear ubiquitin linkages. We found that necroptosome-bound RIP1 is modified with linear ubiquitin chains in an HOIP/HOIL1-dependent manner. Earlier reports suggested that HOIP or HOIL1 knockdown or KO augment TNFα-stimulated cell death. However, we found that in IAP antagonist-pretreated cells linear ubiquitination does not significantly affect necroptosome formation or necrotic cell death. IAP antagonist pretreatment inhibits LUBAC recruitment and RIP1 ubiquitination within the TNFR1-associated complex. Therefore, pretreatment with IAP antagonists dissociates LUBAC function within the necroosome from its role in the TNFR1 signaling complex and explains the differences between the important role HOIP deficiency exerts in TNFα-mediated cell death and IAP antagonist-stimulated necroptosis. Given that downregulation of HOIP and HOIL1 decreases linear ubiquitination of necroptosome-bound RIP1 without affecting necroptosome formation, linear ubiquitination of RIP1 in necrotic cells might not be crucial for IAP antagonist-mediated necrotic cell death.

Sharpin is important for LUBAC activity and the absence of sharpin limits linear ubiquitination in TNFR1 signaling complex and decreases NF-κB signaling. To our surprise, cpdm MEFs or cells with sharpin knockdown showed no decrease in RIP1 linear ubiquitination in necroptosis. In agreement with the reported role of sharpin in TNFR1-mediated signaling, we observed a decrease in RIP1 ubiquitination in the TNFR1 signaling complex upon sharpin knockdown. Nevertheless, it seems that in the IAP antagonist-stimulated necroptosome sharpin is not needed for LUBAC activity. Given that cpdm MEFs can form HOIP-HOIL1 complexes with E3 ligase activity, sharpin may be dispensable for LUBAC activity in certain conditions. In prior studies, cpdm mice and cells were found to be sensitive to TNF-induced cell death to various degrees, which could be explained by the destabilization of HOIP and HOIL1 protein levels in the absence of sharpin. However, in our study, sharpin absence did not negatively affect the levels of HOIP, and accordingly, IAP antagonist and TNFα-driven necroptosome-associated RIP1 ubiquitination was also unchanged.

In summary, this study reveals the presence of a second wave of RIP1 ubiquitination during necroptosis and the complex role for c-IAP proteins in the regulation of IAP antagonist-mediated necrotic cell death. c-IAP1 controls TNFα-stimulated necroptosome formation without affecting RIP1 ubiquitination. On the other hand, linear RIP1 ubiquitination in the necroosome is mediated by HOIP, although its functional relevance remains elusive. Further studies are needed to fully elucidate the importance of RIP1 ubiquitination in necroptosis.
Materials and Methods

Reagents and transfections. The following materials have been used: human recombiant soluble TNFα (Genentech, South San Francisco, CA, USA), mouse recombinant soluble TNFα (R&D, Minneapolis, MN, USA), Flg-TNFα (Enzo, Farmingdale, NY, USA), BV6 (Genentech), z-VAD-Fmk (MBL, Woburn, MA, USA), Nec-1 (Sigma, Atlanta, GA, USA), MG132 (UBPBio, Aurora, CO, USA) and LPS (Invivogen, San Diego, CA, USA). The primary antibodies used were directed against: RIP1, XAP, TRAF2, FADD (BD Biosciences, San Jose, CA, USA), human and mouse RIP1 (Immunex, San Diego, CA, USA), human c-IAP1 and mouse pan-c-IAP (R&D), human c-IAP2 (Novus, Littleton, CO, USA), actin, IgG (Santa Cruz, Santa Cruz, CA, USA), ubiquitin (P4D1; Cell Signaling, Danvers, MA, USA), human caspase-8 (Cell Signaling and Enzo), mouse caspase-8, mouse c-IAP1 and FLIP (Enzo), human shaparin, p100/p52, HDAC2, IAK1 (Cell Signaling), K11, K48, K63 and linear ubiquitin chains, and mouse RIP3 (Genentech), HDIP (Aviva Systems, San Diego, CA, USA), HOIL1 (Sigma), mouse shaparin (ProteinTech, Chicago, IL, USA), MLKL (Millipore, Bilcera, MA, USA) and P-MKL (Abcam, Cambridge, MA, USA). All siRNA transfections were carried out using Lipofectamine RNAiMax (Invitrogen) for 48 or 72 h; siRNA sequences are included in Supplementary Data.

Cell lines. Human colon carcinoma HT29, LS411N, Colo205, Colo205, stomach Kato III, Im95m, 231238/7 and mouse L289 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown and maintained in 50:50 F12:DMEM medium supplemented with 10% fetal bovine serum, penicillin, streptomycin and 2 mM glutamine at 5% CO2. WT and c-IAP1 and LUBAC−/− MEFs were cultured in DMEM medium supplemented with 10% fetal bovine serum, penicillin, streptomycin and 2 mM glutamine at 5% CO2. WT and c-IAP1 and LUBAC−/− MEFs were cultured in DMEM medium supplemented with 10% fetal bovine serum, penicillin, streptomycin and 2 mM glutamine at 5% CO2.

Viability assays. Cell viability was assessed using CellTiter-Glo (Promega) following the manufacturer’s specifications. Cell death was monitored by Incucyte Zoom (Essen BioSciences, Ann Arbor, MI, USA) using the cell death dye Sytox Green nucleic acid stain (Life Technologies, Carlsbad, CA, USA) and the dye Nuclear-ID red DNA stain (Enzo) to count the total number of cells.

Western blot analysis, immunoprecipitation and nuclear extracts. Western blot analyses and immunoprecipitations were performed using Western blot analysis, immunoprecipitation and nuclear extracts. Immunoprecipitations followed the manufacturer’s recommendations. Dephosphorylation of RIP3 was performed by treating cellular lysates with λ-phosphatase at 30 °C for 2 h.

Real-time quantitative PCR. RNA was isolated from cells with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following standard protocols. An on-column DNase treatment was included: cDNA was generated from each RNA sample using a Taqman Gene Expression Cells to Ct Kit (Life Technologies). Gene expression assays were synthesized in-house: RPL19-FW, 5′-AGCGGATCTTCAGGACAA-3′ and RPL19-Rv, 5′-CTGGTGACCGAGCAGCTT-3′; RPL19-Probe, 5′-FAM-TCCCAACGTGGACGACAGGAT-3′; ihcIAP1-FW, 5′-TGGTTGGTGAACATTATATGAATGTCGATAC-3′; ihcIAP1-Rv, 5′-GAAAGAAGACCAACTGCGATCCTCT-3′; ihcIAP1-Probe, 5′-FAM-AAGGGAGTACTGTCCTGTTRTGCA TACATT-TAMRA-3′; or from Life Technologies: hciAP2-Hs00985031_g1, mciAP2-Mm00431800_m1 and mGAPDH-Mm99999915_m1. Target gene levels were normalized against RPL19 or GAPDH gene expression.

Statistical analysis. Data are presented as the mean ± S.E.M. Statistical analysis was performed using Student’s t-test. Results were considered significant if *P<0.05, **P<0.01 or ***P<0.005.

Acknowledgements. We thank Eugene Varfolomeev, Soren Warming, Meron Roose-Girma, Kerry Zobel, Kurt Deshayes, Wayne Fairbrother, Vishva Dixit, Ali Zarin, John Silke, members of the Early Discovery Biochemistry Department and the Oligo Synthesis and Screening facilities at Genentech that provided help with insightful discussions, suggestions and reagents. All authors are employees of Genentech.
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