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Cullin3-Based Polyubiquitination and p62-Dependent Aggregation of Caspase-8 Mediate Extrinsic Apoptosis Signaling

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SUMMARY

Cell-surface death receptors such as DR4 and DR5 trigger apoptosis through a death-inducing signaling complex (DISC) that recruits the apical protease caspase-8. Apoptosis commitment requires efficient activation and autocatalytic release of caspase-8 into the cytoplasm to engage executioner caspases. While DISC recruitment initiates caspase-8 stimulation, full activation of the protease depends on further molecular aggregation events that are not fully understood. Here, we show that death receptor ligation induces polyubiquitination of caspase-8, through a previously unknown interaction of the DISC with a cullin3 (CUL3)-based E3 ligase. CUL3-mediated caspase-8 polyubiquitination required the RING box protein RBX1, whereas the deubiquitinase A20 reversed this modification. The ubiquitin-binding protein p62/sequestosome-1 promoted aggregation of CUL3-modified caspase-8 within p62-dependent foci, leading to full activation and processing of the enzyme and driving commitment to cell death. These results identify a mechanism that positively controls apoptosis signaling by polyubiquitination and aggregation of a key initiator caspase.

INTRODUCTION

Apoptosis is a highly regulated and morphologically distinct process of cell suicide that helps multicellular animals to eliminate unneeded, damaged, or infected cells. Apoptosis plays important roles in embryonic development and adult tissue maintenance, and its aberration contributes to cancer as well as to neurodegenerative and autoimmune diseases (Elmore, 2007; Hanahan and Weinberg, 2000). Caspases are cysteinyl aspartate proteases that function as apical initiators or distal effectors to orchestrate the apoptotic cell death program (Boatright et al., 2003; Green, 2005). Two distinct signaling pathways trigger apoptosis: the intrinsic pathway, activated from within the cell by developmental cues or cellular damage (Zornig et al., 2001), and the extrinsic pathway, stimulated from the cell's outside by specific proapoptotic ligands that belong to the tumor necrosis factor (TNF) superfamily (Ashkenazi, 2002; Peter and Krammer, 2003). These pathways use different initiator caspases yet converge on a single set of executioner caspases. In the intrinsic pathway, the "apoptosome" mediates multimerization of the apical protease caspase-9, facilitating its full activation (Jiang and Wang, 2004; Riedl and Salvesen, 2007).

Proapoptotic ligands signal through cell-surface "death" receptors: Fas/CD95/Apo1 ligand (FasL) acts via Fas/CD95, while apoptosis 2 ligand/TNF-related apoptosis-inducing ligand (Apo2L/TRAIL) signals through DR4 (TRAIL-R1) and/or DR5 (TRAIL-R2) (Ashkenazi and Dixit, 1998; Nagata, 1997; Peter and Krammer, 2003). An initial, trimeric ligand-receptor complex recruits the adaptor protein Fas-associated death domain (FADD) through homophilic interaction of death domains in the receptor and adaptor. FADD also contains a death-effector domain (DED), which recruits the initiator protease caspase-8 via its two tandem N-terminal DEDs, thus forming a DISC. The DISC mediates activation and self-processing of caspase-8, and the liberated protease moves into the cytosol, where it cleaves and activates executioner caspases such as caspase-3 and -7. In "type I" cells, extrinsic signaling generates sufficient caspase-8 and consequent effector caspase activity to commit the cell to apoptotic death. In "type II" cells, further signal amplification is required; this occurs by caspase-8-mediated processing of the Bcl-2 homology domain 3 protein Bid, which engages the mitochondria to promote further caspase activation (Peter and Krammer, 2003). TNFα can induce apoptosis through the death domain-containing receptor TNFR1, which requires inhibition of dominant TNFα prosurvival signaling via nuclear factor (NF)-κB and is further modulated by c-Jun N-terminal kinase (JNK) (Wallach et al., 1999; Karin and Lin, 2002; Varfolomeev and Ashkenazi, 2004). TNFα activates caspase-8 via secondary signaling complexes, assembled downstream of primary complexes that induce NF-κB and JNK activation (Micheau and Tschopp, 2003; Wang et al., 2008).

Proximity-induced dimerization of caspase-8 supports its initial stimulation (Boatright et al., 2003). However, apoptosis commitment depends on full activation and autocatalytic

processing of the protease, which requires its further aggregation. Specific posttranslational modifications of death receptors can facilitate membrane trafficking events that augment DISC activation: palmitoylation of the Fas intracellular domain supports translocation and clustering of Fas into plasma membrane lipid rafts (Feig et al., 2007). Alternatively, O-glycosylation of the extracellular domains of DR4 and DR5 facilitates ligand-induced receptor clustering (Wagner et al., 2007). Other types of posttranslational modification, including phosphorylation, nitrosylation, oxidation, and ubiquitination, also contribute to apoptosis regulation. Ubiquitination involves covalent attachment of the 76 amino acid polypeptide ubiquitin (Ub) to proteins; Ub is added as monoubiquitin, or as polyubiquitin (pUb) chains linked via internal lysines (Pickart, 2004). K48-linked pUb chains frequently provide a signal for proteasome-mediated degradation, while K63-linked chains often affect functional or spatial protein regulation. A wide array of Ub-binding proteins controls the fate or activity of the conjugated substrates: one of these proteins—p62/sequestosome-1—is implicated in mediating the formation and/or clearance of ubiquitinated-protein aggregates (Komatsu et al., 2007; Pankiv et al., 2007), as well as in regulation of several intracellular signaling pathways (Kim and Rao, 2006; Moscat et al., 2007).

Ubiquitination requires coordinated action of a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2), and a Ub ligase (E3). Cullin-RING ligases (CRLs) comprise the largest group of E3 enzymes in eukaryotes (Bosu and Kipreos, 2008; Petroski and Deshaies, 2005). CRLs, including CUL3, operate as multisubunit complexes that contain a cullin, a RING H2 finger protein, and a substrate-binding moiety. CUL3 typically connects the RING H2 finger protein Ring-box (RBX) 1 or 2 and a substrate-binding subunit containing a BTB (Bric-a-brac, Tramtrack, Broad-complex) domain (Bosu and Kipreos, 2008). Ubiquitination is implicated in the regulation of both prosurvival and proapoptotic signals. Activation of the IKK complex by interleukin-1 and Toll-like receptors involves polyubiquitination of TNFR-associated factor 6 (TRAF6) (Israel, 2006). TNFα requires K63-linked polyubiquitination of key signaling mediators, including the kinase RIP1 and the scaffolding protein NEMO/IKKγ, to activate prosurvival genes through NF-κB (Chen, 2005). The deubiquitinase A20 rapidly reverses RIP1 ubiquitination, decreasing the amplitude of NF-κB activation by TNFα (Wertz et al., 2004), while deubiquitination by CYLD augments RIP1 binding to FADD and promotes apoptosis (Wang et al., 2008). The E3 ligase ITCH ubiquitinates the caspase-8 inhibitor FLIP, inducing its proteasomal destruction and enhancing proapoptotic TNFα signaling (Chang et al., 2006). Inhibitor of apoptosis (IAP) proteins, which inhibit caspases by direct binding, can act as RING E3 ligases to promote ubiquitination and degradation of effector caspases (Deveraux and Reed, 1999).

Caspase-8 has been intensely studied as a critical initiator of the extrinsic apoptosis pathway; however, the events that control its activation are not fully defined. Here, we show that stimulation of death receptors 4 and 5 induces CUL3/RBX1-dependent polyubiquitination of caspase-8. In turn, the Ub-binding protein p62 promotes caspase-8 aggregation, augmenting the activation and full processing of the enzyme and thereby leading to robust stimulation of effector caspases and apoptosis. These results identify a mechanism that positively controls apical caspase activation by polyubiquitination and aggregation.

RESULTS

Apo2L/TRAIL Induces Caspase-8 Ubiquitination

Caspase-8 immunoblot (IB) after cell extraction with sodium dodecyl sulfate (but not the milder detergent Triton X-100) revealed apparent ubiquitination of the protease within 10 min after stimulation of H460 or H2122 cells with Apo2L/TRAIL (Figure 1A). Caspase-8 immunoprecipitation (IP), performed under denaturing conditions to disrupt binding to other proteins, followed by IB with caspase-8 or Ub antibody, confirmed the ubiquitination of caspase-8 (Figure 1B).

To identify which cell compartment harbors this modification, we used an established subcellular fractionation approach (Sabio et al., 2005). In unstimulated H460 cells (0 min), DR4 was present mostly in the membrane fraction, while FADD and full-length caspase-8 (p55/53) were primarily in the cytosol (Figure 1C). After ligand stimulation, the cytosolic levels of FADD and caspase-8 gradually declined, while increasing in the membrane and in a third compartment containing membrane lipid-raft platforms and cytoskeletal proteins. Notably, ubiquitinated caspase-8 appeared mainly in the raft/cytoskeleton fraction. Autocatalytic processing of caspase-8 involves initial cleavage near the C terminus between the p18 and p10 domains (generating p10 and p43/41 fragments), followed by proteolytic separation between p18 and the two N-terminal DEDs. The resulting DED fragment (p26/24) remains associated with the DISC for a period of time, while p18 and p10 form the liberated active enzyme heterotetramer. After ligand stimulation, most of the DED fragment was present in the raft/cytoskeleton fraction (Figure 1C), suggesting that complete caspase-8 processing takes place mainly in this compartment. The ubiquitinated caspase-8 bands peaked at 10 min and then diminished, whereas the DED fragment accumulated over time. p18 was undetectable in the cytosolic fraction at 10 min, but it appeared and built up over 30–90 min. H2122 cells showed similar results, whereas two cell lines resistant to Apo2L/TRAIL—HeLa and ADR-RES—did not exhibit caspase-8 ubiquitination or processing (Figure S1A available online). Caspase-8 ubiquitination occurred also in HT1080 cells (Figure S1B), which express DR5 but not DR4, as well as several other cell lines (data not shown). siRNA knockdown of Bid in H460 cells, which exhibit type II signaling, did not diminish caspase-8 ubiquitination (Figure S1C), indicating that the modification occurs upstream of Bid and the mitochondria.

After ligand stimulation, the DISC forms high molecular weight (HMW) aggregates that can be detected by size-exclusion chromatography (SEC) (Feig et al., 2007; Wagner et al., 2007). SEC of stimulated H460 cells confirmed that caspase-8 shifts to HMW fractions, and revealed the ubiquitinated caspase-8 and processed DEDs (Figure 1D), as well as most of the catalytic caspase-8 activity (Figure 1E), in these fractions. Pretreatment of H460 cells with the proteasome inhibitor MG132 led to a general increase in ubiquitinated proteins and a concomitant decrease in free Ub (Figure S1D); however, it did not cause accumulation of caspase-8 (Figure 1F). Thus, ligand-induced ubiquitination

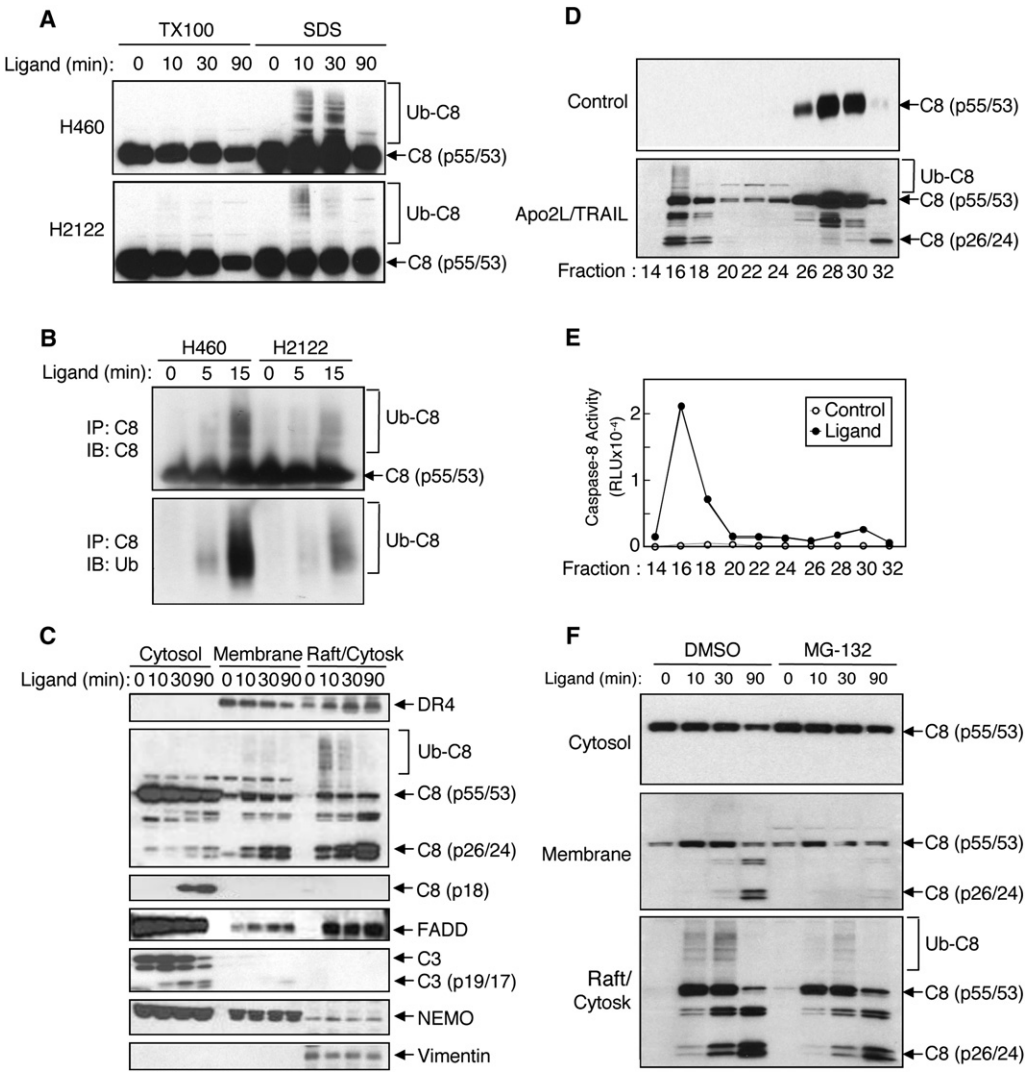


Figure 1. Ligand Stimulation of DR4 and DR5 Induces Ubiquitination of Caspase-8

(A) H460 and H2122 cells were treated with Apo2L/TRAIL (250 ng/ml), lysed in sample buffer containing either 1% Triton X-100 or 1% SDS, cleared by centrifugation, and analyzed by caspase-8 (C8) IB.

(B) H460 and H2122 cells were treated as in (A), lysed in buffer containing 1% SDS, and boiled for 10 min. Lysates were diluted 1:10 with DISC IP buffer and subjected to immunoprecipitation (IP) with C8 antibody. IP samples were analyzed by IB with antibody to C8 or ubiquitin (Ub).

(C) H460 cells were treated as in A and subjected to subcellular fractionation. Cellular fractions containing cytosol, membranes, or lipid rafts and cytoskeleton (raft/cytosk) were analyzed by IB with antibodies to DR4, C8, FADD, caspase-3 (C3), NEMO, and vimentin.

(D and E) Cell lysates with similar protein concentration from buffer or Apo2L/TRAIL-treated H460 cells were resolved by SEC on a Superdex 200 column, and the eluted fractions (0.5 ml) were analyzed by C8 IB (D) or caspase-8 activity assay (E) (open circles, control; closed circles, Apo2L/TRAIL; representative experiment shown).

(F) H460 cells were preincubated with DMSO or MG132 (10 mM) for 1 hr, treated with Apo2L/TRAIL (250 ng/ml), and subjected to subcellular fractionation and C8 IB.

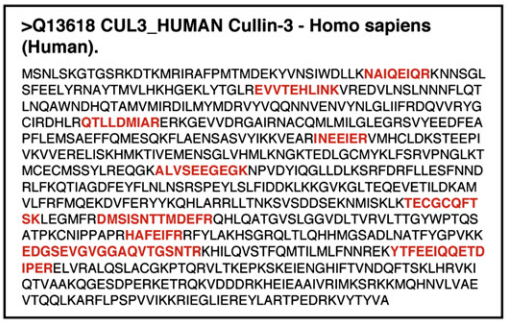
promotes aggregation but not rapid proteasomal degradation of caspase-8.

Apo2L/TRAIL treatment at low temperature rather than 37°C allows initial DISC formation, but attenuates subsequent caspase-8 cleavage and effector-caspase activation (Austin et al., 2006; Kohlhaas et al., 2007). After ligand stimulation of *Bax*$^{+/-}$ HCT116 cells at 37°C, ubiquitinated caspase-8 appeared only transiently in the raft/cytoskeleton fraction; however, at 4°C, it progressively accumulated in this compartment over 2 hr (Figures S2A and S2B). Cytosolic processing of downstream caspase-3 occurred at 37°C but not at 4°C (Figure S2A). Markedly more caspase-8 activity was generated at 4°C than 37°C, whereas the converse was true of caspase-3/7 activity (Figure S2C). Furthermore, substantially greater caspase-8 activity appeared in the HMW SEC fractions of cells stimulated at 4°C as compared to 37°C (Figure S2D). These results indicate

A

	Untreated		Apo2L/TRAIL treated	
	Isotype control	Anti-DR4 IP	Isotype control	Anti-DR4 IP
Apo2L/TRAIL	0	0	0	28
DR4	1	262	0	86
DR5	0	0	0	170
FADD	0	0	0	19
Caspase-8	0	0	0	120
Caspase-10	0	0	0	30
FLIP	0	0	0	10
CUL3	0	0	0	15

B



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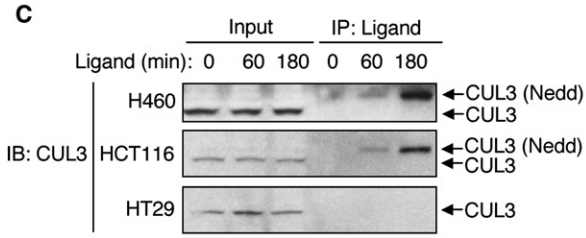

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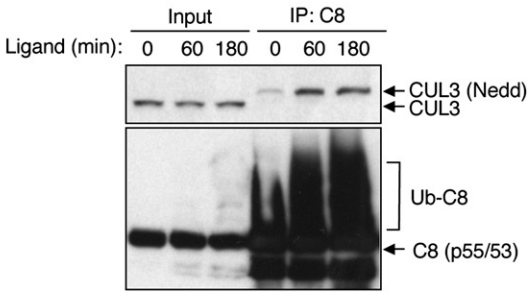

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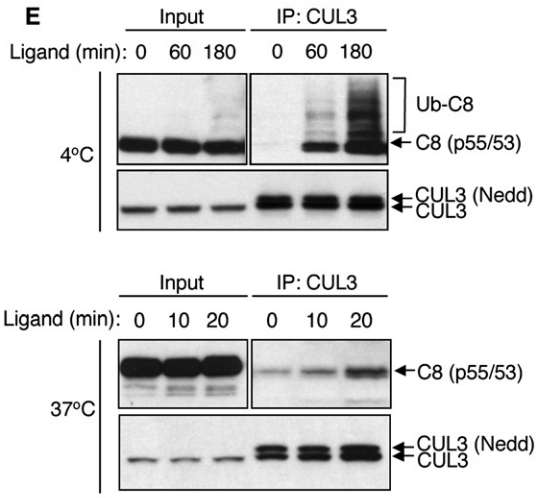

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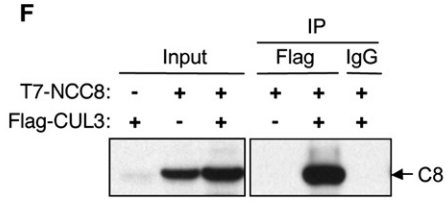

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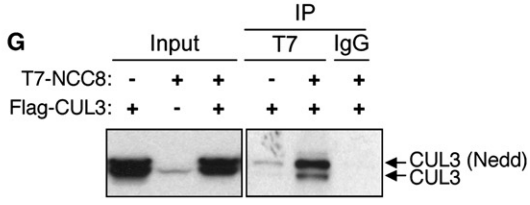


Figure 2. CUL3 Associates with the DISC

(A) H460 cells were treated with buffer or Apo2L/TRAIL (1 μg/ml) for 2 hr at 4°C. The cells were lysed and resolved by SEC, and HMW fractions were subjected to IP with DR4 antibody or an isotype-matched control. Associated proteins were eluted and analyzed by tandem mass spectrometry (MS/MS). The table lists the most abundant proteins identified; numbers indicate the detection frequency of peptides recovered for each protein.

(B) MS/MS sequence coverage of CUL3 after data analysis by the Mascot search algorithm. Identified peptides are highlighted in red.

(C) H460, *Bax*$^{+/-}$ HCT116, and HT29 cells were incubated with Flag-epitope-tagged Apo2L/TRAIL (1 μg/ml) at 4°C. Cell lysates were subjected to DISC IP with anti-Flag M2 antibody, followed by IB with antibody to CUL3 (Nedd, Neddylated).

that stimulation at low temperature permits DISC formation and caspase-8 ubiquitination; however, proteolytic processing and Ub removal from the ubiquitinated enzyme are attenuated, causing it to accumulate and building up its catalytic potential. Consistent with this possibility, stimulation of Apo2L/TRAIL-resistant *Bax*$^{-/-}$ HCT116 cells at 4°C followed by a temperature shift to 37°C led to massive cleavage of caspase-8 and to more complete processing of caspase-3, overcoming resistance caused by *Bax* deletion in these type II cells (Figure S2E).

Exposure of H9 cells to FasL induced rapid apoptosis, which was enhanced by pretreatment at 4°C followed by a shift to 37°C (data not shown). FasL induced detectable caspase-8 ubiquitination by 20 min at 37°C and stronger ubiquitination after stimulation for 2 hr at 4°C (Figure S3A). TNFα also induced ubiquitination of caspase-8 (Figure S3B). These results suggest that, similar to Apo2L/TRAIL, FasL and TNFα are capable of inducing caspase-8 ubiquitination.

CUL3 Associates with the DISC

To identify which E3 ligase supports caspase-8 ubiquitination, we treated H460 cells with Apo2L/TRAIL for 2 hr at 4°C and isolated the DISC from HMW SEC fractions by IP with DR4-specific or control antibody. Tandem mass spectrometry (MS/MS) revealed the expected DISC components, namely Apo2L/TRAIL, DR4, DR5, FADD, caspase-8, caspase-10, and FLIP, as well as another DISC-associated component—the E3 ubiquitin-ligase subunit CUL3 (Figure 2A). CUL3 was identified by ten different tryptic peptides and showed comparable abundance to other DISC-bound proteins, such as FADD or FLIP (Figures 2A and 2B). Control IP recovered neither the known DISC proteins nor CUL3, and DR4 IP from nonstimulated cells contained only DR4. Hence, CUL3 associates with the DISC in a ligand-dependent manner.

To verify this interaction, we stimulated H460 cells at 4°C with Flag-tagged ligand and pulled down the DISC with anti-Flag antibody. CUL3 specifically associated with the DISC, appearing to migrate more slowly than free CUL3 (Figure 2C). CUL3 can be modified by neddylation—a process that conjugates the Ub-like protein NEDD8 to cullins and augments CRL activity (Bosu and Kipreos, 2008; Petroski and Deshaies, 2005). Indeed, restaining of the CUL3 blot with NEDD8 antibody indicated that the DISC-bound CUL3 was neddylated (Figure S4A). Ligand stimulation induced DISC association with CUL3 in Apo2L/TRAIL-sensitive H460 and *Bax*$^{+/-}$ HCT116 cells, but not in resistant HT29 cells (Figure 2C). IP of caspase-8 from H460 cells further confirmed the interaction with neddylated CUL3 (Figure 2D). Moreover, IP of CUL3 demonstrated ligand-induced CUL3 association with caspase-8 in H460 and H2122 cells (Figures 2E and S4B); the interaction was also detectable, albeit more weakly, after stimulation at 37°C as compared to 4°C (Figure 2E). Lysis of H460 or H2122 cells by Triton X-100 or subcellular fractionation revealed most of the unmodified CUL3 in the detergent-soluble cell fraction or cytosolic fraction, whereas neddylated CUL3 was found mostly in the insoluble fraction or the raft/cytoskeleton compartment (Figures S4C and S4D). Ligand stimulation did not significantly change this distribution, suggesting that neddylation of CUL3 controls its distribution to the insoluble fraction, and hence its access to the DISC. To focus our analysis on the events that occur upstream of caspase-8 processing, we used a noncleavable caspase-8 mutant (NCC8), in which critical asparagines within the first cleavage site were replaced by alanines (Boatright et al., 2004). Cotransfection of differentially tagged NCC8 and CUL3 versions followed by reciprocal IP and IB confirmed the ability of the two proteins to interact (Figures 2F and 2G). The N-terminal region of CUL3, which interacts with BTB domain-containing proteins, was sufficient for association with caspase-8 (Figure S4E).

CUL3 Depletion Inhibits Ubiquitination and Full Activation of Caspase-8, Preventing Apoptosis

Transfection of H460 cells with different CUL3 siRNAs specifically depleted the CUL3 protein (Figure S5A). CUL3 knockdown did not alter the amount of unmodified caspase-8, as detected by caspase-8 IP and IB; however, it prevented caspase-8 ubiquitination, as evident from both caspase-8 and Ub IB (Figure 3A). No ubiquitinated caspase-8 was detectable in the raft/cytoskeleton fraction of CUL3-depleted cells as compared with controls (Figure 3B). CUL3 knockdown did not significantly alter the cellular amounts (Figure 3C, left panels) or recruitment to the DISC (right panels) of DR4, DR5, FADD, or caspase-8, nor did it change the cellular levels of various IAP proteins (Figure S5B); however, it markedly decreased the amount of ubiquitinated caspase-8 in the DISC (Figure 3C, right panels). These results indicate that CUL3 is important for ligand-induced caspase-8 ubiquitination, but not for initial DISC assembly.

CUL3 knockdown in H460 cells also inhibited ligand-induced processing of caspase-8, as indicated by an attenuated loss of the full-length p55/53 protein and diminished appearance of the p26/24 fragment (Figure S5C). Moreover, generation of caspase-8 activity and, correspondingly, caspase-3/7 activity was substantially blocked by CUL3 siRNA (Figures 3D and S5D), as was apoptosis stimulation measured at varied ligand incubation time or concentration (Figures 3E and S5E). CUL3 knockdown similarly inhibited ligand-stimulated caspase-8 activation and cell death in H2122 cells (Figures S6A and S6B). CUL3 knockdown markedly decreased the amount of caspase-8 activity in both the HMW and low molecular weight (LMW) fractions of ligand-stimulated H460 cells (Figure 3F), without significantly affecting the rate of ligand-induced DR4 internalization (Figure S6C). Furthermore, siRNA knockdown of the CUL3-associated RING box protein RBX1 substantially blocked ligand-induced ubiquitination of caspase-8, as well as activation or processing of caspase-8 and generation of caspase-3/7 activity (Figures 3G, 3H, and S7A), providing independent

(D and E) H460 cells were incubated with Apo2L/TRAIL (500 ng/ml) at 4°C, lysed in DISC IP buffer, and subjected to C8 IP and CUL3 or C8 IB (D) or CUL3 IP and C8 or CUL3 IB (E).

(F and G) Expression plasmids encoding T7-epitope-tagged, noncleavable caspase-8 (T7-NCC8) and Flag-tagged CUL3 were transfected individually or together into HEK293T cells. After 24 hr, the cells were lysed and subjected to IP with anti-Flag (F) or anti-T7 (G) antibody and analyzed by C8 IB (F) or CUL3 IB (G).

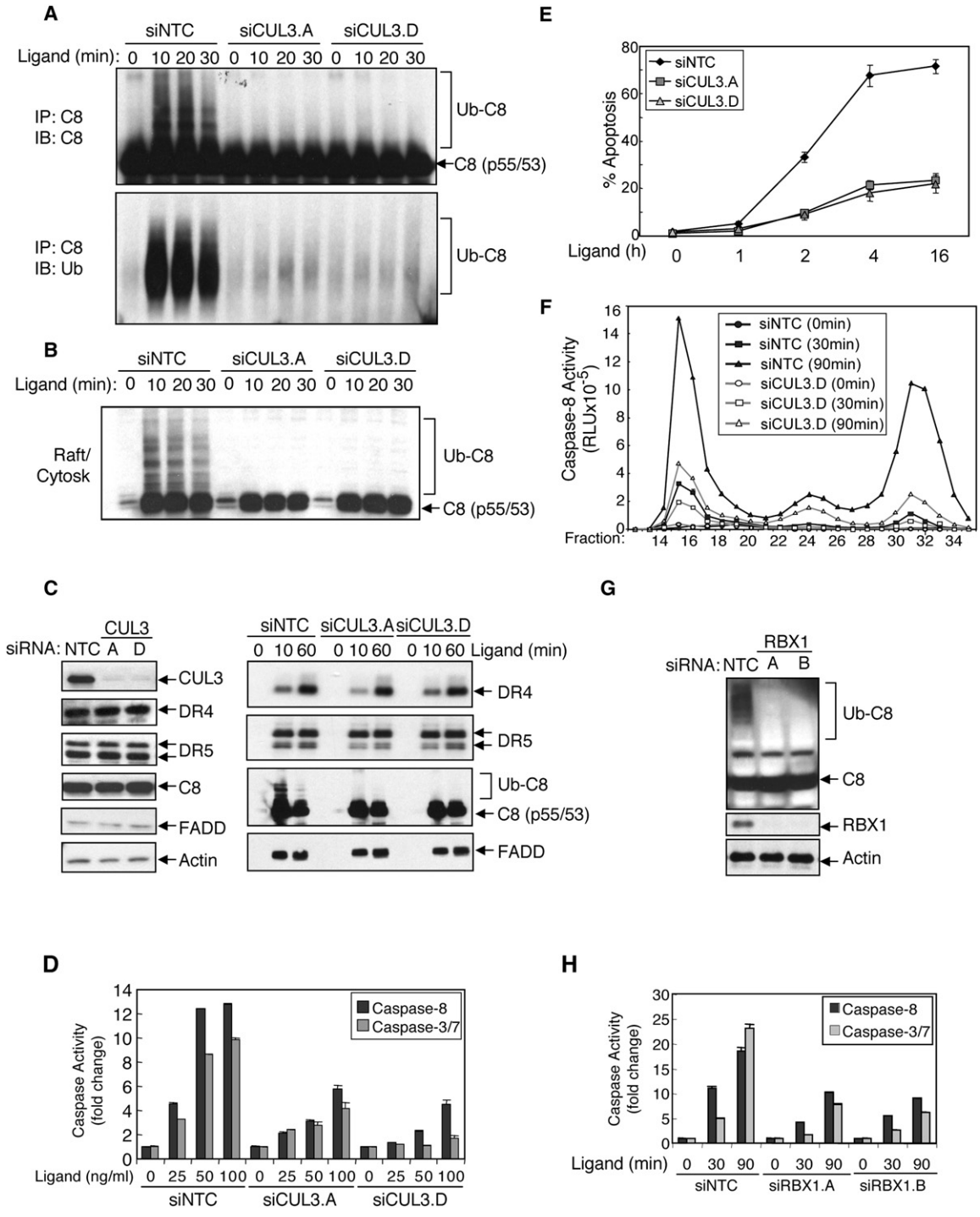


Figure 3. CUL3 Knockdown Inhibits Ligand-Induced Caspase-8 Ubiquitination and Activation

(A) H460 cells were transfected with a nontargeted control (NTC) siRNA or siRNAs against CUL3 (SiCUL3.A, SiCUL3.D) for 48 hr, followed by Apo2L/TRAIL treatment (250 ng/ml) at 37°C. Cell lysates were subjected to C8 IP under denaturing conditions and analyzed by C8 or Ub IB.

(B) H460 cells were transfected with NTC or CUL3 siRNA, treated as in (A), and subjected to subcellular fractionation and C8 IB.

(C) H460 cells were transfected with NTC or CUL3 siRNA and analyzed directly by IB for CUL3, DR4, DR5, C8, or actin (left panles), or treated with Flag-tagged Apo2L/TRAIL (1 μg/ml) at 37°C and subjected to DISC IP with anti-Flag antibody followed by IB as indicated.

(D) H460 cells were transfected with NTC or CUL3 siRNA and treated with Apo2L/TRAIL for 2 hr at 37°C. Cell lysates were assayed for caspase-8 or caspase-3/7 enzymatic activity.

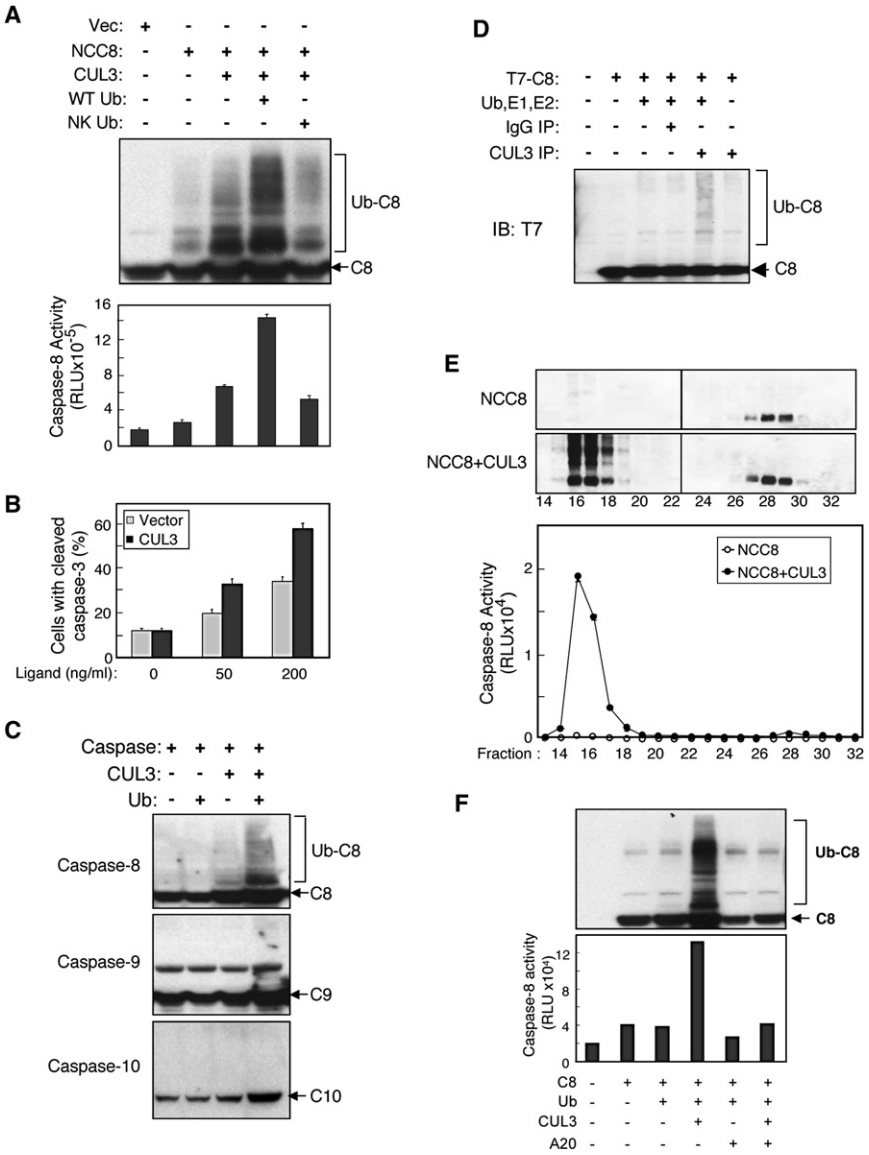


Figure 4. CUL3 Overexpression Promotes Ubiquitination and Activation of Caspase-8

(A) HEK293T cells were transfected with combinations of plasmids expressing noncleavable caspase-8 (NCC8), CUL3, wild-type ubiquitin (WT Ub), or fully lysine-substituted mutant ubiquitin (NK Ub) as indicated. After 24 hr, cells were lysed and analyzed by C8 IB or enzymatic activity assay.

(B) *Bax*$^{+/-}$ HCT-116 cells were transfected with an EGFP-encoding plasmid in combination with CUL3 or a vector control for 24 hr, followed by Apo2L/TRAIL treatment for 2 hr at 37°C. Apoptosis in GFP-positive cells was assessed by flow cytometric analysis with anti-active caspase-3 antibody.

(C) HEK293T cells were transfected with expression plasmids encoding catalytically inactive mutants of caspase-8, caspase-9, or caspase-10 with or without CUL3 and ubiquitin as indicated. After 24 hr, cells were lysed and analyzed by IB with antibody to each caspase.

(D) In vitro-translated, T7-tagged caspase-8 was incubated with immunoprecipitated CUL3 complexes or a control IgG IP in an ubiquitination reaction mix (see the Supplemental Experimental Procedures), for 1 hr at 30°C. The reaction was analyzed by T7 IB.

(E) HEK293T cells were transfected with expression plasmids encoding NCC8 plus vector control or CUL3. After 24 hr, the cells were lysed and resolved by SEC, and fractions (0.5 ml) were analyzed by C8 IB or enzymatic activity assay.

(F) HEK293T cells were transfected with plasmids encoding noncleavable caspase-8 (NCC8), CUL3, wild-type ubiquitin (WT Ub), or A20 as indicated. After 24 hr, cells were lysed and analyzed by C8 IB or enzymatic activity assay. The graphs in (A), (B), and (E) depict mean ±SD (n = 2) from one representative experiment of three.

verification for the functional importance of CUL3. We did not observe binding of RBX1 to the DISC by immunoprecipitation or MS/MS (data not shown), suggesting that RBX1's interaction with the DISC may be less direct and/or stable than that of CUL3. CUL3 knockdown also inhibited TNFα-induced activation and processing of caspase-8 and stimulation of caspase-3/7, and partially attenuated FasL-induced caspase-8 processing (Figures S7B and S7C).

CUL3 Promotes Polyubiquitination, Aggregation, and Activation of Caspase-8 and Commitment to Apoptosis

To examine whether caspase-8 undergoes mono- or polyubiquitination, we cotransfected HEK293T cells with plasmids encoding NCC8, CUL3, and WT Ub or a Ub mutant in which all seven lysine residues that could support pUb chain formation are substituted by arginine (NK Ub) (Camus et al., 2007). Overexpression of NCC8 was associated with a small yet detectable increase in caspase-8 ubiquitination (Figure 4A, top). Cotransfection with CUL3 substantially enhanced this ubiquitination, which was further augmented by addition of WT but not NK Ub.

(E) H460 cells were transfected with NTC or CUL3 siRNA and treated with Apo2L/TRAIL (200ng/ml) for the indicated time. The cells were stained with propidium iodide and apoptosis was measured by flow cytometric analysis of subdiploid DNA content.

(F) H460 cells were transfected with NTC or CUL3 siRNA and treated with Apo2L/TRAIL (100 ng/ml) at 37°C. Cell lysates were resolved by SEC and caspase-8 activity present in column fractions (0.5 ml) was assayed.

(G) H460 cells were transfected with NTC or RBX1 siRNA and treated with Apo2L/TRAIL (100 ng/ml) for 2 hr at 4°C. Cell lysates were analyzed by caspase-8, RBX1, or actin IB.

(H) H460 cells were transfected with NTC or RBX1 siRNA, treated with Apo2L/TRAIL (100ng/ml) at 37°C, and assayed for caspase-8 or caspase-3/7 enzymatic activity. The graphs in (D)–(F) and (H) depict mean ±SD (n = 2) from one representative experiment of three.

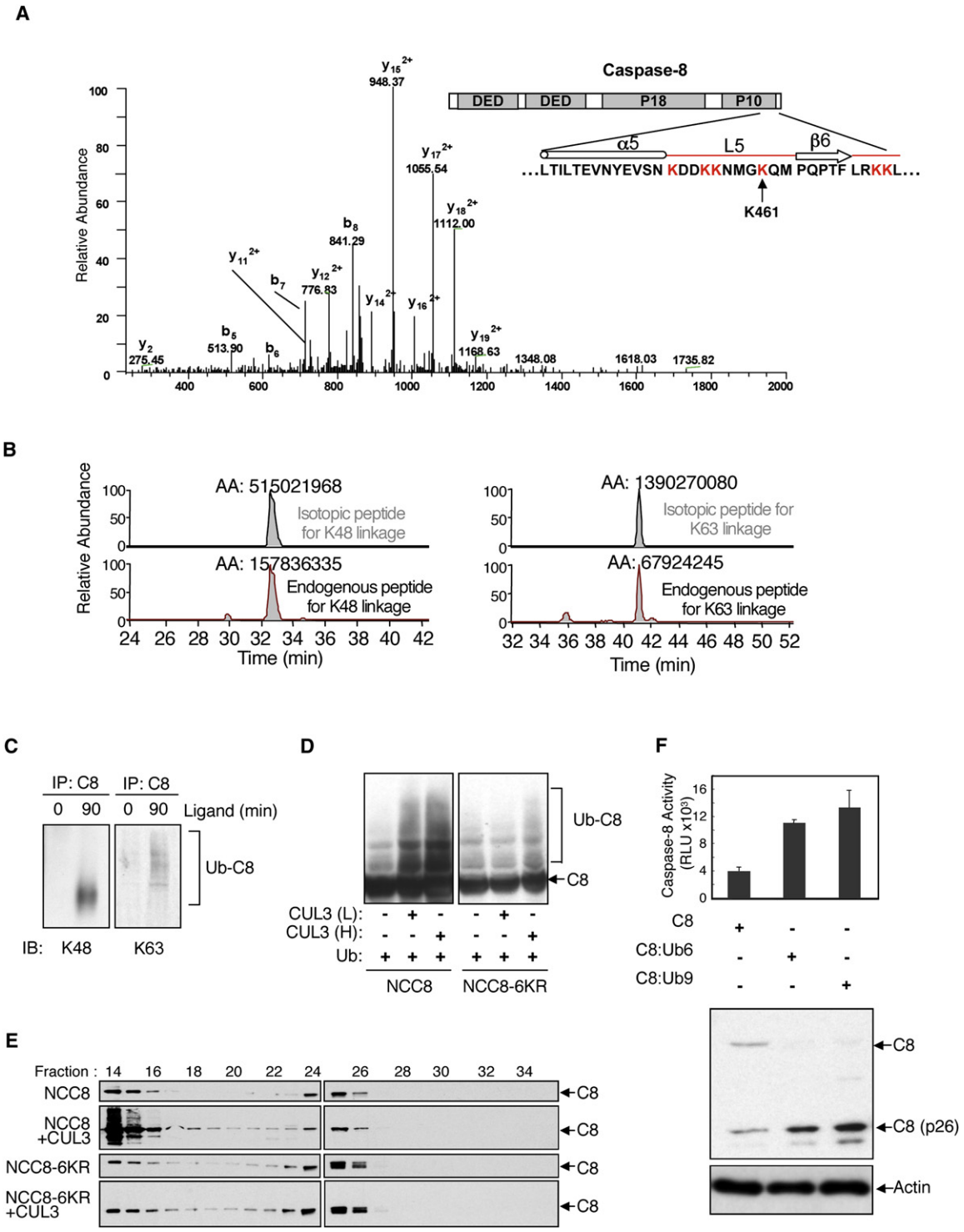


Figure 5. Characterization of the Caspase-8 Polyubiquitination Site, Its Chain Topology, and Its Role in Aggregation

(A) HEK293T cells were transfected with expression plasmids encoding T7-tagged NCC8, CUL3, and ubiquitin, lysed, subjected to T7 IP, and analyzed by tandem mass spectrometry, revealing a principal peptide modification at K461. The parent ion of the triply charged mass, m/z 875.1144, matched the theoretical mass within 7 ppm. A schematic illustration of the caspase-8 domains and location of C-terminal lysines is shown at the top.

(B) Single ion chromatographic traces (top) and full mass spectrum (bottom) for isotopically labeled peptides revealed both K48 and K63 ubiquitin chain linkages (see the Supplemental Experimental Procedures for more details).

Progressively higher caspase-8 activity was generated correspondingly, upon cotransfection of NCC8 with CUL3 alone and in combination with WT, but not NK Ub (Figure 4A, bottom). These results provided additional support for the notion that CUL3-mediated ubiquitination of caspase-8 augments activation; furthermore, the augmentation appeared to require polyrather than monoubiquitination. CUL3 transfection of *Bax*$^{+/-}$ HCT116 cells also enhanced sensitivity to Apo2L/TRAIL (Figure 4B). Cotransfection with CUL3 in HEK293T cells promoted ubiquitination of caspase-8, but not caspase-9 or -10 (Figure 4C), suggesting target selectivity.

To confirm more directly the ability of CUL3-based E3 ligase activity to ubiquitinate caspase-8, we isolated CUL3-containig complexes from cells overexpressing CUL3 by IP and assayed ubiquitination activity toward in vitro translated caspase-8, as described (Furukawa et al., 2003). In the presence of recombinant Ub plus E1 and E2 enzymes (see the Experimental Procedures), the CUL3 complex promoted caspase-8 ubiquitination (Figure 4D). SEC demonstrated abundant caspase-8 protein and corresponding enzymatic activity in the HMW fractions of HEK293T cells cotransfected with NCC8 and CUL3, but not in cells transfected with NCC8 alone (Figure 4E). Thus, upon cotransfection, CUL3-dependent polyubiquitination of caspase-8 can promote aggregation and activation of the protease even in the absence of a ligand-induced DISC.

MS/MS analysis of DISC-associated proteins also revealed the presence of the deubiquitinase A20, and binding of A20 to caspase-8 was confirmed by co-IP (Figures S8A and S8B). Hence, to verify further whether caspase-8 ubiquitination controls activation, we cotransfected HEK293T cells with capspase-8, Ub, CUL3, and A20. A20 reversed CUL3-mediated ubiquitination of caspase-8 and blocked the associated increase in caspase-8 activity (Figure 4F). Moreover, stable transfection of A20 in H460 cells inhibited Apo2L/TRAIL-induced caspase-8 ubiquitination and activation of caspase-8 and caspase-3/7 (Figures S8C and S8D).

CUL3 Mediates Polyubiquitination on the C-Terminal Region of Caspase-8

To identify the site of CUL3-mediated ubiquitination on caspase-8, we cotransfected HEK293T cells with plasmids encoding T7-tagged NCC8, CUL3, and Ub and performed a T7 IP under denaturing conditions. Using MS/MS and database searching, we identified lysine 461 as a likely principal site of CUL3-dependent ubiquitin conjugation (Figure 5A). Further analysis indicated that both K48 and K63 pUb chains were added (Figure 5B); this was confirmed by IP of endogenous caspase-8 under denaturing conditions followed by IB with K48 or K63 pUb chain-specific antibodies (Newton et al., 2008) (Figure 5C). We generated Ub mutants in which lysine 48, lysine 63, or both were substituted by arginine (K48R, K46R, or K48,63R). Upon cotransfection with NCC8 and CUL3, either K48R or K63R supported significant activation of caspase-8, while K48,63R or the NK Ub mutant did not (Figure S9A). Substitution of K461 by arginine decreased but did not abolish CUL3-mediated caspase-8 ubiquitination (Figure S9B). Given previous evidence for ubiquitination-site shifting upon lysine mutation (Petroski and Deshaies, 2003), this raised the possibility that neighboring lysines in caspase-8 may substitute for K461. We therefore replaced all six C-terminal lysines (K453, 456, 457, 461, 472, 473) by arginines (NCC8-6KR). Based on the structure of caspase-8, K472 and K473 are in the C-terminal part of p10, while the other four lysines reside in the connecting loop between the α5 and β6 regions of the p10 subunit. Accordingly, the conservative substitution of these lysines by arginines is unlikely to cause major global structural alterations. Cotransfection of NCC8-6KR with CUL3 resulted in substantially less caspase-8 ubiquitination than seen with the NCC8 control, confirming the C-terminal location of the polyubiquitination site (Figures 5D and S9C). Both NCC8-K461R and NCC8-6KR had less enzymatic activity than NCC8 when cotransfected with CUL3 in HEK293T cells (Figure S9D); however, the activity of recombinant versions of these mutants produced in bacteria was also significantly impaired (Figures S9E and S9F), confounding interpretation of the former results. We therefore examined the aggregation of caspase-8 as an indicator of its activation, based on the tight linkage between these two parameters in the HMW SEC fractions of ligand-stimulated cells (Figures 3F, 4E, and S2D). Cotransfection of NCC8-6KR with CUL3 resulted in much less caspase-8 in HMW SEC fractions than NCC8 plus CUL3 or NCC8 alone, indicating much weaker aggregation (Figure 5E).

To confirm further the importance of ubiquitination for full caspase-8 activation, we generated constructs encoding caspase-8 fused at the C terminus to a chain of six or nine ubiquitins. pUb-fused caspase-8 may mimic the endogenous K63-conjugated protein, given the location of the polyubiquitination site near the C terminus and the linear structure of K63-linked chains. Upon transfection into HEK293T cells, pUb-fused caspase-8 showed markedly stronger enzymatic activity and processing than did WT caspase-8 (Figure 5F).

p62 Binds the DISC and Promotes Aggregation, Activation, and Processing of CUL3-Modified Caspase-8

Analysis of DISC-associated proteins by MS/MS also revealed the presence of the Ub-binding polypeptide p62 (Figure S8A). Both DR4 IP (Figure 6A) and p62 IP (Figure 6B) demonstrated ligand-dependent p62 association with the DISC. The interaction appeared to be dependent on caspase-8 modification by CUL3, because CUL3 knockdown, which abolished caspase-8 ubiquitination but not DISC assembly (Figures 3A and 3D), attenuated

(C) H460 cells were treated with Apo2L/TRAIL (500 ng/ml) at 4°C, lysed, subjected to C8 IP under denaturing conditions, and analyzed by IB with K48- or K63-specific antibody.

(D) HEK293T cells were transfected with expression plasmids encoding CUL3 (L or H, 0.5 or 2 μg/ml plasmid DNA, respectively), WT Ub, and NCC8 or NCC8-6KR (the latter has arginine substitutions of the six C-terminal lysines). After 24 hr, cells were lysed and analyzed by C8 IB.

(E) HEK293T cells were transfected with the plasmids indicated to the left. After 24 hr, cells were lysed, resolved by SEC, and analyzed by C8 IB.

(F) HEK293T cells were transfected with expression plasmids encoding caspase-8 N-terminally tagged with Flag and C-terminally unchanged (C8) or fused to a 6- or 9-ubiquitin chain (C8.Ub6 or C8.Ub9) The graph depicts mean ± SD (n = 2) from one representative experiment of two. After 24 h, cells were lysed and analyzed by Flag IB or C8 activity assay.

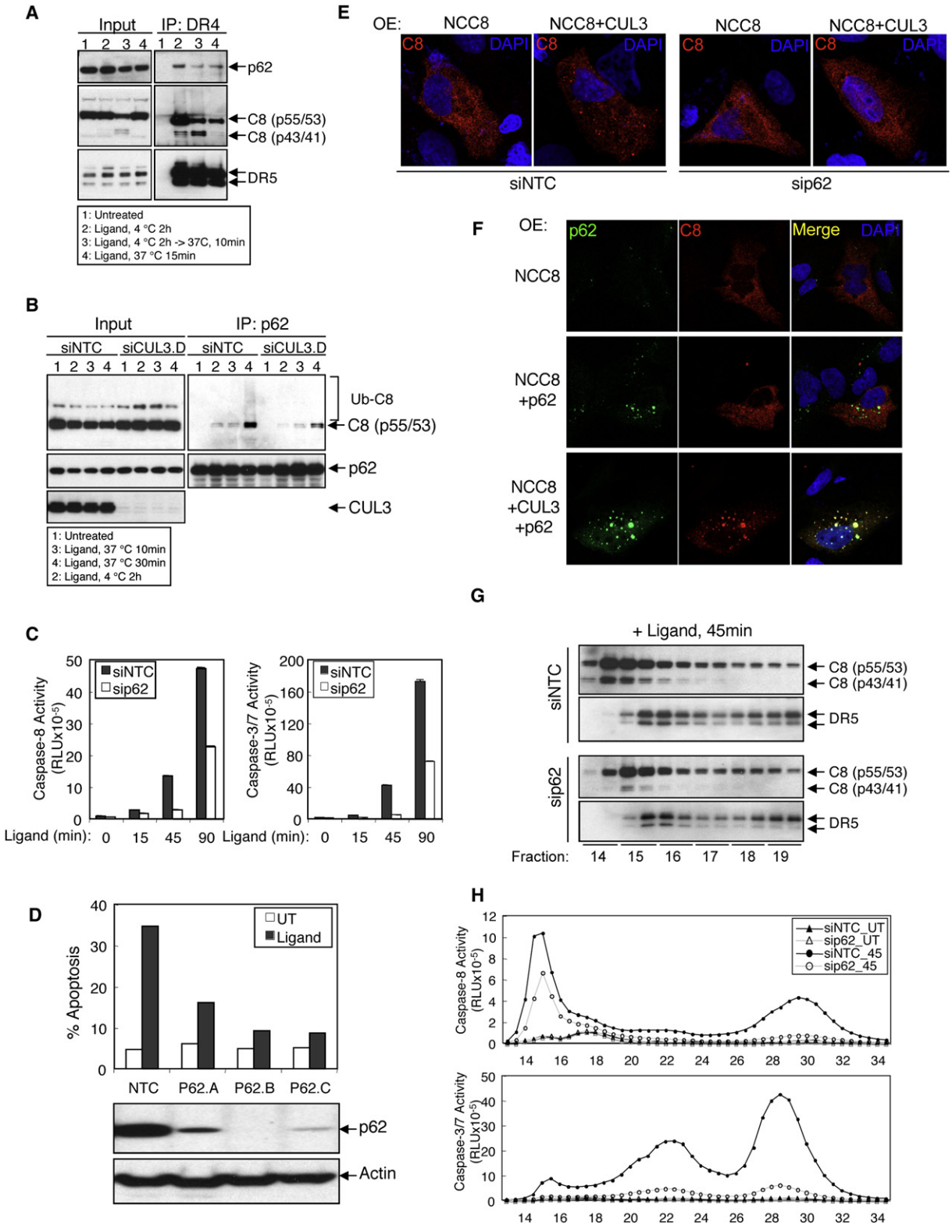


Figure 6. p62 Associates with the DISC and Promotes Aggregation of Caspase-8

(A) H460 cells were incubated with Apo2L/TRAIL (500 ng/ml) as indicated (box). Cell lysates were subjected to DISC IP with anti-DR4 antibody. After extensive washing by DISC IP buffer containing up to 500 mM NaCl, associated proteins were detected by IB with the indicated antibodies.

the association of p62 with the DISC (Figure 6B). siRNA knockdown of p62 substantially decreased ligand-induced activation of caspase-8 and caspase-3/7 (Figure 6C), as well as apoptosis (Figure 6D). Unlike CUL3 siRNA (Figures 3A and 3B), p62 knockdown did not prevent caspase-8 ubiquitination (Figure S10A), suggesting that p62 regulates caspase-8 downstream of its CUL3-mediated modification. Confocal microscopy indicated diffuse immunofluoresence staining of NCC8 after transfection into U2OS cells, while NCC8 cotransfection with CUL3 rendered the staining markedly more speckled, indicating aggregation (Figure 6E, left panels). p62 siRNA reversed the apparent aggregation (right panels), indicating a p62 requirement for this event. While NCC8 cotransfection with p62 alone did not induce significant NCC8 aggregation, cotransfection with both p62 and CUL3 did, resulting in colocalization of NCC8 and p62-positive aggregates (Figure 6F). Moreover, p62 cotransfection further augmented the activation of NCC8 by CUL3 (Figure S10B). These data suggested that p62 mediates the aggregation of CUL3-modified NCC8. To verify this possibility, we analyzed the DISC by SEC, while increasing the resolution of collected column fractions by 2-fold. Caspase-8 peaked at higher MW fractions than did DR5 (Figure 6G, top), suggesting further caspase-8 aggregation beyond the receptor-associated DISC. p62 knockdown did not change the distribution of DR5; however, it significantly attenuated caspase-8 recruitment into the highest MW fractions (Figure 6G, bottom). p62 siRNA also decreased the amount of caspase-8 activity in the HMW fractions (Figure 6H, top) and prevented the development of caspase-8 activity in the LMW fractions representing active caspase-8 in the cytosol (p18/p10), as well as downstream caspase-3/7 activity (Figures 6H and S10C).

p62 Controls Translocation of Caspase-8 into Ub-rich Foci

We next examined the cellular distribution of endogenous caspase-8 by confocal immunofluorescence microscopy. In nonstimulated H460 cells, caspase-8 displayed diffuse cytoplasmic staining, while DR4 appeared to associate with the plasma membrane and Golgi, with little colocalization of the two proteins (Figure 7A, top). Upon ligand stimulation at 4°C, a fraction of caspase-8 was recruited to the plasma membrane, where it strongly colocalized with DR4 (Figure 7B, top). Temperature shift from 4°C to 37°C for 30 min drove caspase-8 and DR4 into speckled structures, some of which contained both proteins (Figure 7C, siNTC, triangles). Additional caspase-8-positive speckles, which appeared to be larger, did not colocalize with DR4 (Figure 7C, siNTC, arrows). Staining of nonstimulated cells with Ub antibody indicated the presence of Ub-rich foci (Figure S11). After ligand stimulation, caspase-8 colocalized with these structures (Figure 7D, siNTC, triangles). Hence, ligand stimulation recruits caspase-8 to the DR4 DISC at the plasma membrane, followed by association of caspase-8 with Ub-rich foci. CUL3 or p62 siRNA did not affect the recruitment of caspase-8 to DR4 (Figures 7A–7C). CUL3 knockdown prevented association of caspase-8 with Ub-rich foci, but not the formation of such structures (Figure 7D); by contrast, p62 knockdown blocked both events (Figures 7D and S12). The requirement of p62 for generation of Ub-rich foci is consistent with recent evidence (Bjorkoy et al., 2005; Komatsu et al., 2007; Pankiv et al., 2007; Tan et al., 2008). Costaining for caspase-8, p62, and DR4 confirmed the presence of speckles containing (1) caspase-8 and p62 without DR4, (2) caspase-8 and DR4 without p62, and (3) caspase-8, DR4, and p62 (Figure 7E). Together, these results suggest that CUL3-mediated polyubiquitination drives p62-dependent caspase-8 translocation from the receptor-associated DISC into Ub-rich foci.

DISCUSSION

Metazoans have evolved tight mechanisms to regulate the irreversible process of apoptosis. Unlike the effector caspases, which depend strictly on proteolytic cleavage for activation, the basic functional unit of initiators such as caspase-8 or -9 is a noncleaved dimer (Boatright et al., 2003). While caspase-8 activation likely begins with dimerization at the initial DISC, full apoptosis commitment necessitates more massive stimulation and processing, achieved through further aggregation of the enzyme. Caspase-9 activity also intensifies upon higher-order oligomerization within the apoptosome (Bao and Shi, 2007).

Our findings show that after proapoptotic stimulation by Apo2L/TRAIL, caspase-8 undergoes CUL3/RBX1-dependent polyubiquitination, which promotes p62-mediated aggregation and full activation of the protease. These observations identify a mechanism of positive apoptosis control that operates directly at the level of a crucial initiator caspase in the extrinsic pathway. In TNFα signaling, JNK stimulation induces ITCH-dependent ubiquitination and proteasomal degradation of the apical caspase inhibitor FLIP, indirectly augmenting caspsae-8 activation (Chang et al., 2006). CUL3 knockdown in H460 cells did not significantly alter FLIP levels (data not shown), confirming that

(B) H460 cells were transfected with NTC or CUL3 siRNA and treated with 500 ng/ml Apo2L/TRAIL as indicated (box). Cell lysates were subjected to IP with p62 antibody and IB with the indicated antibodies.

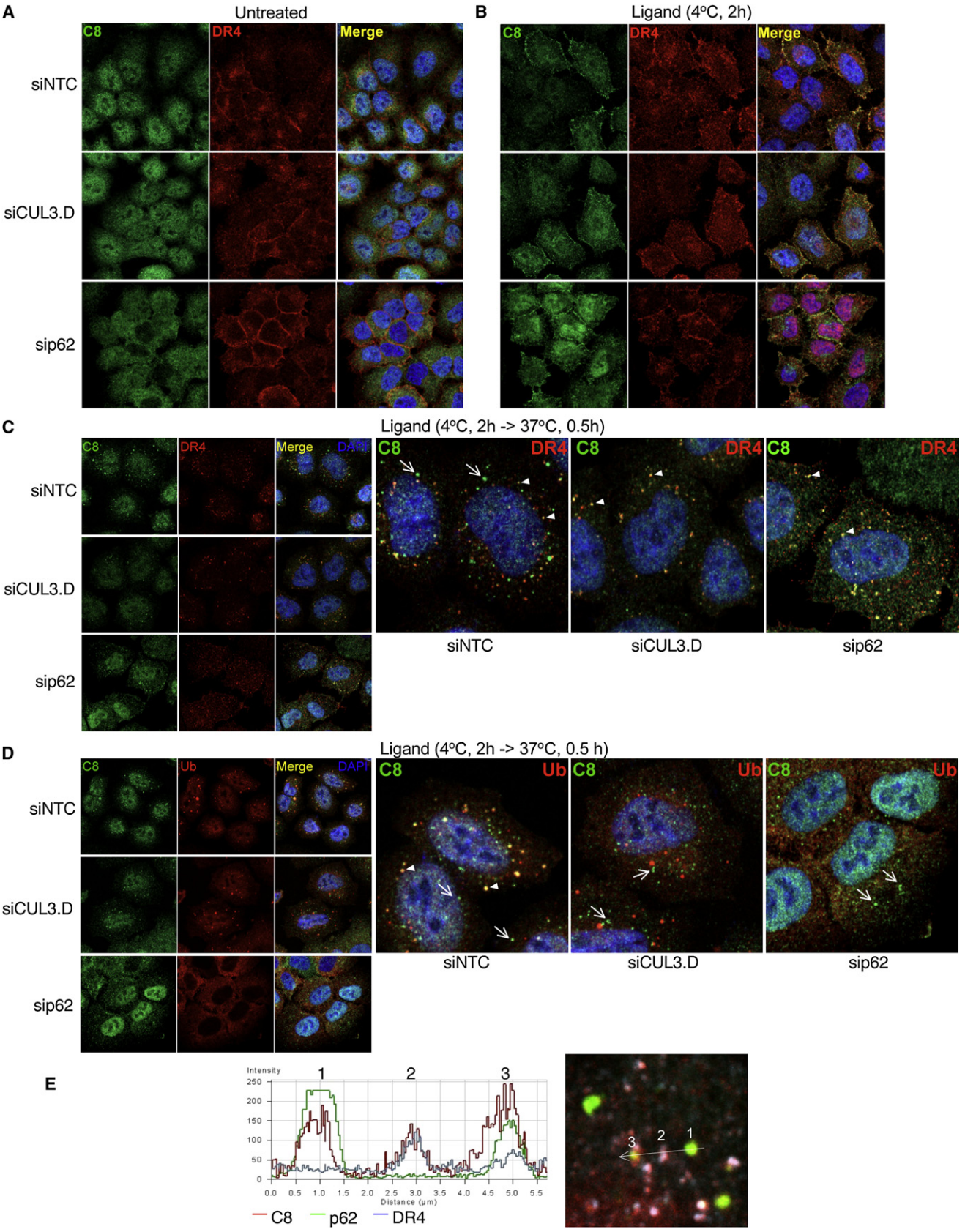
(C) JHH6 cells were transfected with NTC or p62 (oligo C) siRNA, treated with 100 ng/ml Apo2L/TRAIL at 37°C, and assayed for caspase-8 and caspase-3/7 enzymatic activity. The graph depicts mean ±SD (n = 2) from one representative experiment of three.

(D) MG63 cells were transfected with siNTC or various p62 siRNA oligonucleotides for 48 hr, followed by treatment with Apo2L/TRAIL-Flag (250 ng/ml) plus anti-Flag M2 antibody for 3 hr at 37°C. Apoptosis was assessed by flow cytometric analysis of subdiploid DNA. Lysates of the untreated (UT) cells were also analyzed by p62 or actin IB.

(E) U2OS cells were transfected (OE) with NTC or p62 siRNA (oligo C) for 48 hr, followed by transfection with Xpress-NCC8 and CUL3 plasmids. After overnight incubation, the cells were fixed and stained with anti-Xpress antibody (for NCC8) (red) and DAPI (blue) and analyzed by confocal microscopy.

(F) U2OS cells were transfected with plasmids encoding Xpress-tagged NCC8, CUL3, p62, or vector as indicated, fixed and stained with anti-Xpress plus AF-595-counjugated anti-mIgG (red), AF-488-conjugated anti-p62 (green) or DAPI (blue), and analyzed by confocal microscopy.

(G and H) JHH6 cells were transfected with NTC or p62 siRNA (oligo C) for 48 hr, followed by treatment with Apo2L/TRAIL (100ng/ml) for 45 min at 37°C. Cell lysates were resolved by SEC, and 0.25 ml fractions were collected and analyzed by casapse-8 and DR5 IB (G) or caspase-8 and caspase-3/7 activity assay (H).


A
Untreated
C8
DR4
Merge
siNTC
siCUL3.D
sip62
B
Ligand (4ºC, 2h)
C8
DR4
Merge
C
Ligand (4ºC, 2h -> 37ºC, 0.5h)
C8
DR4
Merge
DAPI
siNTC
siCUL3.D
sip62
D
Ligand (4ºC, 2h -> 37ºC, 0.5 h)
C8
Ub
Merge
DAPI
siNTC
siCUL3.D
sip62
E
Intensity
1
2
3
250
200
150
100
50
0
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5
Distance (µm)
C8
p62
DR4

FLIP ubiquitination is independent of CUL3. Genetic studies in *Drosophila* implicate a CUL3-based CRL in effector caspase activation during sperm cell differentiation; however, this too is an indirect effect, involving enhanced degradation of the IAP dBruce (Arama et al., 2007). In other potentially relevant work, yeast two-hybrid screening identified two related caspase-8/10-associated RING proteins (CARPs) (McDonald and El-Deiry, 2004). Upon overexpression, CARPs interacted with and promoted degradation of caspase-8 and -10. Recent data show that in response to TNFα, RIP1 polyubiquitination promotes NF-κB signaling, whereas deubiquitination of RIP1 indirectly permits caspase-8 activation (Wang et al., 2008). To our knowledge, the present data provide the first example to date of direct *and* positive regulation of a caspase by its polyubiquitination in response to a proapoptotic stimulus.

On the basis of the new findings, we propose a refined model for cell-extrinsic apoptosis signaling through death receptors 4 and 5. As previously established, ligand binding induces receptor clustering and recruitment of FADD and caspase-8, forming an apical DISC (Kischkel et al., 2000; Sprick et al., 2000). The DISC translocates to lipid-raft platforms, which are linked to the cytoskeleton. Neddylated CUL3 resides in this cell compartment, where it comes into physical contact with the DISC and mediates RBX1-dependent polyubiquitination of caspase-8. The caspase is modified by pUb on its C-terminal region (within the p10 subunit), with K461 as the likely principal conjugation site. The Ub-binding protein p62 promotes translocation of the modified caspase-8 from receptor-associated complexes into higher MW structures corresponding to Ub-rich foci. p62-dependent aggregation enhances activity and drives the autoproteolytic release of caspase-8 into the cytosol to activate executioner caspases and trigger apoptosis.

The p62 scaffold protein is involved in modulation of intracellular signaling events and is implicated both in physiological and pathological settings (Kim and Rao, 2006; Moscat et al., 2007). Consistent with evidence that Ub-chain linkage per se is not critical for determining the fate of proteins bound by p62, we detected both K48- and K63-linked pUb on caspase-8, and both types of chain enabled caspase-8 activation upon overexpression. While caspase-10 structurally resembles caspase-8, that the two enzymes may not be redundant for apoptosis initiation (Jin and El-Deiry, 2006). In that context, it is intriguing that caspase-10 did not undergo CUL3-mediated ubiquitination, despite the conservation of K461 in both caspase-8 and -10.

In support of our model, CUL3 knockdown did not prevent recruitment of caspase-8 to the DISC and DISC translocation to the raft/cytoskeleton compartment. However, it blocked all of the subsequent events, including caspase-8 polyubiquitination, aggregation, activation, and processing. By contrast, p62 knockdown only blocked the events that occurred downstream of caspase-8 polyubiquitination. The critical importance of ubiquitination for control of caspase-8 activation was further demonstrated by the inability of CUL3 to drive aggregation of the ubiquitination-deficient 6KR mutant, the reversal of caspase-8 ubiquitination and activation by the deubiquitinase A20, as well as the marked increase in activation of pUb-fused caspase-8.

In conclusion, we have uncovered a mechanism that controls death receptor-induced activation of caspase-8 via CUL3/RBX1-based polyubiquitination and consequent p62-mediated aggregation. CUL3 and p62 may serve as points of coordination between the extrinsic apoptosis pathway and other cellular signaling cascades. Furthermore, these results identify a link between direct pUb conjugation and activation of a caspase.

EXPERIMENTAL PROCEDURES

In Vitro Ubiquitination Assay

T7-tagged NCC8 was translated in vitro with the TNT System (Promega). CUL3 complexes were immunoprecipitated from CUL3-transfected HCT116 cells treated with Apo2L/TRAIL and incubated with T7 tagged NCC8, recombinant ubiquitin, recombinant human E1 and E2 (UbcH5a, UbcH7, UbcH13/Uev1a) in Ubiquitin Conjugation Reaction Buffer and 1× Mg-ATP solution (Boston Biochem) for 1 hr at 30°C.

Subcellular Fractionation

Subcellular fractionation experiments were performed as described (Sabio et al., 2005), with the ProteoExtract subcellular proteome extraction kit (Calbiochem) with 5 × 10^6 cells per sample. The cytoskeletal fraction was concentrated with acetone.

Size-Exclusion Chromatography

Apo2L/TRAIL-treated cells or transfected cells were lysed in TRIS-buffered saline containing 1% Triton X-100, and protease inhibitor cocktail (Roche). Lysates were spun at maximum speed, and supernatants were loaded onto a Superdex 200 10/300 GL column (GE Healthcare). Fractions were collected at the indicated volumes and analyzed by caspase-8 activity assay, or concentrated by acetone precipitation for IB analysis.

Caspase Activity and Apoptosis Assays

Caspase-8 or caspase-3/7 activity was assayed by the Caspase-Glo 8 or Caspase-Glo 3/7 assay kits (Promega). For active caspase-3 assay, ligand-treated cells were fixed with the Cytofix/Cytoperm kit (PharMingen), incubated with 0.125 μg/μl fluorescently conjugated anti-active caspase-3 antibody (Clone C92-605; PharMingen), and assayed by flow cytometry (Beckman Coulter). Apoptosis was measured by subdiploid DNA content: cells were fixed in 70% ethanol, stained with 50 μg/ml of propidium iodide, treated with RNase A for 30 min at room temperature, and analyzed by flow cytometry.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and 12 figures and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00275-X.

Figure 7. Distribution of Endogenous Capsase-8 upon Ligand Stimulation

(A–D) H460 cells were transfected with NTC, CUL3 (oligo D), or p62 (oligo C) siRNA. After 48 hr, cells were either left untreated (A), treated with Apo2L/TRAIL (500 ng/ml) for 2 hr at 4°C (B), or 2 hr at 4°C followed by 30 min at 37°C (C and D). After fixation, cells were stained with antibody to caspase-8 (green) versus DR4 (red) (A–C) or caspase-8 (green) versus Ub (red) (D). Nuclei were stained with DAPI (blue). Larger panels on the right represent a higher magnification of the corresponding merge panels for cells transfected with siNTC, siCUL3, or sip62.

(E) High-magnification image representing the merged image of ligand-stimulated H460 cells stained with antibodies to caspase-8 (red), p62 (green), and DR4 (blue) (see Figure S12, square). The relative fluorescence intensity distribution of three spots (1, 2, 3) is shown in the left panel.

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