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DIRECT REGULATION OF APOPTOSIS BY LINEAR UBIQUTIN CHAIN ASSEMBLY COMPLEX (LUBAC) AND FEEDBACK REGULATION OF LUBAC FUNCTION BY CASPASES

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A

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Dedication

This dissertation is dedicated to my lovely wife, Goeun Bae, and my dearest son, Yeonjoon Anthony Joo. This study is also dedicated to my supportive parent, In Taek Joo and Ki Ja An and my sister & her family, Donghee Joo, Sung Tae Kim, Do Yeon Kim and Min Joon Kim.

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DIRECT REGULATION OF APOPTOSIS BY LINEAR UBIQUTIN CHAIN ASSEMBLY COMPLEX (LUBAC) AND FEEDBACK REGULATION OF LUBAC FUNCTION BY CASPASES

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Tumor Necrosis Factor-alpha (TNF-α) is a cytokine that plays a role in various cellular processes such as proliferation, differentiation (mainly through NF-κB signaling) and death (via apoptosis signaling). Recently, linear ubiquitination by LUBAC (linear ubiquitin chain assembly complex) was reported to have a regulatory function in TNF-α mediated NF-κB activation. Although LUBAC is suggested to control not only NF-κB signaling but also the apoptosis pathway, the precise mechanism of apoptosis regulation remains unknown. Moreover, NF-κB and apoptosis pathways have opposed but fundamental functions for various cellular processes. Although these two pathways actively interplay to balance the death and survival, the reciprocal regulation between these two are not completely established. Here, I report direct regulation of apoptosis by LUBAC and the novel crosstalk mechanism between these two pathways. First, I investigated the role of RNF31 in mouse development with RNF31 knockout mouse. RNF31 deletion resulted in embryonic lethality of mouse around 8.5 days. Second, I examined the direct regulation of apoptosis by linear ubiquitination and RNF31. RNF31 inhibited TNF-α induced apoptosis via regulating degradation of antiapoptotic molecule, cellular FLICE-like inhibitory protein (cFLIP). Finally, I determined the negative feedback to regulate function of LUBAC from apoptosis. Effector caspases induce RNF31 cleavage in apoptotic condition, which suppresses its role in NF-κB activation. This study is the first to demonstrate the molecular mechanism of how LUBAC governs the apoptosis pathway via linear ubiquitination and a novel regulatory loop between cell death and survival signal. Therefore, this will present the first insight into the physiological role of RNF31 and linear ubiquitination in the apoptosis pathway and may provide a novel therapeutic strategy for cancers.

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Abbreviations

TNF-α: Tumor necrosis factor alpha

TNFR1: Tumor necrosis factor receptor 1

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NEMO: NF-kappa-B essential modulator

RIP: Receptor-interacting protein

IκB-α: inhibitor of kappa B- alpha

IKK α/β: IκB kinase alpha/beta

TRADD: TNFRSF1A-associated via death domain

LUBAC: Linear ubiquitin chain assembly complex

RNF31: Ring Finger Protein 31

HOIP: HOIL-1–interacting protein

HOIL-1: Heme-Oxidized IRP2 Ubiquitin Ligase 1

Sharpin: SHANK-associated RH domain interactor

OTULIN: OTU Deubiquitinase With Linear Linkage Specificity

CYLD: Cylindromatosis (turban tumor syndrome)

NOD2: Nucleotide-binding oligomerization domain-containing protein 2

Cpdm: chronic proliferative dermatitis

TRAF6: TNF receptor-associated factor 6

MEFs: Mouse Embryo Fibroblasts

CRISPR: The Clustered Regularly Interspaced Short Palindromic Repeats

TRAIL: TNF-related apoptosis-inducing ligand

PARP: poly ADP ribose polymerase

TAK1: Transforming growth factor beta activated kinase-1

CHX: cycloheximide

BCL2: B-cell lymphoma 2

cFLIP: Cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP)

JNK: c-Jun N-terminal kinases

cIAP: Cellular Inhibitor of apoptosis

MLKL: Mixed lineage kinase domain-like protein

NLRP: NOD-like receptor family, pyrin domain containing protein

ASC: Apoptosis-associated speck-like protein containing CARD

DOX: Doxorubicin

CPT: Camptothecin

LPS: Lipopolysaccharide

CK2: Casein Kinase II

ZF domain: Zinc finger domain

RING domain: Really Interesting New Gene domain

Chapter I: Introduction

1.1 TNF-α signaling pathway

1.1.1 History of TNF-α

Tumor Necrosis Factor-alpha (TNF-α) was initially discovered as an anti-tumor molecule, as its name implies: Tumor Necrosis Factor. Before the identification of TNF-α, many independent observations have indicated the existence of anti-tumor molecule. In 1725, Deidier observed that syphilis patients developed less malignant tumors [1]. In 1867, the German physician Busch noted that in the erysipelas developed patient, there was a regression in tumor. After identifying streptococcal organism as a cause of erysipelas, French physician P. Bruns injected streptococcal organism into a tumor patient and observed the disappearance of tumor [1]. Based on these reports, Coley developed Coley's toxin, a mixture of bacterial organisms that treated over one thousand cancer patients. Although this treatment caused severe toxicity in his patients, he reported that the injection of toxin efficiently shrunk his patients' tumors, including sarcoma and bone tumors [1]. However, a later study by O'Malley *et. al.* showed that the bacterial component does not directly induce the tumor regression, but instead promotes the release of a direct mediator into serum, which he named tumor-necrotizing factor in 1962 [2]. Later, Old group identified the main source of this mediator and renamed the mediator as tumor-necrosis factor (TNF) in 1975 [3]. Then, several groups cloned TNF-α and TNF-β (also called LT-α) and investigated its role in tumor regression [4-9]. After identifying these cytokines, researchers isolated and identified the enormous cytokine family, TNF superfamily. However, later studies observed the opposite effects of TNF-α on tumor development and formation. TNF not only activates cell death but also NF-κB signaling, which is critical for tumor cell growth and migration [10]. This contradictory function of TNF on tumor will be more precisely described in a later section.

1.1.2 TNF-α superfamily

Up to now, researchers have identified 19 TNF super family ligands [11-14]. **Tumor necrosis factor alpha (TNF-α)** is the most well known ligand of the TNF superfamily. The main sources of this cytokine are immune cells and epithelial cells. TNF-α is expressed as pro-inactive form (which is a transmembrane protein) and is later released from membrane via the TNF-converting enzyme (TACE) induced cleavage. After trimerization of released TNF-α, the soluble trimeric ligands bind with TNFRI or TNFRII and activate downstream signaling pathways such as NF-κB, AP-1, MAPKs, and apoptosis. Through the regulation of these signaling pathways, TNF-α governs fundamental cellular events including proliferation, inflammation, immune responses, and cell death [11, 12]**. Lymphotoxin-alpha (LT-α)** and **Lymphotoxin-beta (LT-β)** are also categorized in the TNF superfamily. Alpha form is a soluble protein, while beta form is a transmembrane protein. Activated CD4+ T cells and NK cells produce them and trimetric formation is essential to be activated. LT-α is also known as TNF-β and shares receptors with TNF-α (TNFRI and TNFRII). However, the effects of these two cytokines are different, which may result from different affinity. **FasL (CD95L)** is a transmembrane protein that dominantly regulates apoptosis pathway. This cytokine is expressed in T cell and NK cell, and activates apoptosis of target cells by binding with its receptor, Fas (CD95). The ligation between Fas and FasL induces cell specific apoptosis that is a key mechanism in regulating immune responses [15]. **CD27 Ligand (CD70)** is expressed in and activates proliferation in NK cells, T cells, and B cells. In B cells, CD27 ligation inhibits the differentiation of plasma cells and maintains memory B cell characteristics [16]. **CD30 Ligand (CD153)** is expressed in monocytes, T cells, and B cells and regulates the functions of these immune cells [17]. **4-1BB Ligand** is expressed in activated antigen-presenting cells (APCs) including dendritic cells, B cells. It regulates proliferation of T cell by binding with 4-1BB **(CD137)** [18]. **CD40 ligand (CD154)** is expressed primarily in activated CD4+ T cells, but is

also expressed in NK cells and mast cells. It is critical for T cell and B cell activation as a costimulatory molecule through binding with CD40 on APCs. Most importantly, antigen recognition by B cell induces the expression of CD40L and further activates B cells for proliferation, cytokine production, isotype switching, and memory responses [19]. **A proliferation-inducing ligand (APRIL)** and **B cell activating factor (BAFF, CD257)** play a role in B cell function by regulating differentiation, proliferation and maintenance of plasma cells [20, 21]. **TNF-related apoptosis-inducing ligand (TRAIL, CD253)** is expressed in most normal cells and strongly induces the apoptosis of target cells by binding with death receptors 4 (DR4) and DR5 [21]. **Glucocorticoid-induced TNFR family related gene Ligand (GITRL)** is primarily expressed in vascular endothelial cells and suppresses the inhibitory function of regulatory T cells [22]. **LIGHT (CD258)** is a costimulatory factor for the activation and proliferation of T cells. It is expressed in DC and T cells and binds with LT beta-receptor, Herpesvirus-entry mediator (HVEM), and decoy receptor 3 (DcR3). **OX40L** (CD252) is expressed in DCs and plays a role in T cell homing, proliferation, and differentiation. The ligation with receptor, OX40 regulates cytokine production from various immune cells. **Receptor activator of nuclear factor kappa-B ligand (RANKL)** plays a critical role in osteoclast differentiation and activation. It also regulates immune responses, bone remodeling, and regeneration. This cytokine has a pivotal role in bone formation, and it is reported that this cytokine is a key signal mediator in tumor-related bone diseases [23]. **Vascular endothelial growth inhibitor (VEGI)** plays an essential role in mucosal immunity. It is produced in large quantities by myeloid cells and endothelial cells of the gut and lung barriers, and it regulates immune responses and endothelial cell death [24]. **Tumor necrosis factor-like weak inducer of apoptosis (TWEAK)** governs cell growth, inflammation, apoptosis, and angiogenesis. **Ectodysplasin-A1 (EDA-A1)** and **EDA-A2** regulate the morphogenesis and maintenance of

specialized structures of skin such as sweat gland, hairs, nails and teeth. It is expressed in pro-form (inactive) and the cleavage by Furin leads to activation of this cytokine [25].

There are 29 TNF superfamily receptors that bind with TNF-α superfamily ligands and they are classified into 3 groups: death domain (DD)–containing receptors, TNF receptor-associated factor (TRAF) binding receptors and decoy receptors. DD-containing receptors, including TNFRI and Fas, trigger apoptosis through the association and activation of DD-containing mediators, while TRAF binding receptors like TNFRII activate proliferation or growth related signaling such as NF-κB and MAPK pathways through interaction with TRAFs. In addition, there are four decoy receptors such as DcR1, DcR2, DcR3 and osteoprotegerin (OPG). They interact with functional ligands to prevent their binding with conventional receptors that inhibit signaling activation. For instance, DcR1 and DcR2 compete with DR4 and DR5 for interacting with TRAIL. Binding of TRAIL with DcRs is not capable of activating apoptosis in target cells, leading to abnormal proliferation or growth. Various tumors utilize this mechanism to escape immune surveillance [12-14, 26].

Most ligands are expressed in various immune cells including T cells, B cells, NK cells, dendritic cells, and monocytes. There are some exceptions. For instance, VEGI is expressed mainly in endothelial cells [27]. However, receptors of TNF superfamily are expressed in all different cells. For example, TNFRI are expressed in all cell types that leads to the numerous effects of TNF-α. Generally, each ligand recognizes one receptor in order to activate corresponding, designated signaling. However, there are some exceptions [12] such as association of TNF-α with TNFRI and TNFRII. TRAIL binds to five receptors: DR4, DR5, DcR1, DcR2, and OPG. BAFF binds to three receptors: BAFFR, B-cell maturation antigen (BCMA), and transmembrane activator and cyclophilin ligand interactor (TACI) [28]. These different interactions can lead to different cellular consequences as a result of the different strength in signaling activation.

1.1.3 The biology of TNF-α

TNF-α is predominantly expressed in T cells, NK cells, and macrophages. It is produced in pro TNF-α form (inactive transmembrane form) that is 26kDa. After the cleavage of pro-form by TACE, it is released from the cellular membrane and forms a homotrimer to be a functional ligand. Although TNF binds with TNFRI and TNFRII, TNFR1 mediated signaling is dominantly regulates cellular events. This is because TNFRI is expressed in all cell types, while TNFRII expression is mainly produced in immune cells [12]. Since the death domain of TNFRI contains the death domain, it strongly induces the apoptosis pathway, while death domain is absent in TNFRII. TNF-α and TNFRI ligation activates not only apoptosis, but also survival signaling such as a NF-κB signaling pathway. Once TNF-α binds with TNFRI, TNFRIs form trimers, and silencer of death domain/BAG family molecular chaperone regulator 4 (SODD/BAG4) is released from TNFRI that allows the recruitment of TNFR-associated death domain (TRADD). TRADD's association with receptor complex further promotes the recruitment of receptor interacting protein 1 (RIP1) and TRAF2 for a NF-κB signaling pathway and Fas-associated death domain (FADD) for apoptosis activation. First, TNFR, RIP1 and TRAF2 complex activates IκB kinase (IKK) complex (composed of IκB kinases α, β, and γ). Although this step is not clearly elucidated, some posttranslational modifications such as phosphorylation of IKK α and β and ubiquitination of IKKγ play critical roles in this activation of IKK complex. Activated IKKs then, phosphorylate inhibitor of κB-α (IκB-α), followed by its degradation. Since NF-κB transcriptional factors such as p65 and p50 are sequestered to cytoplasm by binding with IκB-α in the unstimulated condition, this degradation process allows NF-κB transcriptional factors to enter into nucleus and regulate gene expression [26]. At the same time, TNFRI triggers the programmed cell death pathway: apoptosis. Recruited FADDs further bind with pro-caspase 8, and homodimerization of caspase 8 subsequently activates itself

through the cleavage of pro-domain that exposes the caspase 8 proteolytic active sites. Activated caspase 8 initiates activation of caspase cascade and further executes apoptosis process [12, 29] (Figure 1). Since two pathways triggered by TNF cause contradictory cellular events, cell death and survival, they actively interplay to maintain the homeostasis [30]. The detail of this interplay will be discussed in a later section.

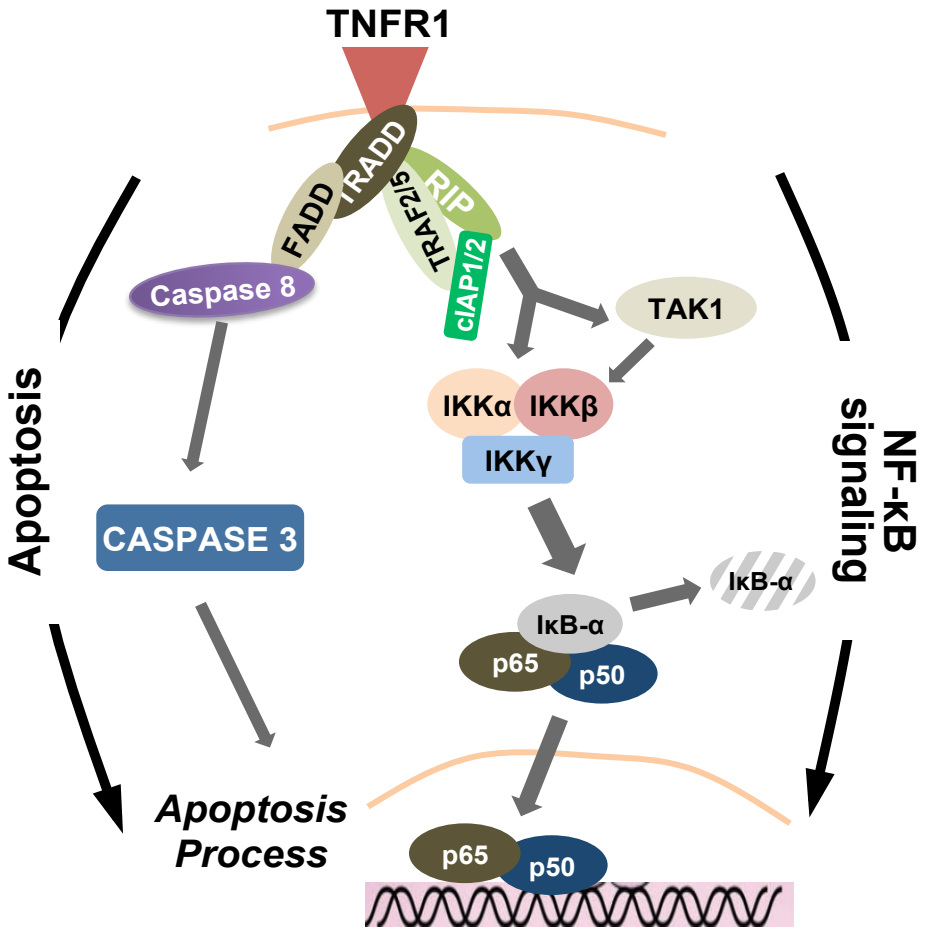


Figure 1 TNF-α mediated signaling pathways

TNF-α signaling simultaneously activates two different pathways including NF-κB and Apoptosis signaling. Upon stimulation, RIP1 and TRAF2/5 are recruited to receptor complex to transfer the activated signaling to IKK complex. Also, TGF-β activated kinase-1 (TAK1) is also activated by RIP and TRAFs complex and triggers the activation of IKK complex. Activated IKK complex further induce phosphorylation of IκB-α, followed by its ubiquitination-dependent degradation. Then, free p65 and p50 transcriptional factors enter into the nucleus and regulate target gene expression. At the same time, FADD is recruited to receptor complex through interaction with TRADD. FADD further recruits and activates caspase 8 and active caspase 8 induce the cleavage of caspase 3 to execute apoptosis process.

1.1.4 Tumor promoting function of TNF-α

As mentioned above, TNF-α triggers two different signaling pathways that lead to the activation of contradictory cellular events: cell death and survival. In previous research, the ability of TNF-α to induce cell death had been actively studied. However, later studies identified the tumor-promoting potential of TNF-α. First, researchers found that TNF-α induces further TNF-α expression in breast cancer cells [31], confirmed that human malignant and stromal cells produce TNF-α, and the level of TNF-α in blood increased in some tumor patients. Furthermore, clinical observations and experimental models have indicated its tumor-promoting role. The TNF-α deficient mouse model showed increased resistance to skin cancer initiation and development of carcinogens, indicating that TNF-α promotes skin cancer carcinogenesis [32]. Moreover, administration of TNF-α enhanced the lung metastasis of human fibrosarcoma cells in xenograft mouse model. Additionally, blocking TNF-α by etanercept or Infliximab (FDA-approved TNF-α inhibitor drug or neutralizing antibody for rheumatoid arthritis treatment) suppressed chemically induced colon cancer development or pancreatic ductal adenocarcinoma (PDAC) growth and metastasis respectively [33, 34]. The mechanism behind this action is not fully elucidated, but it has been suggested that TNF-α may govern the tumor microenvironment by regulating inflammation and cytokine production (which promotes tumor development and progression) and control the transition of EMT of malignant cells [35]. Also, DNA damage and genetic mutation caused by TNF-α promotes cancer development [36, 37]. For example, TNF-α stimulates macrophages or monocytes to build up the blood vessel around tumor to provide more nutrients for tumor cells growth [38, 39].

1.1.5 Clinical application of TNF-α in the cancer treatment

Although TNF-α shows dual effects on tumor, it is clearly demonstrated that TNF-α is a good therapeutic target in malignant disease. Especially, the application of infliximab to renal cell carcinoma, metastatic breast cancer and advanced cancer are currently under clinical trials [40-42]. Because TNF-a simultaneously activates survival signaling, it is critical to augment the tumor killing ability of TNF-α. As a result, combination therapy with other agents has been actively studied to achieve this enhancement. Watanabe *et. al.* reported that conventional chemotherapy drug synergistically enhanced the cytotoxicity effects to tumorigenic mouse fibroblast LM cells [43]. Synergistic cytotoxic and antitumor effect with other agents including interferon gamma, tamoxifen, heat-stress and diphtheria toxin (DTX) was also examined with various cancer cell lines. Since severe toxicity such as hypotension, fever, hepatotoxicity, and rigors has been reported in various clinical studies, not only enhancing cytotoxicity and improving specificity is another key issue in cancer therapy with TNF superfamily [44-47]. To alleviate the non-specific effects of systemic TNF treatment, local administration of TNF to tumor region has been examined. Limb perfusion with high dose TNF in combination with melphalan (anti-cancer drug) is approved in Europe to treat melanoma and soft tissue sarcomas. Although TNF administration shows promising effects on some cancer patients, the type of responsive cancer is very limited and results to severe systemic toxicity. Long-term treatment may lead to recurrence of resistant tumor, sometime even more aggressive mutants [48]. To overcome these obstacles, alternatives have been actively investigated. For example, NGR-hTNF, antitumor recombinant protein was designed to target specifically blood vessel around tumor. In this strategy, human TNF-α is fused with C**NGR**CG peptide that specifically binds with CD13 (selectively expressed in the angiogenic vessel but not normal vessel [49]). Conjugated NGR peptide guides TNF-α to newly generated blood vessel around tumor and

suppresses angiogenesis [50], which inhibits tumor growth [51]. Trimeric formation of TNF-α is critical in activating TNF signaling pathway. Therefore, single-chain TNF that contained three TNF-α monomers with linker peptide were artificially constructed and this recombinant protein decreased the non-specific cytotoxicity and enhanced the specific anti-tumor activity [52]. Manipulating the microenvironment to sensitize tumor cells to TNF-α induced cell death is an alternative approach to treat cancers. Many tumor cells express a certain level of TNF-α, but this TNF-α actually promotes tumor cell growth. This is because apoptosis pathway is suppressed or survival pathway is activated in these cells. Thus, breaking the balance between cell survival and death would be a promising strategy to control cancer. For instance, SMAC mimetic sensitized tumor cells to TNF-α induced apoptosis by eradicating the inhibitory pathway of cell death signaling. Therefore, treatment of SMAC mimetic promotes tumor cell death by autocrine TNF-α [53]. Furthermore, the treatment with other TNF superfamily members such as FasL and TRAIL shows more encouraging results on cancer treatment [54].

1.2 NF-κB signaling pathway

1.2.1 Overview of NF-κB signaling

After the discovery of NF-κB by Ranjan Sen and David Baltimore in 1986 [55], the biology of NF-κB signaling pathway has been actively explored. Once the NF-κB signaling pathway is activated by various cytokines and external stimuli, it regulates almost every cellular events including survival, inflammation, proliferation, and immune responses [56]. NF-κB transcriptional factors (TFs) are composed of 5 proteins including p65 (RelA), RelB, c-Rel, p50, and p52. All these TFs contain Rel Homology Domain (RHD) that binds with DNA [56]. They enter into the nucleus after forming homodimers or heterodimers and regulate gene expression. In the resting status, cytosolic NF-κB dimers bind with inhibitor of κB (IκB) proteins and are sequestered in the cytoplasm. There are eight IκB proteins: IκB-α, IκB-β, IκB-ε, IκB-ζ, BCL-3, IκB-δ, p100 (precursor of p52), and p105 (precursor of p50). They all contain multiple ankyrin repeat motifs and interact with NF-κB proteins [57]. Typical IκBs including IκB-α, IκB-β, and IκB-ε suppress the NF-κB activation. IκB-α binds with p65:p50 dimer and prevents them from entering into the nucleus by using their nuclear exporting sequence or masking nuclear localization sequence of p65 [57]. IκB-α and IκB-β interact with p65 homodimer and c-Rel: p65 heterodimer. This inhibition is released by proteasome dependent degradation of IκB protein after stimulation. As a prerequisite for the degradation, phosphorylation of two serine residues (D<u>S</u>GXX<u>S</u>) by IKK complex is important [58]. Then, the phosphorylated IκB-α proteins are degraded by E3 ligase complex, Skp-Cullin-F-box protein (SCF) complex / β-TrCP [59]. The essential function of IκB-ε in NF-κB signaling is demonstrated with IκB-ε deficient cells. This molecule has a similar function as IκB-α, but it shows delayed kinetics in terms of degradation and synthesis. [60]. The function of IκB-β is still unclear. Although phosphorylated IκB-β leads to DNA binding of p65 and c-Rel, newly synthesized and unphosphorylated form inhibits NF-κB activation by covering NLS sequence of p65 [61, 62]. IκB-β knockout mouse illustrate the

conflicting functions of IκB-β in NF-κB activation [63]. There are three atypical IκBs: IκB-ζ, BCL-3, and IκB-δ. BCL-3 binds with p52 heterodimer or p50 homodimer and activates these dimers to initiate transcription [64]. At the same time, BCL3 suppresses NF-κB by stabilizing inhibitory NF-κB dimers and this alternative function is dependent on its PTM status including phosphorylation and ubiquitination [65, 66]. Inducible activator, IκB-ζ enhances gene expression in a similar way to BCL-3. But its expression level is more critical than the modification. Induced IκB-ζ by NF-κB activation further augments NF-κB target gene expression by interacting with p50 homodimer [67]. IκB-δ suppresses the expression of proinflammatory cytokines through the stabilization of κB site bound p50 homodimer [68]. Processed IκB proteins, p50 and p52 are involved in transcriptional activation of NF-κB TFs while precursors, p100 and p105 inhibit the activity of other NF-κB TFs through interacting with them [57].

After stimulation by proper stimuli, released NF-κB proteins enter into the nucleus and regulate gene expression of various target genes. Diverse outcomes from NF-κB activation result from the various combinations of NF-κB TFs and different binding affinity to DNA of these complex. NF-κB binds to promoters and enhancers of DNA and controls transcriptional regulation and chromatin remodeling [69]. The activity of NF-κB transcriptional factors is manipulated by various PTMs and this modification is responsible for associating molecules. For example, phosphorylation of IκBs is mainly regulated by IKKα and β and its kinase activity is regulated by the regulatory subunit, NEMO (IKK gamma). The function of NEMO is depends on PTMs such as phosphorylation and ubiquitination [70]. Recently, a novel function of NEMO was proposed as a guider of IKKs to IκBs for their phosphorylation. Activated and guided IKKs directly induce phosphorylation of IκBs and regulate NF-κB activation [71].

1.2.2 Types of NF-κB signaling

NF-κB signaling pathway is classified into two pathways: canonical and non-canonical pathways. The canonical pathway is dependent on IKKβ and NEMO mainly leading to phosphorylation of IκBα and nuclear translocation of heterodimers containing p65. In contrast, the non-canonical pathway depends on IKKα-mediated phosphorylation of p100. Phosphorylation of p100 leads to partial processing of p100 that generates p52: RelB complexes. Non-canonical signaling activation is induced by specific members of the TNF cytokine family, such as CD40 ligand, BAFF and lymphotoxin-β2 while canonical pathway is activated by inflammatory cytokines such as TNF and IL-1, pathogen-associated molecular patterns (PAMPs) including LPS, flagellin and lipoproteins. Antigen receptors such as T cell receptor (TCR) and B cell receptor (BCR) trigger activation of canonical NF-κB pathway as well.

In the canonical NF-κB pathway, IKKβ is critical to induce phosphorylation of IκB-α and IκB-β. In the resting status, NF-κB transcriptional factors are sequestered in the cytoplasm as an inactive form through binding with inhibitory IκB proteins. After stimulation, the master kinase complex (composed of IKKβ along with IKKα and γ) is activated and phosphorylates IκB-α and IκB-β leading to degradation in a proteasome dependent manner [72]. Then, released NF-κB dimers translocate into nucleus and regulate gene expression. Deletion of IKKβ promotes embryonic lethality in mouse that results from the defect of NF-κB activation by TNF-α [73]. Various biochemical experiments have demonstrated that TNF-α or IL-1 induced NF-κB activation is defective in IKKβ deficient cells [74, 75]. However, the function of IKKα in canonical pathway is unclear. Recently, it is proposed that IKKα is involved in the canonical NF-κB pathway by phosphorylating p65 and histone H3 [76]. For a clear understanding of IKKα function, further studies are required.

In the non-canonical pathway, activation of IKKα is essential. In resting cells, NF-κB-inducing kinase (NIK) is unstable by TRAF2/3 and cIAP1/2 mediated ubiquitination and degradation. IκB protein, p100 inhibits translocation of Rel-B into nucleus by binding with it. After stimulation with TNF super family members such as LT-beta and CD40L, TRAF2/3 and cIAP1/2 complex recruited to the receptors. This recruitment promotes activation of TRAF2 and activated TRAF2 conjugates K-63 polyubiquitin chains to cIAP1/2. Then, cIAP1/2 conjugates K-48 ubiquitin chain to TRAF3, leading to a proteasome dependent degradation. The absence of TRAF3 leads to the accumulation of its target, NIK, and these accumulated NIKs phosphorylate and activate IKKα that promotes the phosphorylation of p100. Phosphorylated p100 is partially processed by E3 ligase complex, SCF / β-TrCP to p52. Then, this processed p52 forms a heterodimer with RelB and the dimer enters into the nucleus to regulate target gene expression. Currently, only IKKα but not IKKβ or IKKγ is reported to have a critical role in the non-canonical pathway [77-79].

1.2.3 Stimuli to activate NF-κB signaling

Various external stimuli activate the NF-κB pathway regulating diverse cellular events including survival, proliferation, cell death, differentiation, and immune responses. To sense external stimuli such as microbes, cytokines and antigen, a variety of receptors are developed such as PRRs, cytokine receptors and antigen receptors. TLR4 recognizes bacterial proteins including LPS and initiates immune response by triggering NF-κB signaling. TLR2 detects gram-positive bacteria with lipoteichoic acid (LTA) and fungus with triacylated and diacylated lipoprotein and activates NF-κB signaling [80]. Not only extracellular materials but also intracellular proteins such as D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP) are recognized by PRR and NF-κB signaling is activated. Nucleotide-binding oligomerization domain-containing protein 1 (NOD1) binds with iE-DAP and NOD2 interacts with MDP to trigger activation of NF-κB signaling [81]. Dectin 1 and Dectin 3 that belong to C-type lectin receptors (CLRs) recognize proteins of fungal cell walls such as β-glucans and mannan, respectively and trigger NF-κB activation [82]. RIG-I-like receptors (RLR) including retinoic acid-inducible gene-I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) interact with viral RNA and initiate NF-κB activation [83]. Some viral proteins such as Tax of Human T-lymphotropic virus type I (HTLV-1) and the latent membrane protein 1 (LMP1) of Epstein-Barr virus (EBV) activate NF-κB signaling by direct interaction with IKK complex [84]. Various cytokines including TNF superfamiliy and interleukin family also activate NF-κB signaling to regulate cellular processes. Antigen receptors such as TCR and BCR bind with major histocompatibility complex (MHCs) presenting antigen to activate the NF-κB pathway. In addition, NF-κB can be activated by a diversity of external stresses such as UV, γ-irradiation, ischemia and hyperosmotic shock and oxidative stresses [72].

1.2.4 Ubiquitination in NF-κB signaling

Ubiquitination is the post-transcriptional modification (PTM) that regulates protein stability and activity. In the canonical NF-κB pathway, this modification process plays a pivotal role to transfer the activated signals from the receptor to target gene expression. Once IKKs are activated by various stimuli, IκB proteins are phosphorylated, ubiquitinated, and degraded in proteasome dependent manner. Phosphorylation of IκB proteins leads to exposure of its degron motif (DSGXXS), responsible for binding with SCF complex. Associated SCF/β-TrCP complex then, conjugates polyubiquitin chains to IκB proteins, leading to proteasome dependent degradation [58]. The degradation of IκB-α releases NF-κB dimers that allow them to enter into nucleus. K-48 ubiquitination is the responsible ubiquitination type for this degradation. This K-48 ubiquitination, followed by degradation is fundamental in activation of the non-canonical pathway as well. After stimulation with CD40L or BAFF, SCF / β-TrCP recognizes phosphorylated p100 (by IKK complex) and processes them to p52. p52 forms a dimer with Rel-B and enters into the nucleus to regulate target gene expression. p105 is constitutively processed to p50 by KIP1 ubiquitination-promoting Complex (KPC). Constitutively, KPC recognizes phosphorylated p105 (a kinase for this phosphorylation is unknown.) and process them to p50, which allows them to bind with p65 [85]. K-48 ubiquitination is also essential for NIK degradation that is critical for activation of non-canonical NF-κB signaling. In the resting stage, NIK is constitutively ubiquitinated by TRAF3, TRAF2 and cIAP1/2 complex and degraded in proteasome dependent manner. Therefore, unprocessed p100 inhibits translocation of Rel-B into the nucleus through binding with it. Once CD40L or BAFF binds with their receptors, TRAF3, TRAF2 and cIAP1/2 complex is recruited to receptor complex. In the receptor complex, activated TRAF2 induces K-63 ubiquitination of cIAP1/2. Then, cIAP1/2 promote K-48 ubiquitination of TRAF3, leading to proteasome dependent degradation.

Degradation of TRAF3 results to the accumulation of NIK that is fundamental for activation of IKKα and non-canonical NF-κB signaling [77, 79].

In both canonical and non-canonical pathways, the ligation between ligand and receptor leads to the recruitment of TRAF molecules and forms a receptor complex. All of these TRAF proteins are E3 ligase containing a ring finger domain and a zinc finger motif (except TRAF1) [86]. TRAF family proteins dominantly promote K-63 ubiquitination that generally regulates activity of target, not stability. For example, TRAF2 is a key mediator in TNF-α induced NF-κB activation. Once TNF-α binds with TNFRI, TRADD, TRAF2 and RIP1 are recruited to receptor and form a complex. Although precise mechanism is unclear, TRAF2 is ubiquitinated by itself or unknown E3 ligase and this K-63 ubiquitination of TRAF2 is sufficient to induce canonical NF-κB activation [87]. However, recent study limited the function of TRAF2 in TNF-a induce JNK activation not NF-κB and p38 pathways [88]. Therefore, further study is required to clarify the role of TRAF2 in NF-κB signaling. In TLR4 and IL-1R signaling, TRAF6 is a key mediatory. It is auto-ubiquitinated in K-63 manner and recruit TGF-β activated kinase-1 (TAK1) / TAK1-Binding Protein 2 (TAB2) complex. Then, TAK1/TAB2 complex further activates down stream molecules to regulate NF-κB target genes [89]. Another example is cIAP1/2 which are E3 ligases promoting K-63 ubiquitination of RIP1 in the TNF-α signaling pathway. Ubiquitinated RIP1 provides a platform to recruit downstream molecules to activate NF-κB signaling such as TAK1, TAB2, TAB3, NEMO and linear ubiquitin chain assembly complex (LUBAC) [90].

Recently, researchers discovered linear ubiquitination as a novel type of ubiquitination and reported that it plays an important role in NF-κB singling activation [91-94]. The type of ubiquitination is dependent on the lysine of proximal ubiquitin that the C-terminus of distal ubiquitin binds to. For example, K-63 ubiquitin chains indicate that lysine 63 of proximal

ubiquitin binds with C-terminus of distal ubiquitin and the continuous linkage generates polyubiquitin chains. Linear ubiquitination indicates N-terminus of proximal ubiquitin is recognized by C-terminus of distal ubiquitin to generate polyubiquitin chains. Linear ubiquitin chain assembly complex (LUBAC) is the only E3 ligase for linear ubiquitination [93]. The precise role of linear ubiquitination in NF-κB signaling will be discussed in the later section.

1.3 Ubiquitination

1.3.1 The mechanism of ubiquitination

Ubiquitination is a reversible posttranslational modification regulating protein stability and activity. This is a tightly regulated cellular process that requires the successful completion of several steps [95]. First, ubiquitin is activated by E1 enzyme (ubiquitin-activating enzyme) in an ATP dependent way and transferred to the E2 enzyme (ubiquitin-conjugating enzyme). Then, the E2 enzyme specifically binds with E3 ligase that has a recognizing capacity of their targets. In order to be recognized by an E3 ligase, the target protein often has a prerequisite modification (e.g. phosphorylation) as well [96]. After the recognition of modified targets, E3 ubiquitin ligase transfers the activated ubiquitin from E2 enzyme (RING types) or conjugated ubiquitins on itself (HECT types) to target molecules and induces the degradation by 26S proteasome in an ATP-dependent manner. Moreover, deubiquitinase (DUB) can recognize ubiquitin chains or ubiquitinated target to remove the ubiquitins from it (Figure 2). The type of ubiquitination is categorized by Mono, Multi-mono, Homogenous, Mixed chain, branched and unanchored chain based on the number of ubiquitins and how they are conjugated. So far, the most extensively studied types are mono and homogenous ubiquitination (linear, K-11, K-48 and K-63, Figure 3A).

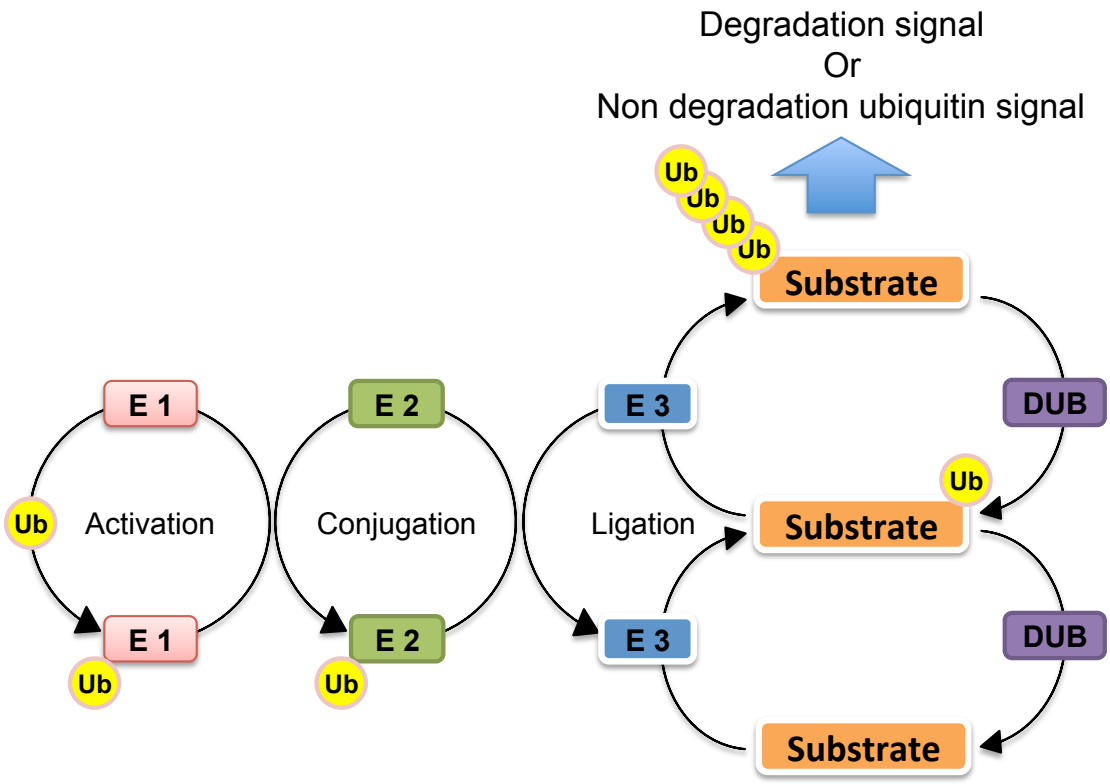


Figure 2 Regulation of ubiquitin chains by enzymatic machinery.

Ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligating (E3) enzyme sequentially regulates ubiquitin proteins to conjugate ubiquitin chains to the substrates. Deubiquitination enzymes (DUBs) break ubiquitin chains and remove ubiquitins from the ubiquitinated substrates.

Adapted from Ubiquitin-binding proteins: decoders of ubiquitin-mediated cellular functions. Husnjak, K. and I. Dikic, Annu Rev Biochem, 2012. 81: p. 291-322 with permission.

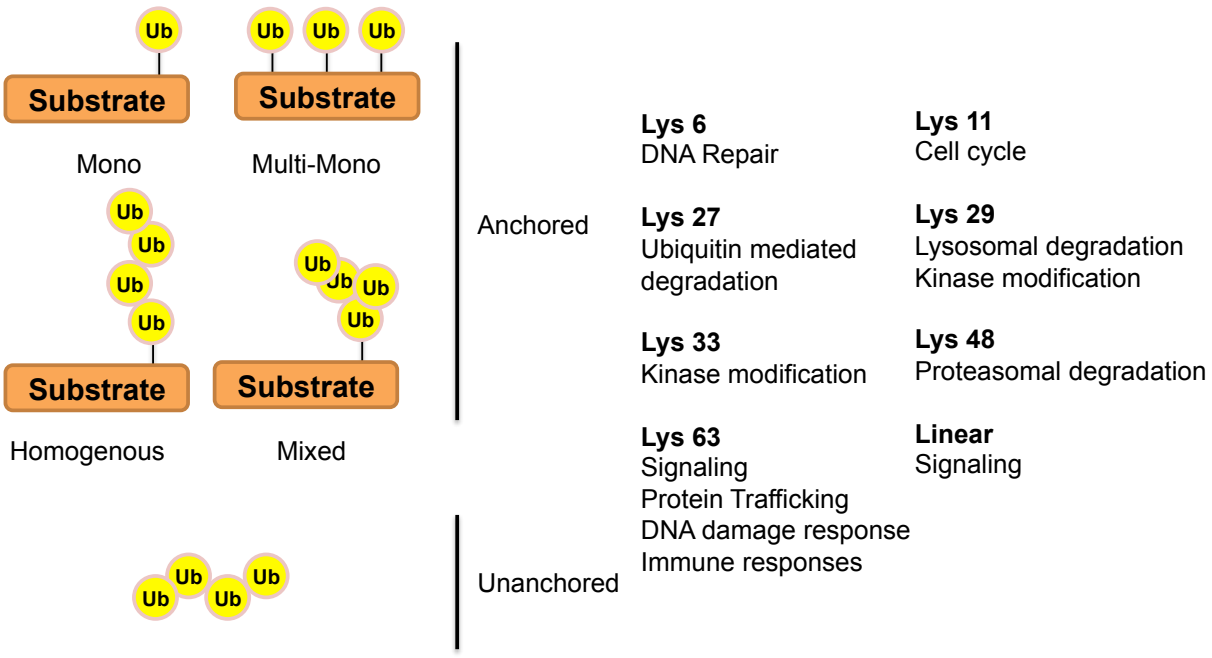


Figure 3 Types of ubiquitin chains.

Target substrates are modified with single ubiquitin (mono), multiple single ubiquitins (Multi-mono), and ubiquitin chains (Homogenous or mixed). Based on Lys residue at which next ubiquitin binds, ubiquitin chains are subcategorized to Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, or Lys63. Moreover, linear ubiquitin chain can be generated through head to tail manner, which is Lys independent conjugation.

Adapted from Ubiquitin-binding proteins: decoders of ubiquitin-mediated cellular functions. Husnjak, K. and I. Dikic, Annu Rev Biochem, 2012. 81: p. 291-322 with permission.

1.3.2 Types of ubiquitination

One ubiquitin molecule has seven lysine residues, Lys-6, 11, 27, 29, 33, 48 and 63 at which the following ubiquitin can conjugate or connect via its c-terminus (Figure 3B). Once the first ubiquitin attaches to the lysine of substrate, it is the lysine binding with the next ubiquitin that decides their type and function (e.g. K-63 or K-48 ubiquitination). Recently, linear ubiquitination is discovered and its function in signaling transduction has been actively studied [97-99].

1.3.2.1 Monoubiquitination

The E3 enzyme for monoubiquitination recognizes lysine residue of substrates and its specificity depends on the components of complex such as the E2, the E3 or substrate-E3 complex. For example, Bim1-RING1 complex induces monoubiquitination of Histone H2A at K119. Once this complex binds to DNA and necleosomes, E2 ligase, Ube2D transfer ubiquitin to lysine 119 of Histone. Due to the stiffness of this complex, further modification is prohibited [100]. E2 ligase, Ube2W and Ube2T (with the E3 ligase Fanconi anaemia complementation group L [FANCL]) conjugate single ubiquitin to the DNA repair protein FANCD2 [101]. The function of monoubiquitination is reported to be involved three major processes; Histone regulation, Endocytosis and virus budding process. Histones H2A and H2B (components of nucleosome) are monoubiquitinated and this modification is critical for normal meiosis, growth and spermatogenesis [102, 103]. Also, the linker Histone H1 is monoubiquitinated by TATA Box Binding Protein (TBP)-Associated Factor 250 (TAF250) and transcriptionally activated [104]. Monoubiquitination also regulates the activity of proteins located at the plasma membrane. First, monoubiquitination promotes the degradation of plasma membrane receptors through lysosomal machinery. In both yeast and mammalian cells, monoubiquitination of membrane protein is sufficient to trigger its internalization into primary endocytic vesicles

[105]. Therefore, they function as a marker for internalization followed by degradation and regulate endocytic machinery. Gag is an inner plasma membrane protein, regulating virus budding [106]. Monoubiquitination of this protein recruits them to membrane to form a complex and promotes viral budding [107]. The monoubiquitination is often involved in complex formation. For example, monoubiquitination of Proliferating cell nuclear antigen (PCNA) is monoubiquitinated by RAD6/RAD18 complex upon DNA damage. This ubiquitination further recruits translesion synthesis (TLS) polymerases and triggers DNA repair [108, 109]. Furthermore, deubiquitinase, USP1 deconjugates the bound ubiquitin protein from monoubiquitinated PCNA, thereby DNA repair machinery revert to the normal status [110].

1.3.2.2 K-48 ubiquitination

The best-studied example of ubiquitination is K-48 polyubiquitination. Among several types of ubiquitination, the role of K-48 ubiquitination; recognition of target proteins for proteasome dependent degradation is the extensively characterized mechanism [111]. Many E3 ligases such as SCF (Skp, Cullin, F-box containing complex) and Fbxw (F-box/WD repeat-containing protein) are involved in this degradation machinery. Consistent with the fact that K-48 is the only fundamental lysine of ubiquitin in yeast, this ubiquitination is the most abundant in all organisms [112]. K-48 ubiquitination modulates signaling pathways in diverse ways. First, inhibitors of signaling are degraded though this ubiquitination, which eventually activate the signaling. For example, the degradation of IκB-α is critical step for NF-κB activation and this degradation is regulated by K-48 ubiquitination by SCF/βTrCP [113]. Proteasomal cleavage of NF-κB precursor also regulates the activity of transcriptional activity. For instance, functional NF-κB subunit p50 and p52 are processed from precursor p105 and p100 in proteasome dependent manner.

1.3.2.3 K-63 ubiquitination

K-63 ubiquitination has critical roles in the diverse cellular functions such as activation of NF-κB, DNA damage repair, and endocytosis regulation. In the NF-κB pathway, it was reported that the activation of IκB kinase (IKK) could be achieved by polyubiquitination through the mechanism independent of proteasomal degradation [114]. And follow up study identified that E3 ligase TRAF6 is critical for NF-κB activation upon IL-1 and TLR stimulation [115]. The TRAF6 complex with E2 ligase, UBC13 catalyzes K63-linked polyubiquitination of target molecules including IRAK4, NEMO and TRAF6 itself to activate NF-κB and JNK signaling pathways. Moreover, TAK1 (upstream of IKK complex) is an important mediator in NF-κB activation [116]. To be activated, TAK1 requires K-63 ubiquitination via its subunits TAB2 and TAB3. Many studies suggested that K-63 ubiquitination of TAK1 mediates oligomerization and autophosphorylation of them, therefore resulting in the activation of downstream signaling molecules [117, 118]. Since TRAF family members are involved in NF-κB activation upon various stimulations, K-63 ubiquitination play a pivotal role in diverse signaling pathways including TNFR, IL-1R, TCR/BCR and Virus or bacteria recognition singling pathways. K-63 ubiquitination is also important for DNA repair. The receptor-associated protein 80 (RAP80) recruits the Abraxas-BRCA1/2-containing complex 36 (BRCC36)- BRCA1-associated RING domain protein 1 (BARD1) complex to DNA damage lesion through specific recognition of K-63 polyubiquitinated Histone H2A and H2AX [119]. Also, PCNA can be polyubiquitinated via K-63 conjugation by Ubc13/Mms2 (E2) and Rad5 (a RING-domain E3). The K-63 ubiquitinated PCNA is vital for error-free repair of the damaged DNA, since mutations of Ubc13, Mms2 and Rad5 or the conserved ubiquitination site of PCNA promotes hypersensitivity to DNA damage inducers [120]. Although monoubiquitination was first reported as a sufficient signal for internalization and endocytosis of receptor, subsequent works

have shown the function of K-63 ubiquitination in regulating the receptor endocytosis. It is reported that uracil permease undergoes K-63 ubiquitination at cell surface by the E3 ligase Npi1/Rsp5, resulting to the endocytosis and degradation [121]. Additionally, DUBs specifically cleaving K-63 ubiquitination chains regulate receptor trafficking by from internalized receptors [122, 123], indicating that K-63 plays a role in endocytosis of membrane proteins.

1.3.3 Linear (Met-1) ubiquitination

Recently, linear ubiquitination was identified as a novel type of ubiquitination, which has a role in NF-κB signaling activation. In 2006, Iwai group first reported that a E3 complex generates linear ubiquitin chains and they called linear ubiquitin chain assembly complex (LUBAC) [99]. Since then, the biology of linear ubiquitination has been intensively investigated [91, 92, 94, 124]. In the linear ubiquitin chains, the C-terminal glycine of distal ubiquitin is linked to the N-Terminal methionine of proximal ubiquitin [93]. The activation signaling from binding between TNF-α and its receptor leads to formation of a large complex called the TNF–receptor signaling complex (RSC) (consisting of TRADD, RIP1, TRAF2 and cIAP1/2). It is proposed that the LUBAC functions as a bridge between TNF-RSC and IKK complex. So far, three components of this complex are characterized: HOIL-1, Sharpin and RNF31 (Figure 4). Either associated molecules, HOIL-1, Sharpin or both bind with a key E3 ligase, RNF31 to generate linear ubiquitination of substrates. The deficiency of RNF31 and HOIL-1 suppressed the recruitment of IKKα and NEMO to the TNF-RSC [125]. Moreover, overexpression of RNF31 with HOIL-1, Sharpin, or both induces the ubiquitination of NEMO, which is important for activation of NF-κB signaling. Although RNF31 and HOIL-1 contain RBR (RING in-between RING) domains, only the RBR domain of RNF31 is essential for LUBAC activity. This date indicates that RNF31 requires binding with these associated molecules to acquire E3 ligase activity for linear [91, 92, 97]. cIAP1/2 is fundamental for the effective recruitment of LUBAC to TNF-RSC by providing the binding sites [125]. Additionally, genetic studies describe that the defect of LUBAC components affects overall NF-κB signaling events and functions. Sharpin or HOIL-1 deficient MEFs show the attenuated level of phosphorylation and degradation of IκB-α and delayed translocation of p65 upon stimulation with TNF-a and IL-1. Furthermore, the induction of NF-κB target genes is reduced in these cells and TNF-a triggered

cell death is specifically enhanced in Sharpin deficient cells. [91, 92]. All these observations support the idea that linear ubiquitination of NEMO is critical to effective NF-κB activation. The importance of LUBAC in NF-κB signaling is further supported by clinical observation that the mutation of NEMO that abrogates its linear ubiquitination in patients harboring X-linked ectodermal dysplasia and immunodeficiency, caused by impaired NF-κB signaling [126].

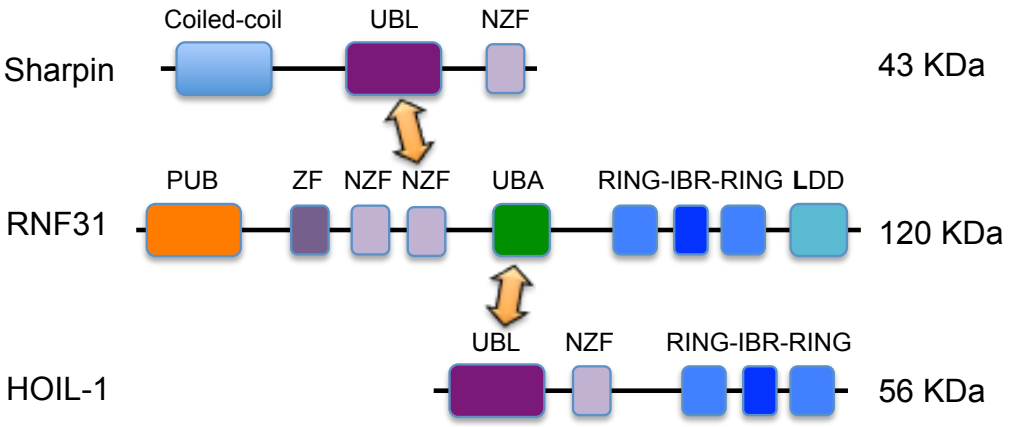


Figure 4 Schematic diagram of LUBAC

LUBAC is composed of three different molecules, RNF31 (120KDa), HOIL-1 (56KDa) and Sharpin (43KDa). RNF31 and HOIL-1 or Sharpin interacts through UBA of RNF31 and UBL of HOIL-1 or Sharpin and this association is required to generate linear ubiqutination.

1.4 Apoptosis

1.4.1 The history of apoptosis

Although Apoptosis was first named in 1972 to illustrate the different cell death from necrosis [127], many researchers have observed this unique cell death more than a century earlier. In 1842, Carl Vogt observed that tadpole cells naturally die during developmental process [128]. Since he reported the first observation, others described unique cell death features different from necrosis with different terms such as Necrobiosis (Rudolph in 1858), Chromatolysis (Walther Flemming described the morphological features of apoptosis such as shrinkage chromatin condensation nuclear fragmentation with this term in 1885). Karyopyknisis (Glucksmann proposed this term and reported the involvement of this process in embryogenesis and development of vertebrates in 1951) and Shrinkage necrosis (John F. Kerr in 1965) [128]. Finally, Kerr together with Andrew Wyllie and Professor Alastair Currie coined the term, "APOPTOSIS" in 1972, which brought up huge attentions in the biology field [127]. They proposed that the deregulation of apoptosis, rather than mitosis could result to hyperplasia, which represent the vitality of apoptosis in cancer development. Later, degradation of DNA was observed during apoptosis indicating that a certain biochemical process controls apoptosis [129] and a series of intensive study with *C. elegans* allowed us to expand our knowledge of apoptosis, this process is exquisitely regulated by signaling cascades [130]. Importantly, David Vaux *et. al.* reported the first apoptosis regulating mammalian gene, BCL-2 and its anti-apoptotic and tumorigenic functions in 1988 [131]. Furthermore, human proteins homologous to *C. elegans* proteins regulating apoptosis were identified and named "Caspase". Diverse caspases activation and their function were discovered through these studies [132-134]. Additionally, assorted viral proteins that suppress apoptosis including IAP and FLIP and its human homologs were distinguished [135, 136]. Later studies investigated the role of apoptosis regulation in human

diseases such as the function of p53 in cancer and the role of Fas/FasL ligation in immune disease [137, 138]. Afterward, a variety of researches have examined and emphasized the importance of apoptosis regulation in the human developmental process and diseases [139-142].

1.4.2 Types of apoptosis

Generally, Apoptosis is classified into two pathways depending on the sources of inducers; Intrinsic and Extrinsic pathways [143]. Intrinsic pathway is activated by internal inducers such as DNA damage, oxidative stress, radiation, malfunction of mitochondria, and hypoxia. Extrinsic pathway is triggered through external stimuli such as TNF superfamily members: TNF-α, TRAIL and FasL (Figure 5).

1.4.2.1 Intrinsic pathway

In the intrinsic apoptosis pathway, mitochondrial outer membrane permeabilization (MOMP) is a vital event to initiate cell death process and regulation of this event depends on BCL-2 family members [144]. Among these members, pro-apoptotic BCL-2 associated X protein (BAX) and BCL-2 antagonist or killer (BAK) are important to initiate MOMP. In unstimulated condition, BAK is localized in membrane of mitochondria and BAX stay in the cytosol as a monomer. Once intrinsic apoptosis is activated, these molecules are recruited to the outer membrane and activate mitochondria through conformational change resulting in the generation of mitochondrial apoptosis-induced channel (MAC). The mechanism how pro-apoptotic members, BAK and BAX are activated is not clearly understood, but there are two theories that explain this process. First, other pro-apoptotic BCL-2 family; BH3-only proteins (BID, BIM, BAD, BIK, NOXA and PUMA) directly interact to BAK or BAX and activate them. The other is indirect regulation. Anti-apoptotic proteins including BCL-2, BCL-XL and MCL-1 localize on the outer membrane of mitochondria and inhibit conformational change of BAX and BAK. Upon activation, BH3-only proteins associate with these anti-apoptotic proteins and release their inhibitory functions, resulting in the activation of BAX and BAK [145]. Through MACs, cytochrome c is released from mitochondria to cytosol and initiates caspases activation. Released cytochrome c binds to Apoptotic protease activating factor 1 (APAF-1) and induce

heptamerization of this protein (called Apoptosome). Then, pan-caspase 9 is recognized and activated by this complex, leading to the following activation of effector caspase 3 or 7 [146].

1.4.2.2 Extrinsic pathway

Extrinsic pathway is triggered through death receptor mediated signaling such as Fas, TFNRI, DR4 and DR5. Once designated ligands including FasL (binds with Fas), TNF-α, LT-α (bind with TNFRI) and TRAIL (binds with DR4 and DR5) bind with corresponding receptors, extrinsic apoptosis is activated [147]. First, the ligation between TNF-α and TNFRI lead to the trimerization of TNFRI that further promote recruitment of TRADD to the cytoplasmic domain of receptor complex. Then, FADD interacts with TRADD, followed by the recruitment of Caspase 8 to TNFRI and TRADD complex. In Fas, DR4 and DR5-mediated signaling, TRADD is not necessary to recruit caspase 8 to receptor complex. FADD directly binds to Fas or DRs and facilitate the recruitment of caspase 8 [148]. This engagement of death-inducing signaling complex (DISC) prompts to homo-dimerization of Caspase 8 and further self-cleavage at D216 and D374/384 that produce active form of caspase 8; p18 and p10 [149]. Then, this activated heterodimeric p10 and p18 (two p10 and two p18) complex further cleaves effector caspases including caspase 3 and caspase 6 and generates active forms; p20 and p12 (for caspase 3) and p20 and p10 (for caspase 6) [149]. These activated effector caspases target and cleaves vital proteins such as structural proteins (e.g. actins, tau, tubulin, lamins and tpr), cell adhesion components (e.g. APC, catenins, cadherins, and paxillin), cell cycle regulators (e.g. CDCs, Cyclins, p21 and p27) and proteins involved in DNA synthesis (ATM, BRCA-1, PARPs, polymerases, and RADs) [150]. Cleavage of these proteins finally executes cell death. Researchers also proposed the crosstalk between intrinsic and extrinsic signaling; activated caspase 8 through death receptor ligation results in cleavage of BH-3 only protein, BID and

truncated BID (tBID) translocates to mitochondria and activates BAX and BAK leading to the formation of pore on the outer membrane [151].

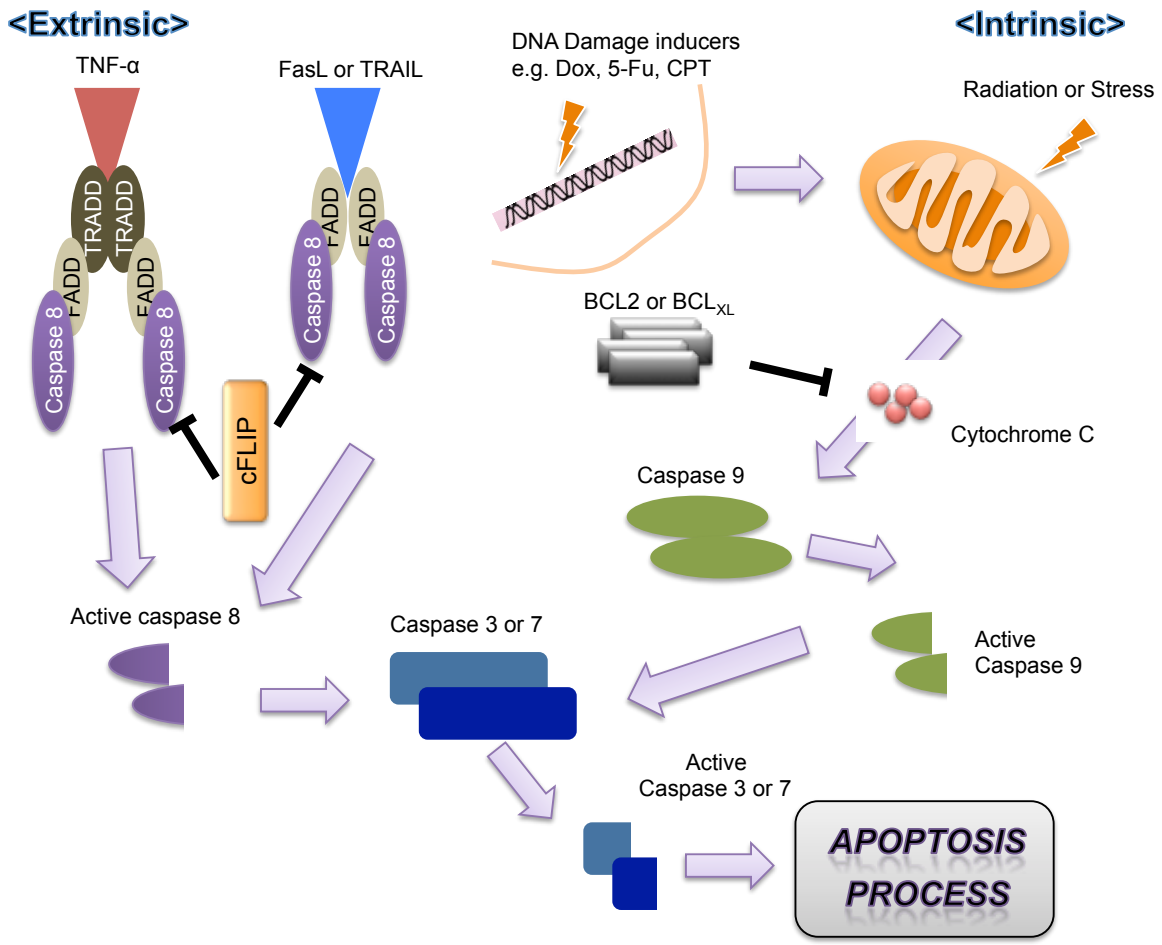


Figure 5 The intrinsic and extrinsic cell-death pathways.

Apoptosis is activated through two different pathways (extrinsic and intrinsic), based on the source of inducers. First, extrinsic factors such as TNF-α, FasL and TRAIL activate caspase 8 through receptor complex including FADD, caspase 8 and/or TRADD. Active caspase 8 then, induce the cleavage of caspase 3 or 7 and these cleaved caspases (active forms) execute apoptosis. cFLIP inhibits this extrinsic pathway through suppression of caspase 8 activity. In intrinsic pathway, apoptosis inducers such as DNA damage inducers, radiation or stresses induce the release of cytochrome C from mitochondria. Released cytochrome C further activates caspase 9 by the cleavage of inactive form. Active caspase 9 further induce the activation of caspase 3 or 7 and the execution of apoptosis. In this pathway, BCL2 family members including BCL2 and BCL$_{XL}$ suppress the release of cytochrome C and block the activation of the intrinsic pathway.

1.4.3 Apoptosis in diseases

Since maintaining the balance between death and survival is critical in all of cellular processes, apoptosis is tightly regulated and deregulation of this pathway often results in severe diseases such as cancer, neurodegenerative diseases, and autoimmune diseases.

First, tumor is developed when cells are resistant to apoptosis. Diverse mutations that lead to hyperactivation or amplification of anti-apoptotic proteins or suppression or silencing of pro-apoptotic molecules play critical roles in the development of tumor. For example, amplification of anti-apoptotic molecule, BCL-2, is observed in follicular lymphoma [152, 153], prostate [154], breast [155], lung cancers [156] and chronic lymphocytic leukemia [157]. And loss of function mutation in p53 (the pivotal tumor suppressor) is observed in various cancers such as lung [158], stomach [159], breast [160], prostate cancers [161] and leukemia [162]. This p53 is critical for cellular stress induced apoptosis so, the absence of p53 activity frequently leads to resistance to apoptosis and tumor formation. Since many conventional cancer treatments such as radiation and chemotherapy utilize activation of apoptosis, the tumors harboring these mutations are often resistant to conventional approaches [163]. Therefore, clear understanding of apoptosis is fundamental to develop the therapeutic approaches to control apoptosis oriented diseases.

Reversely, the unwanted activation of cell death results in neurodegenerative diseases. In contrast to the normal cells that actively proliferate and die at the same time, neuron cells survive for the whole lifetime for the specialized function such as memories and muscle control [164]. Therefore, deregulation of neuron cell death frequently leads to neurodegenerative diseases including Parkinson's, Alzheimer's or Huntington's diseases [165]. Alzheimer's disease involves the accelerated cell death of hippocampal and cortical neurons, responsible for spatial memory, navigation, and perception. The most obvious symptom of Parkinson's disease;

an uncontrollable movement is responsible for excess apoptosis of dopaminergic neurons in the brain. Huntington's disease results from the damage of striatum critical for movement, and cognitive function [166].

Deregulation of apoptosis results in many autoimmune diseases such as rheumatoid arthritis (RA), autoimmune lymphoproliferative syndrome (ALPS) and Insulin-dependent diabetes mellitus (IDDM) as well. Enhanced proliferation and resistance to apoptosis of synovial cells involves in RA. The synovial tissue from RA patients shows hyperplasia of synovial surface, resulted from accumulated synovial cells and macrophages. Additionally, the accumulation of macrophages and lymphocytes in bone-cartilage damages to joint. Deregulation of apoptosis through high level of anti-apoptotic molecules, cFLIP and BCL-2 in synovial cells contributes to the accumulation of these immune cells and destroy joint regions [167-169]. ALPS patient harbors Fas or FasL mutation leading to defect of Fas/FasL mediated apoptosis in T lymphocytes. Due to this defect, T cell and B cells are hyper-proliferated and generate excessive immune responses such as immunoglobulin production [170]. IDDM is caused by uncontrolled death of insulin-secreting pancreatic beta cells. Uncontrolled antigen presenting cells (dendritic cells [DCs] and macrophages), NK cells and T cells induces the generation of beta-cell specific T cells that specifically kill beta islet cell [171].

1.5 The feedback between NF-κB and apoptosis pathways

As shown in the previous section, TNF-a activates two contrary signaling pathways: survival pathway (NF-κB signaling) and cell death pathway (apoptosis signaling). Since these two pathways are essential for intact development and homeostasis by growth of necessary cells and clearance of unwanted cells, these signaling pathways are thoroughly controlled. The crosstalk between the two signaling helps to maintain the balance between cell death and survival. Since this balance is fragile and instability of this balance often leads to severe diseases, it is critical to have precise mechanism in single signaling regulation and crosstalk between them is important to control apoptosis related diseases [172].

1.5.1 Regulation of NF-κB signaling by apoptosis

Many signaling components in NF-κB pathway are regulated by apoptosis. The key event in apoptosis is the activation of caspase that enables protease activity. Therefore, various NF-κB components are cleaved and inactivated by activated caspases. For example, NF-κB transcriptional factor p65, p50 and c-Rel are substrates of active caspase 3. Under apoptosis condition, activated caspase 3 interacts and inactivates its substrates by cleaving them [173-175]. Regulatory molecule, IKKγ is cleaved by caspases and its role in NF-κB activation is suppressed [176]. Additionally, signaling mediators such as RIP1 and TRAF1 are recognized by activated caspase 8 and lose their function in NF-κB activation [177, 178]. Caspase 7 is specifically associated with TNFRI and controls the function of TNFRI [179]. IκB-α, a principal inhibitory component for NF-κB signaling is a target of caspase 3, but this cleavage enhances the function of IκB-α by generating super repressor form [180].

1.5.2 Regulation of apoptosis by NF-κB signaling

Genetically modified mouse models demonstrate the importance of NF-κB signaling in the regulation of apoptosis. The deletion of NF-κB components such as RelA, IKKβ or IKKγ result in excessive apoptosis of liver cells and lethality of mouse embryo [181]. Therefore, NF-κB signaling suppresses apoptosis signaling in order to prevent excessive cell death. Generally, NF-κB signaling regulates apoptosis pathway through the induction of apoptosis regulating proteins such as IAP family members, BCL-2 family members, FLIP, and TRAFs. NF-κB target genes, cellular inhibitors of apoptosis (cIAPs), directly bind with caspase 3, 6, 7 and 9 and inhibits their protease activity. Activation of NF-κB signaling induces cIAP expression that further suppresses apoptosis activation [182]. Cellular FLICE inhibitory protein (cFLIP) is also induced by NF-κB activation. This inhibitory protein associates with Caspase 8 and inhibits its dimerization, which is critical for the activation of extrinsic apoptosis pathway [183]. Inhibitory molecules of mitochondria mediated apoptosis, BCL-2 family members, such as BCL-2 and BCL-XL are target genes of NF-κB signaling. Induced BCL-2 and BCL-XL prevent activation of BAK and BAX and suppress the formation of cytochrome C channels [184, 185]. Expression of adaptor proteins, TRAF1 and 2, are triggered by activated NF-κB pathway. The induced proteins augment NF-κB activation, which enhances the expression of other apoptosis regulating proteins [186].

Chapter II: Materials and methods

2.1 Genotyping of RNF31 and TNFR1 knockout mouse

To isolate mouse genomic DNA, mouse-tail was incubated overnight in 400ul of extraction buffer containing 10mM Tris-HCl (pH8.0), 100mM NaCl, 10mM EDTA (pH8.0), 0.5% SDS and 0.2 mg/ml proteinase K. Then, 200ul of 5M NaCl was added to precipitate proteins. After spin down at 13,000 rpm, 400ul of supernatant was transferred and DNA was precipitated by adding 800ul of cold ethanol, followed by centrifugation at 13,000rpm. Precipitated DNA was resuspended with TE buffer after washing with 70% ethanol. PCR amplification was performed with isolated DNA to analyze the genomic status. The primers for each mouse are following; to analyze RNF31 mouse, RNF31-5F (CACAGCCATTCTTAAG TCCAGTGC) and RNF31-5R WT (ACTTGGTTACAGGCTGGACAGTG) for WT allele, RNF31-5F and en2-R (CCAACTGACCTTGGGCAAGAACAT) for RNF31 KO allele were used. For detect TNFR1 alleles, TNFR1 WT1 (GGATTGTCACGGTGCCGTTGAAG) and TNFR1 WT2 (TGACAAGGACACGGTGTGTGGC) for TNFR1 WT allele and TNFR1 KO1 (TGCTGATGGGGATACATCCATC) and TNFR1 KO2 (CCGGTGGATGTGGAATGTGTG) for TNFR1 KO allele were used. The PCR products were electrophoresed on 1% agarose gel.

2.2 Reagents and plasmid

The human TNF-α recombinant protein RTNFAI was purchased from Thermo Scientific. The *de novo* protein synthesis inhibitor CHX (ALX-380-269), proteasome inhibitor MG132 (BML-PI102), pan-caspase inhibitor Z-VAD-FMK (ALX-260-020), and necroptosis inhibitor Nec-1 (BML-AP309) were purchased from Enzo Life Sciences. The translocation inhibitor of the NF-κB complex sc-3060 was purchased from Santa Cruz Biotechnology. The JNK inhibitor SP600125 (S5567) was obtained from Sigma. 5-Fluorouracil (F6627; Sigma-Aldrich) and doxorubicin (BML-GR319; Enzo Life Sciences) were stored at -20°C after dissolution in

dimethyl sulfoxide and water, respectively. Antibodies against RNF31 (ab85294) and Sharpin (ab125188) were purchased from Abcam. Antibodies specific for PARP (9542), caspase 3 (9665), cleaved caspase 3 (9661), caspase 8 (9746), caspase 9 (9508), IκB-α (4814), phosphorylated IκB-α (9246), phosphorylated TAK1 (4508), phosphorylated IKKα/β (2697), phosphorylated JNK (9255), and JNK (4672) were obtained from Cell Signaling Technology. Antibodies against $cFLIP_{S/L}$ (SC-5276), actin (sc-8432), TNF receptor 1 (sc-8436), IKKα (sc-7218), p65 (sc-109), lamin B (SC-6219), β-tubulin (sc-5274), Myc (sc-7899), FLAG (SC-807), and ubiquitin (SC9133) were obtained from Santa Cruz Biotechnology. An antibody specific for RIP1 (610459) and FADD (F36620, BD bioscience) was obtained from BD Biosciences. A K-48 linkage-specific polyubiquitin antibody (05-1307) was purchased from Millipore. An anti-linear polyubiquitin antibody was provided by Genentech. WT RNF31, HOIL-1, and Sharpin were cloned from Jurkat cDNA. All mutants of RNF31 (including WT, C885S, NT, CT, ΔRL, RL, RBR, D390A, D348/390A and 348/387/390A) were generated using polymerase chain reaction with WT cDNA and verified via sequencing. Phosphorylated EF4-FLAG-cFLIP was provided by Dr. Jianke Zhang (Thomas Jefferson University). Mutant constructs were generated using polymerase chain reaction and cloned into pcDNA3.1 plasmid. ShRNA clones for human RNF31 and Sharpin were obtained from Open Biosystems through MD Anderson's shRNA and ORFeome Core. The RNF31 and Sharpin clone numbers were V2LHS_284762 (GACAATAACGTCATGTTTA) and V3LHS_390302 (GGTGTAGGAAACTGACTCC), respectively.

2.3 Cell cultures and transfection

HEK293T, Phoenix, HeLa, A431, HepG2, BxPC-1, Panc-1 A549, HT29 and HCT116 cells were purchased from ATCC and cultured in DMEM supplemented with 10% FBS and 1%

antibiotics. Jurkat was obtained from ATCC and Caspase 8 deficient Jurkat and FADD deficient Jurkat were kindly provided by Dr. Jianke Zhang (Thomas Jefferson University). WT Jurkat and mutant cells were maintained in RPMI 1640 supplemented with FBS and the antibiotics. Cells were starved for 16 hours with DMEM or RPMI containing 0.5% FBS before the induction of apoptosis by the treatment of the indicated inducers. For overexpression experiments, 293T cells were transfected using the calcium phosphate transfection method. Briefly, 7.5×10^5 cells were plated into 6 well plate and add CaCl2/HBSS/DNA precipitate in next day (1~4ug of DNA per well). After 24 hours, cells were lysed for further experiments.

2.4 Viral production and infection

A lentiviral supernatant was collected 48 hr after cotransfection of pGIPZ (for shRNA) or LentiCRISPR (for the CRISPR system) with packaging plasmids (pCMV-VSV-G and pCMV-dR8.2 dvpr) into HEK293T cells. pMX plasmid for rescue experiments was transfected into Phoenix cells with pCMV-VSV-G for retroviral production. Viral supernatants were collected after 48 hr, and target cells were incubated with the supernatant in the presence of polybrene for 8-12 hr. After infection with virus, viral supernatants were replaced with fresh medium. After 24 hr, infected cells were selected using puromycin (2 ng/ml) or sorted using flow cytometry. The efficiency of the infection was determined using Western blot (WB) analysis.

2.5 KO of cFLIP and RNF31 using the CRISPR system

Single guide RNAs were designed using CRISPR Design (http://crispr.mit.edu/) and inserted into a LentiCRISPR vector expressing hSpCas9 and guide RNA [187]. The single guide RNA sequence for human RNF31 was TTGACACCACGCCAGTACCG, and that for human cFLIP was ATGAAGGATTACATGGGCCG.

2.6 Ubiquitination assay

Twenty-four hours after transfection of them with FLAG-cFLIP, FLAG-RIP1, and myc-LUBAC, 293T cells were lysed with 2% sodium dodecyl sulfate lysis buffer (2% sodium dodecyl sulfate, 150 mM NaCl, 10 mM Tris-HCl, pH 8.0) containing 2 mM sodium orthovanadate, 5 mM sodium fluoride, and 1 mM N-ethylmaleimide under denaturing conditions. After 10 min of boiling and sonication, samples were diluted with a dilution buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 1% Triton) up to 10 times and incubated on ice for 30 min. Lysates were then immunoprecipitated with prewashed anti-FLAG M2 Affinity Gel (A2220; Sigma). Immunoprecipitates were analyzed using a WB assay.

For an *in vitro* ubiquitination assay, recombinant cFLIP protein was incubated with or without lysine KO ubiquitin, E1, E2 (Boston Biochem), or LUBAC proteins at 37°C for 2 hr in *in vitro* ubiquitination buffer (20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 2 mM ATP). The reaction was stopped by adding 2× loading buffer to the incubated followed by boiling, and the level of ubiquitination was monitored using a WB assay.

2.7 *In vitro* cleavage assay

Human recombinant RNF31 WT and D348/387/390A mutant protein conjugating Myc tag were expressed in 293 T cell. After 24 hours, cell lysates were prepared with lysis buffer (50mM Hepes (pH 7.4), 150mM NaCl, 1% NP-40, 1mM EDTA) containing 1mM sodium orthovanadate , 1 mM sodium fluoride, 1mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Roche). Then, overexpressed RNF31 protein was precipitated with EZview™ Red Anti-c-Myc Affinity Gel (Sigma). Recombinant caspases were obtained from Biovision (Active Recombinant Caspase Set III, K232-8-25) and prepared according to the manufacturer's

recommendation. 2 units of recombinant caspases and precipitated RNF31 protein were incubated at 37°C in a reaction solution (50mM Hepes, pH 7.2, 50mM NaCl, 0.1% Chaps, 10mM EDTA, 5% Glycerol, and 10mM DTT). After 1~2 hours, the reaction was terminated by adding 4X loading buffer, followed by boiling for 5 min. Then, the cleavage band was analyzed by Western Blot assay.

2.8 Western Blot

Western Blot assay was performed as previously described. Briefly, cells were lysed in lysis buffer (50mM Hepes (pH 7.4), 150mM NaCl, 1% NP-40, 1mM EDTA) containing 1mM sodium orthovanadate , 1 mM sodium fluoride, 1mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Roche). After 20 min incubation, samples were centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was transferred and mixed with 4X loading buffer. For immunoprecipitation experiment, lysates from transfected 293 cells were incubated with anti-FLAG M2 Affinity Gel for 16 hours. After washing with lysis buffer four times, the proteins eluted with 2X SDS loading buffer. Then, cell lysates or Immunoprecipitates were separated by SDS-PAGE and transferred onto nitrocellulose membrane (Bio-Rad). The membrane was probed with primary antibodies, followed by HRP conjugated secondary antibodies. The bands were then visualized with ECL substrates (Pierce).

2.9 Electrophoretic Mobility Shift Assay

Nuclear proteins were extracted from 1×10^6 to 4×10^6 cells of HeLa or Jurkat cells after the indicated treatment. Nuclear extracts (2 mg of each) were incubated with ^{32}P-labeled, double-stranded NF-κB or an OCT1-specific probe (Promega) for 15 min at room temperature.

Samples were separated on a native Tris-borate-ethylenediaminetetraacetic acid polyacrylamide gel and visualized using autoradiography.

2.10 Luciferase assay

HEK293T cells were transfected with reporter plasmids encoding NF-κB-luc and pEF-Renilla-luc together with the expression vectors. Each transfection was performed in triplicate. Cells were lysed 24 hours after transfection and luciferase activities were measured with Dual-Luciferase assay kits (E1980, Promega). NF-κB activities in each lysates were determined by ratios of Renilla luciferase readings to Firefly luciferase readings and the average of measured activity in each group were normalized to the activity of the empty construct.

2.11 MTT assay

1~3x10^3 cells were plated to 96 well plates with 100ul of complete media. After 16 hours starvation (0.5% FBS), cells were treated with each agent for indicated time and MTT solution was added to each well at a final concentration of 0.5mg/ml (Sigma). After 2hours, the media was aspirated and formazan was dissolved with DMSO. The absorbance was measured at 570nm by using spectrophotometric microplate reader (BioTek) and measured value of each group was normalized to the value of untreated cells. All experiments were performed in triplicates.

2.12 Annexin V-Staining apoptosis assay

HeLa cells (3×10^5) were seeded on six-well plates and incubated in starvation medium (0.5% FBS) for 16 hr. After treatment of the cells with the indicated inducers of apoptosis (TNF-α alone or with CHX), suspended cells in the supernatant were collected, and adherent

cells on the plates were combined after detaching them using 0.05% trypsin (Sigma). Also, 1×10^6 Jurkat cells were stimulated with apoptosis inducers. After washing with phosphate-buffered saline, the collected cells were stained with APC Annexin V (550474; BD) and analyzed using flow cytometry.

2.13 Statistical analysis

Statistical significance were analyzed using Prism6 software (GraphPad) with the independent two-sample student t-test

Chapter III: Deletion of RNF31 results in the embryonic lethality in mouse.

3.1 Introduction

Ring Finger Protein 31 (RNF31) is the E3 ligase as a member of RING (Really Interesting New Gene) finger protein family. It contains three zinc finger domains, ubiquitin-associated (UBA) domain, RING1-in between ring-RING2 (RBR) domain and linear ubiquitin chain Determining Domain (LDD). This molecule is also called HOIP (Heme-Oxidized IRP2 ubiquitin ligase-1 [HOIL-1] interacting protein) or zibra (zinc, in-between-ring finger, ubiquitin-associated domain) and was first cloned from a transformed breast cancer cell line [188]. After the first cloning, only few studies have examined the biological function of RNF31 like its regulation of the DAX-1 transcriptional activity. The study of this molecule had been limited before researchers discovered its pivotal role in the novel type of ubiquitination, linear ubiquitination. In 2006, RNF31 and associated protein: HOIL-1 was reported to form a complex in order to generate a novel type of ubiquitination [99]. Subsequently, NEMO was identified as a substrate of linear ubiquitination, this modification playing an important role in NF-κB activation [189]. Later, three independent papers discovered another associated molecule, Sharpin and reported that RNF31, HOIL-1 and Sharpin forms complex (which called linear ubiquitin chain assembly complex [LUBAC]) to conjugate linear ubiquitination to NEMO and RIP1 [91, 92, 97]. Among these three components, RNF31 is the main E3 ligase because the complex containing catalytic dead RNF31 was not able to generate linear ubiquitination. LUBAC regulates TNF-α, IL-1, CD40, lymphotoxin β, Toll-like receptor (TLR), and nucleotide-binding oligomerization domain containing 2 (NOD2)-mediated cellular events, but it is still controversial whether it regulates T cell receptor (TCR) or B cell receptor (BCR)-mediated signaling pathways. Additionally, OTU domain-containing deubiquitinase with linear linkage specificity (OTULIN) was identified as a linear ubiquitin chain-specific deubiquitinase which affects NF-κB responses. Although the majority of studies have focused on its role in NF-

κB signaling, the data indicates that linear ubiquitination regulates other pathways, including apoptosis that is also activated by TNF-α stimulation. Moreover, Rodgers MA *et. al.* demonstrated the regulation of NF-κB independent signaling pathway such as inflammasome mediated cytokine production. They found that LUBAC induces linear ubiquitination of apoptosis-associated speck-like protein containing a carboxyl-terminal CARD (ASC) and regulates NLR family, pyrin domain containing 3 (NLRP3) activation that is critical for IL-1β secretion [124]. The biological significance of LUBAC and its components was investigated with genetically modified mouse model. Specifically, the role of LUBAC in mouse development was analyzed with *Sharpin* deficient mouse (*cpdm* mouse). *Cpdm* mouse, arose through spontaneous genetic mutations that showed hyperproliferation, neutrophil infiltration, and a chronic persistent inflammatory reaction in the skin. Mouse embryonic fibroblasts (MEFs) from *cpdm* mouse showed suppressed NF-κB activation upon TNF-α and CD40 treatment. And HOIL-1$^{-/-}$ MEFs also exhibited repressed activation of NF-κB signaling by TNF-α, and IL-1β stimulation although HOIL-1 knockout mouse did not show significant skin problem like *cpdm* mouse. However, the role of RNF31 in mouse development was not reported yet. Therefore, I generated RNF31 knockout mouse and examined the role of RNF31 in mouse development.

3.2 Results

3.2.1 **Knockout of RNF31 Results in embryonic lethality of mouse.**

In order to investigate the role of RNF31 in mouse development, I studied RNF31 deficient C57BL/6 mouse obtained from KOMP (KO-2896, Figure 6). The mouse I received was a heterozygous knockout mouse, so I bred them in a manner that produced a homozygously deleted mouse. Six pairs of adult mice were crossed and 42 pups were obtained from them. I expected about 10 homozygous KO mice (I expected 1:2:1 ratio of $RNF31^{+/+}$: $RNF31^{+/-}$: $RNF31^{-/}$ mice according to Mendel's law), but I did not have any homozygous knockout mouse. I obtained 13 wild type mice and 29 homozygous mice, indicating that the deletion of RNF31 resulted in embryonic lethality (Figure 7A). To identify the lethal date, I collected mouse uteri from pregnant female that were 12.5, 9.5 and before 7 days postcoitum. To begin, I found two dead embryos from the uterus on day 12.5 of pregnancy. These dead embryos were already shrunken so I could not isolate them. 9 days after pregnancy, I observed one bloody embryo among 9 implantation sites. Finally, I could not observe any abnormal fetus sites in uterus isolated from pregnant female earlier than 7 days of pregnancy (Figure 7B), implying that the lethal date of RNF31 knockout mouse is between 7 days and 9.5 days.

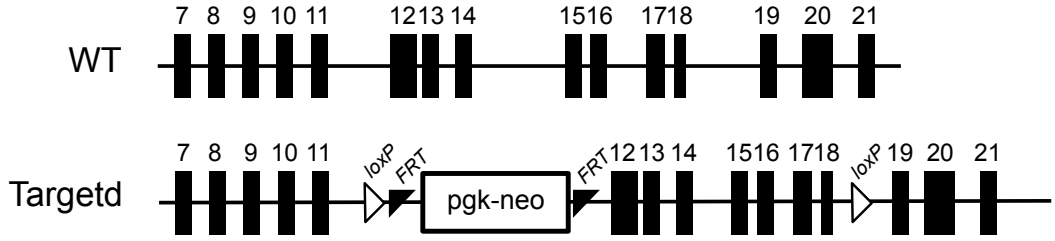


Figure 6 The structures of the WT HOIP genomic allele and mutated allele.

Inserted pgk-neo cassette between exon 11 and 12 leads to the interference of RNF31 expression. Black boxes indicate the coding exons.

A

$RNF31^{+/-}$ X $RNF31^{+/-}$				
	$RNF31^{+/+}$	$RNF31^{+/-}$	$RNF31^{-/-}$	Total
Expected	10.5	21	10.5	42
Observed	13	29	0	42

B

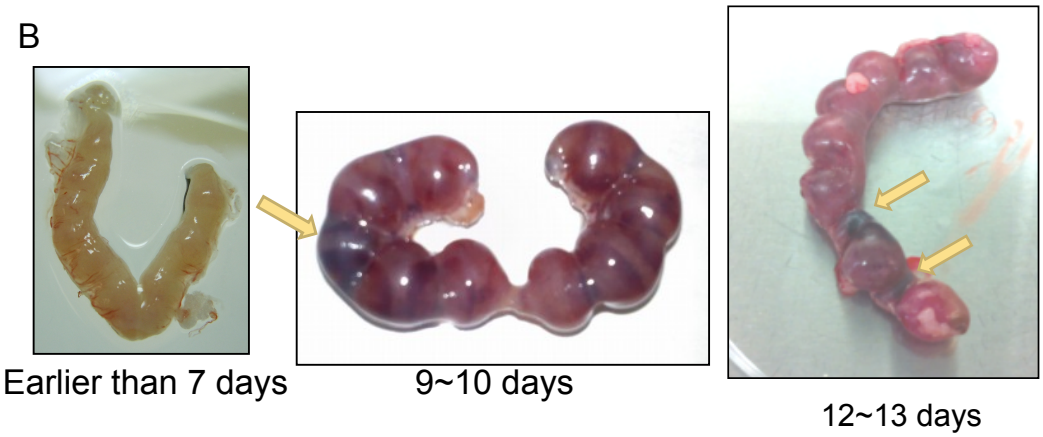


Figure 7 Knockout of RNF31 leads to embryonic death before E 9.5 days.

(A) Genotype of mice obtained from breeding between $RNF31^{+/-}$ parental mice (B) Uteri were excised from pregnant mice at 12.5, 9.5, and before 7 days of pregnacny.

3.2.2 Deletion of TNFR1 dose not rescue the lethality of RNF31 KO mouse.

Since it is suggested that RNF31 regulates TNF-α mediated cell death, I crossed $RNF31^{+/-}$ mouse with TNFR1 knockout mouse. I bred over 8 pairs of $TNFR1^{-/-}$, $RNF31^{+/-}$ parental mice and obtained 44 offspring. However, none of them were TNFR1-/-, RNF31-/- mouse. I obtained 14 $TNFR1^{-/-}$, $RNF31^{+/+}$ mice and 30 $TNFR1^{-/-}$, $RNF31^{+/-}$ mice among 44 pups (Figure 8). This data indicated that deletion of TNFR1 did not prevent the lethality of RNF31 knockout mouse.

$TNFR1^{-/-}\ RNF31^{+/-}$ X $TNFR1^{-/-}\ RNF31^{+/-}$				
	$TNFR1^{-/-}\ RNF31^{+/+}$	$TNFR1^{-/-}\ RNF31^{+/-}$	$TNFR1^{-/-}\ RNF31^{-/-}$	Total
Expected	11	22	11	44
Observed	14	30	0	44

Figure 8 Deficiency of TNFR1 did not rescue the lethality of RNF31 knockout mouse.
Genotype of mice obtained from breeding between $TNFR1^{-/-}$, $RNF31^{+/-}$ parental mice

3.3 Summary

RNF31 is the E3 ligase for linear ubiquitination. It must be associated with either Sharpin or HOIL-1 to generate linear ubiquitination, which controls NF-κB signaling by regulating IKK activation. Sharpin deficient mouse suggested that LUBAC played a critical role in mouse development. *Cpdm* mouse exhibits a severe skin problem that is a result of hyper apoptosis. Also, researchers demonstrated that Sharpin deficiency leads to suppressed NF-κB activation and enhanced apoptosis in mouse embryonic fibroblasts (MEFs) cells. However, the role of RNF31 in mouse development has not been reported yet. Here, we reported the embryonic lethality of RNF31 knockout mouse. With the observation of embryos, I found that the lethal date of this mouse is between 7 days and 9.5 days. Furthermore, I observed that this lethality of RNF31 knockout mouse was not rescued by crossing with TNFR1 deleted mouse. These data are indicative of a pivotal role: RNF31 has in mouse development and are suggestive of the TNFR- independent roles in cellular events.

CHAPTER IV: Linear Ubiquitination by LUBAC Directly Regulates the Apoptosis Pathway via Stabilizing cFLIP

4.1 Introduction

Tumor necrosis factor (TNF)-α is a cytokine that plays roles in various cellular processes, such as proliferation, differentiation, and death. It has been reported that it mainly activates two different signaling pathways: nuclear factor (NF)-κB signaling and apoptosis [190]. Once TNF-α binds to its receptor, signaling molecules such as the TNF receptor type 1-associated DEATH domain (TRADD), receptor-interacting proteins (RIPs), and TNF receptor-associated factors (TRAFs) are recruited and form the receptor-signaling complex (RSC). This complex triggers activation of the IκB kinase (IKK) complex (composed of IκB kinases α, β, and γ), which further induces the degradation of inhibitor of κB-α (IκB-α) and translocation of NF-κB transcriptional factors, sequentially. At the same time, TRADD/FAS-associated death domain (FADD)/caspase 8 complex is assembled to activate the programed cell death process; apoptosis. This active complex further triggers caspase activation to execute cell death [26]. Because the cell-death process is critical to homeostasis, it is tightly regulated by various inhibitory mechanisms. For example, cellular FLICE-like inhibitory protein (cFLIP) interacts and forms a heterodimer with caspase 8, inhibiting its activation and apoptosis signaling. In addition, B-cell lymphoma 2 (BCL2) family proteins and inhibitor of apoptosis proteins (cIAPs) directly and indirectly suppress caspases activation [172].

Ubiquitination is a key posttranslational modification (PTM) in TNF-α induced signaling. For example, K-48 ubiquitination regulates degradation of IκB-α [191] and FADD [192], and K-63 ubiquitination of NF-κB essential modulator (NEMO, also called IKKγ), RIPs, and TRAFs triggers the functional activation of these molecules [89], which further recruit downstream molecules. Recently, researchers discovered linear ubiquitination to be a novel type of ubiquitination involved in the TNF-α signaling pathway, especially NF-κB signaling [94]. To date, investigators have identified the linear ubiquitin chain assembly complex (LUBAC) as the

only ligase complex for this type of ubiquitination. LUBAC has three components: RNF31 (HOIP), HOIL-1 (RBCK1), and Sharpin. In particular, RNF31 has a major role in activation of NF-κB signaling by conjugating linear ubiquitin chains onto NEMO and RIP1. Two associated molecules, HOIL-1 and Sharpin are involved in the functional activation of RNF31 [93]. Genetic studies demonstrated that defects in LUBAC components attenuate TNF-α–induced NF-κB activation and gene expression [91, 92, 97]. In addition to TNF-α, LUBAC regulates interleukin-1 (IL-1)–, CD40-, lymphotoxin β-, Toll-like receptor (TLR)-, and nucleotide-binding oligomerization domain containing 2 (NOD2)-mediated cellular events, in which the NF-κB pathway plays a role [193]. Recently, authors reported linear ubiquitination to have an NF-κB–independent function, as well [124]. LUBAC regulates inflammasome activation and IL-1 production by conjugating linear ubiquitination chains with apoptosis-associated speck-like protein containing a carboxyl-terminal CARD (ASC). Although biochemical and mouse developmental data have suggested that LUBAC has a role in apoptosis signaling (such as the sensitization of Sharpin-deficient mouse embryonic fibroblasts (MEFs) to TNF-α–induced cell death [92], cell death-dependent skin problems in Sharpin-deficient mice [194, 195], and embryonic lethality in RNF31-knockout (KO) mice [196]), the roles of linear ubiquitination and RNF31 in TNF-α–mediated cell death are largely unknown. Since deregulation of cell survival and death is the main course of many diseases such as cancer and autoimmune disease, the clear understanding of cell death regulation is critical for the therapeutic strategies to control these diseases.

In the present study, I determined the function of RNF31 in the cell death pathway and hypothesized that RNF31 negatively regulates apoptosis. First, I found that LUBAC directly regulated apoptosis. Moreover, the complex dominantly suppressed TNF-α–induced extrinsic but not intrinsic apoptosis in an NF-κB–independent manner. As a mechanism of this regulation,

RNF31 as a major ligase in LUBAC conjugated linear ubiquitination with the antiapoptotic molecule cFLIP and stabilized it upon stimulation with TNF-α. Analysis of a genetic model generated by using CRISPR further confirmed regulation of apoptosis by RNF31 and linear ubiquitination. These findings provided insight into the regulatory mechanism of apoptosis by linear ubiquitination and that the disruption of this mechanism may be a novel therapeutic target for diseases oriented from deregulation of cell death.

4.2 Results

4.2.1 LUBAC is an E3 ligase that regulates TNF-α–induced apoptosis.

Although previous studies suggested that LUBAC plays a role in not only NF-κB signaling but also apoptosis, whether it directly regulates apoptosis or indirectly controls it via NF-κB signaling is unclear. To determine the function of LUBAC and RNF31 in apoptosis, I generated stable HeLa cells in which RNF31 was silenced using a short hairpin RNA (shRNA) system and compared them with control HeLa cells. I then monitored the pattern of apoptosis in these cells after cotreatment of them with TNF-α and cycloheximide (CHX). CHX is a *de novo* protein synthesis inhibitor, so it blocks TNF-α–induced antiapoptotic molecule expression and allows for activation of the apoptosis pathway. Although both groups of cells were responsive to TNF-α/CHX-induced apoptosis, RNF31-silenced cells exhibited earlier cleavage of initiator caspases (caspases 8 and 9) and the effector caspase (caspase 3) as well as their substrates (poly[ADP-ribose] polymerase [PARP], a substrate of caspase 3, and RIP1, a substrate of caspase 8) (Figure 9A). In particular, a single stimulation with TNF-α induced apoptosis in RNF31-silenced HeLa cells, whereas control HeLa cells were resistant to this single stimulation (Figure 9B). A higher number of annexin V-stained cells (Figure 9C) and increased incidence of shrunken morphology in TNF-α/CHX- and TNF-α–treated RNF31-silenced cells under microscopy (Figures 10A and B) than those in control cells demonstrated that RNF31 suppressed activation of apoptosis under TNF-α stimulation. Then, I treated shCon and RNF31 silenced HeLa cells with TNF-related apoptosis-inducing ligand (TRAIL), another death receptor (DR) mediated apoptosis inducer. This stimulation dominantly activates the apoptosis pathway, not the NF-κB signaling pathway since TRAIL binds with DR4 and DR5 and these receptors less efficiently bind with TRADD and thus, activate the NF-κB signaling compared with the TNFR1 signaling pathway. Upon TRAIL treatment, I observed the identical activation pattern of apoptosis in shCon and RNF31 silenced HeLa and HCT116 (Figure 11A and B).

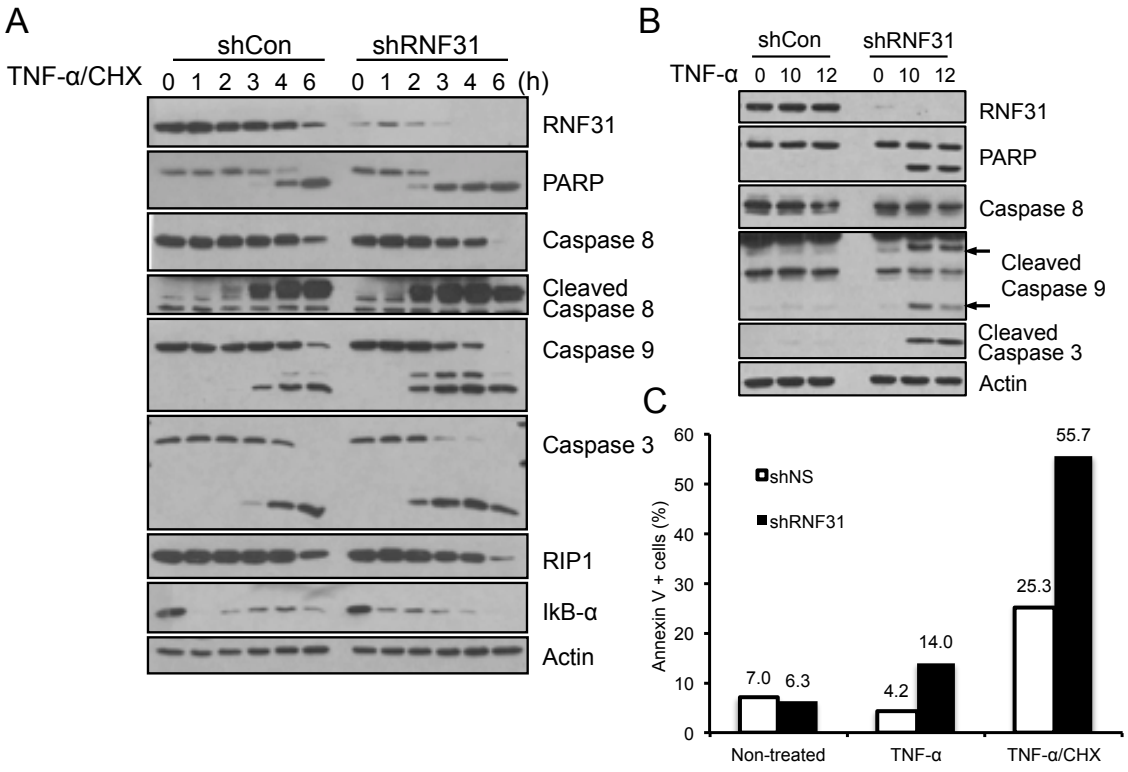


Figure 9 RNF31 silenced cells are more sensitive to TNF-α/CHX induced Apoptosis

(A) WB analysis of the indicated proteins in lysates of control and RNF31-silenced HeLa cells treated with TNF-α (10 ng/ml) and CHX (10 mg/ml). (B) WB analysis of the indicated proteins in control and RNF31-silenced HeLa cells after treatment with TNF-α (40 ng/ml). (C) Analysis of apoptotic cells upon stimulation of control and RNF31-silenced HeLa with TNF-α (40 ng/ml, 12 hr) or TNF-α and CHX (10 ng/ml and 10 mg/ml, respectively; 4 hr) according to annexin V staining.

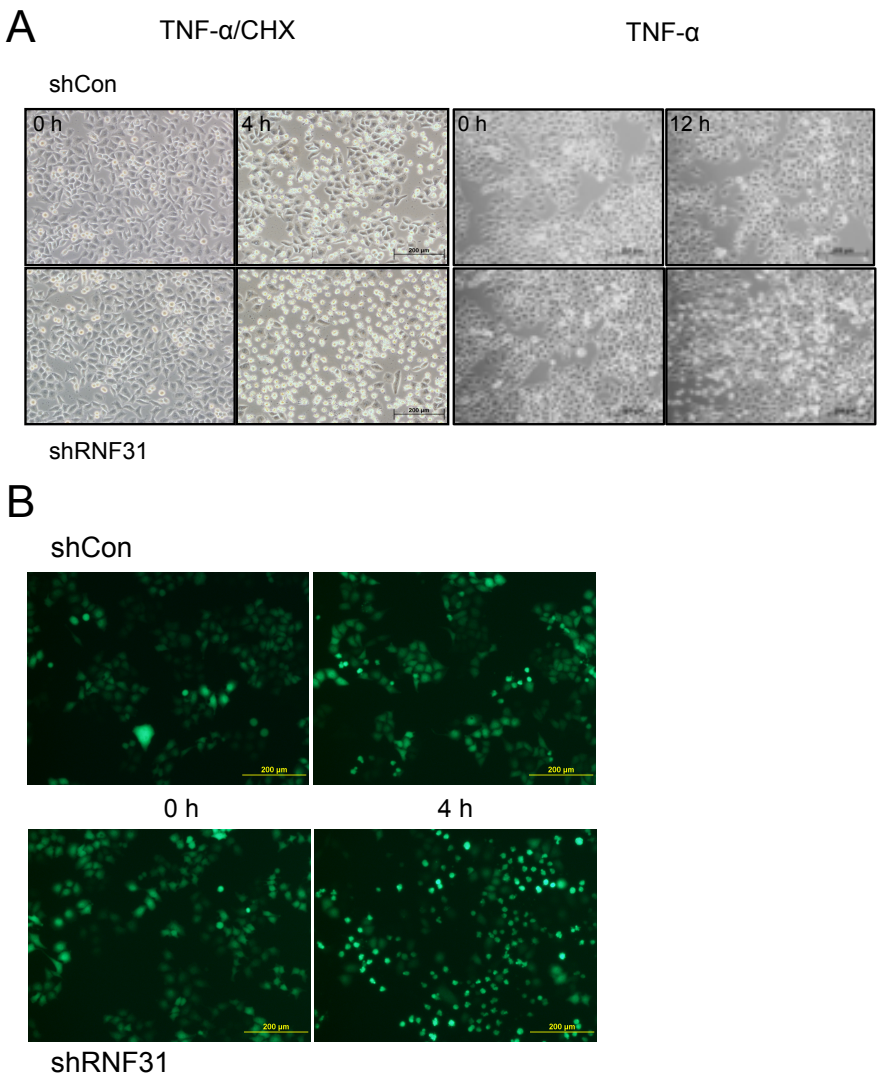


Figure 10 Morphological diffence of Control and RNF31 silenced HeLa cells after induction of apoptosis

(A and B) After being infected with non-target shRNA control and an shRNF31 virus encoding for green fluorescent protein expression, control and RNF31-silenced HeLa cells were stimulated with TNF-α and CHX (10 ng/ml and 10 μg/ml, respectively), and induction of apoptosis in the cells was monitored under a microscope.

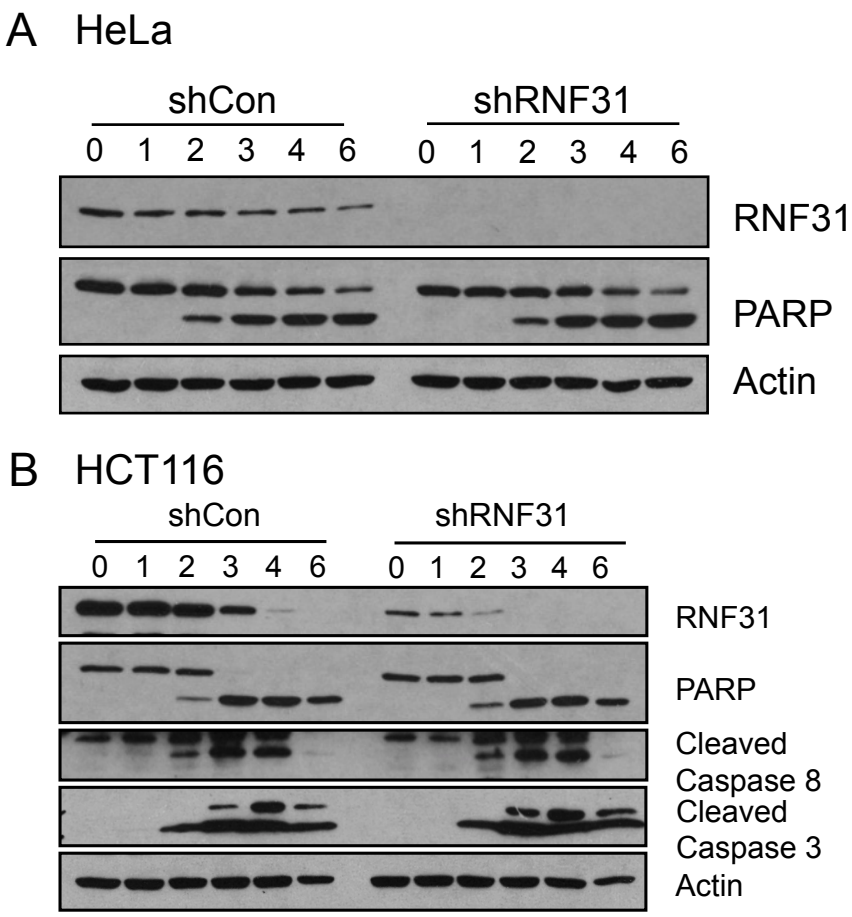


Figure 11 RNF31 silencing did not change the sensitivity of HeLa and HCT116 cells to TRAIL-induced apoptosis.

WB analysis of the indicated proteins in lysates of control and RNF31-silenced HeLa cells (A) or HCT116 (B) treated with TRAIL (200ng/ml).

To exclude the possibility that this sensitization is a result of the HeLa cells' characteristics, I generated four different stable cell lines in which RNF31 was silenced (the pancreatic cancer cell lines BXPC-3 and PANC-1, the lung cancer cell line A549, and the colorectal cancer cell line HCT116) and found that silencing of RNF31 generally sensitized the cells to TNF-α/CHX-induced apoptosis (Figure 12). Consistent with MEFs obtained from chronic proliferative dermatitis (*cpdm*), which harbor an inactivating mutation of *Sharpin*, silencing of Sharpin sensitized HeLa cells to TNF-α/CHX-induced apoptosis, as well (Figure 13). To further confirm the role of RNF31 and its ligase activity in apoptosis, I rescued RNF31 expression in RNF31-silenced HeLa cells using a retroviral system after modifying constructs to be resistant to the shRNA target sequence. TNF-α stimulation did not induce cleavage of caspase 3 or its substrate PARP in these HeLa cells. However, reconstitution using the catalytically dead mutant C885S [197] did not restore the cells' resistance to TNF-α–induced apoptosis, implying that RNF31 indeed regulates apoptosis and that the catalytic activity of RNF31 is critical for regulation of apoptosis by LUBAC (Figure 14A and B). Taken together, these data suggested that LUBAC regulates apoptosis in a catalytic activity-dependent manner.

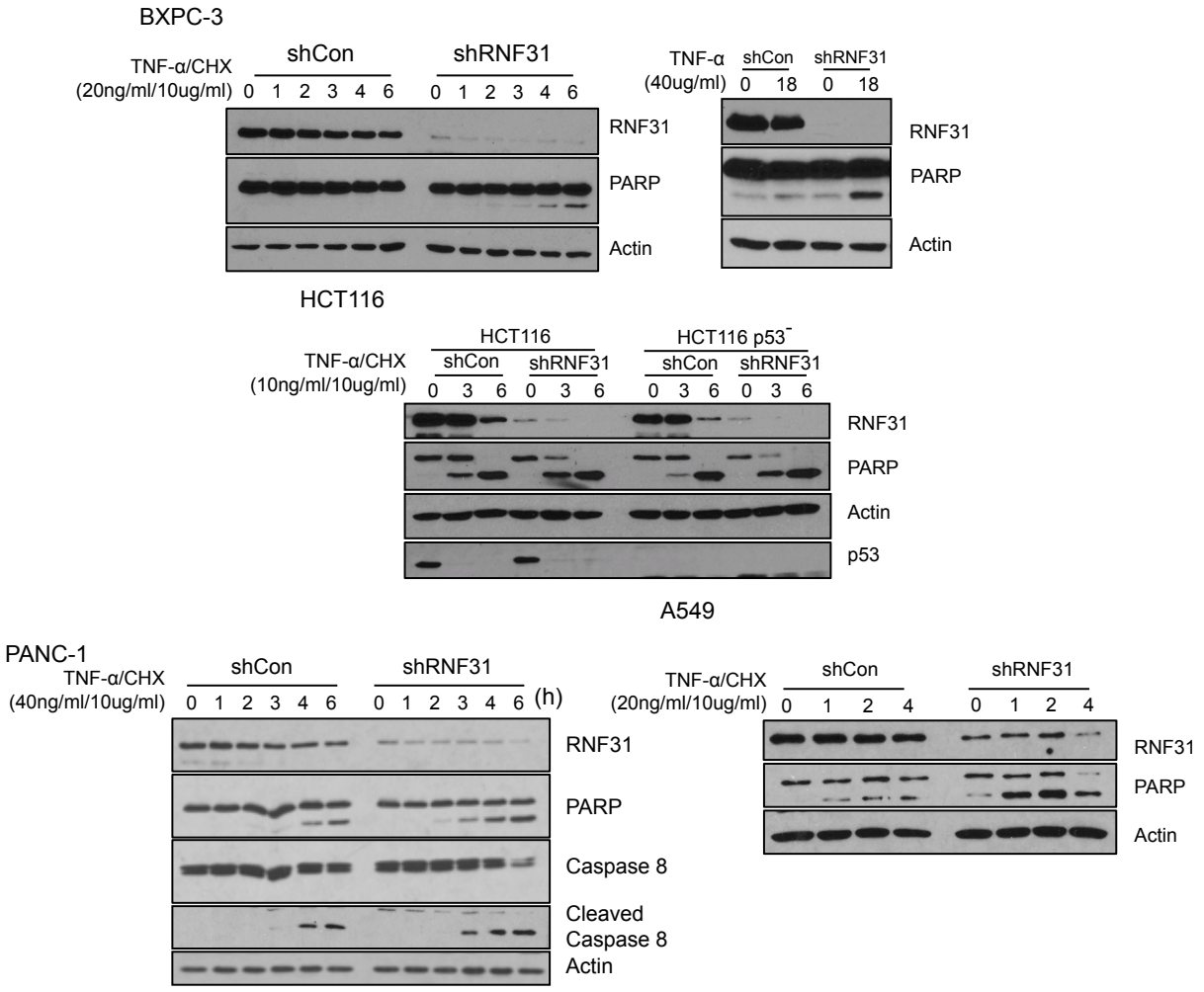


Figure 12 Sensitization of various human cancer cells to TNF-α– and CHX-induced apoptosis via RNF31 silencing

BXPC-3, A549, PANC-1, and WT or $p53^{-/-}$ HCT116 cells were subjected to knockdown using shRNF31), and induction of apoptosis in control and RNF31-silenced cells by treatment with TNF-α and CHX or TNF-α alone was monitored using WB analysis.

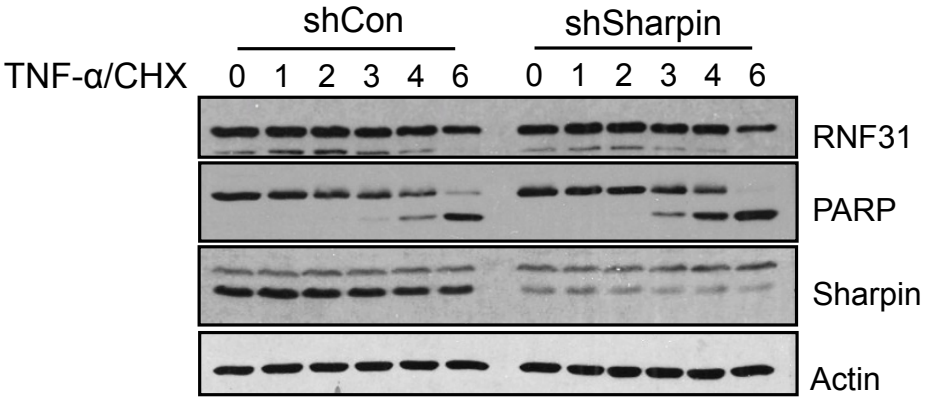


Figure 13 Sharpin silenced cells are more sensitive to TNF-α/CHX-induced apoptosis

WB analysis of the indicated proteins in control and Sharpin-silenced HeLa cells after treatment with TNF-α and CHX (20 ng/ml and 10 μg/ml, respectively) for the indicated periods.

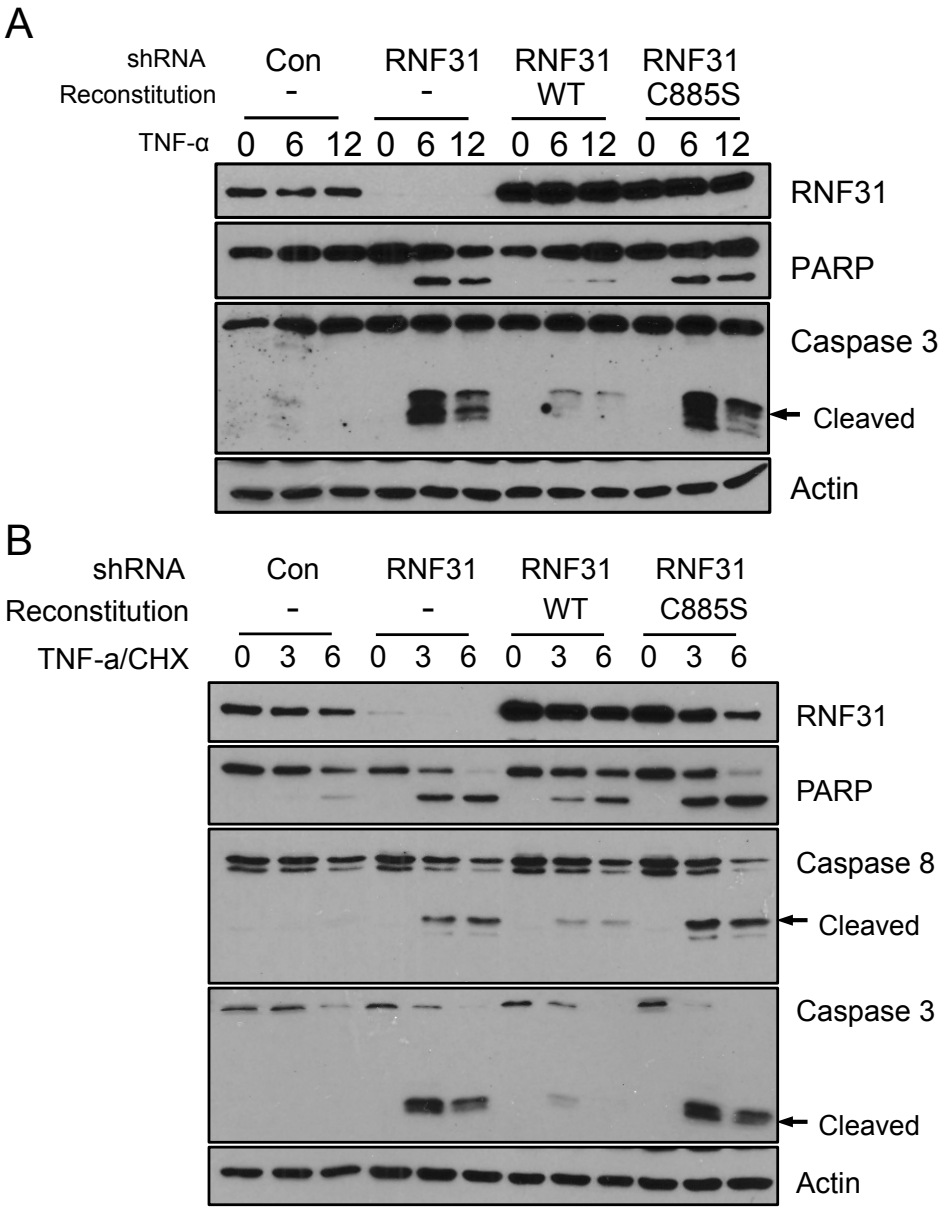


Figure 14 The regulation of apoptosis by RNF31 is dependent on its catalytic activity.

WB assay of the indicated proteins in control, RNF31-silenced, WT RNF31-rescued RNF31-silenced, and C885S RNF31-rescued RNF31-silenced HeLa cells stimulated with or without TNF-α (40 ng/ml) (A) or TNF-α and CHX (B).

4.2.2 Regulation of apoptosis by LUBAC is independent of its role in the NF-κB signaling pathway

Because LUBAC is reported to promote cell survival through activation of the NF-κB pathway that promotes cell survival, a candidate for this sensitization to apoptosis is defective NF-κB activation in RNF31-silenced cells. However, I observed degradation of IκB-α upon treatment with TNF-α and CHX, which indicated that activation of NF-κB is not defective in RNF31-silenced HeLa cells upon activation of the TNF-α pathway (Figure 9A). Moreover, costimulation of CHX with TNF-α prevented the indirect inhibitory effects of NF-κB activation because it blocked new protein synthesis. Based on these data, I hypothesized that sensitization of RNF31-silenced cells is not dependent on the NF-κB signaling pathway. To test this, I examined NF-κB activation in RNF31-silenced A431 and HeLa cells. Upon treatment with 10 ng/ml TNF-α (a level similar to that in our apoptosis experiment described above), the pattern of activation was not significantly different in RNF31-silenced A431 cells from that in control cells. I detected identical levels of TNF receptor 1 in both cells and induction of similar patterns of phosphorylation of TAK1 and IKK α/β. Although delayed, I observed marked degradation of IκB-α in RNF31-silenced cells, as well. Phosphorylation of IκB-α was sustained in silenced cells, resulting from the delayed degradation of total IκB-α (Figure 15).

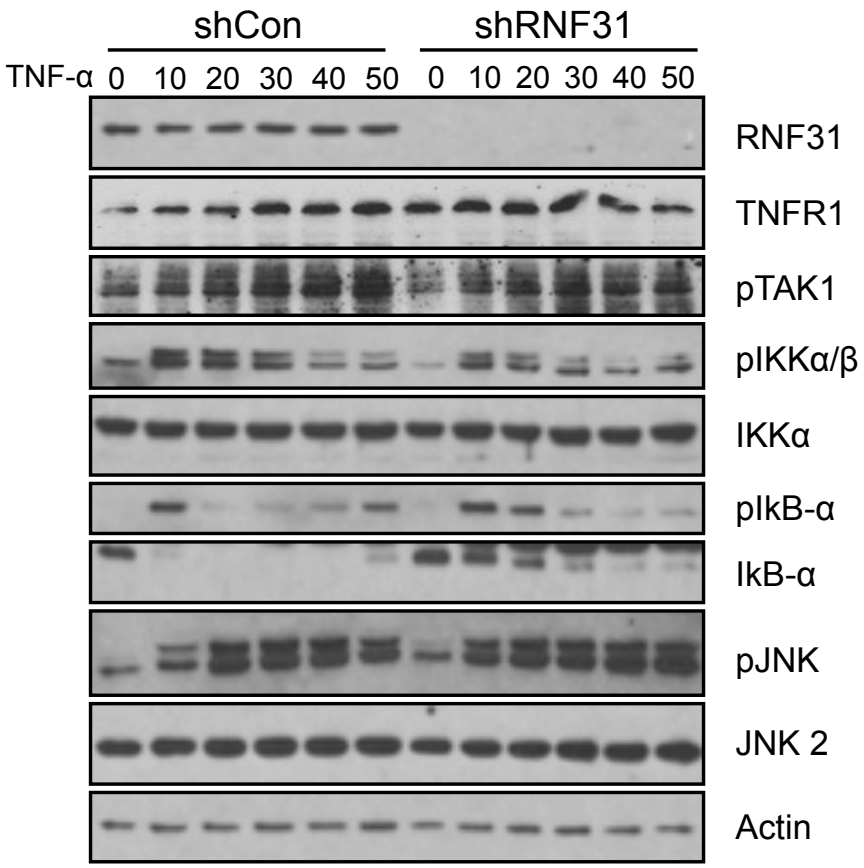


Figure 15 The activation of NF-κB is not defective in RNF31 silenced cells upon activation of TNF-α pathway.

WB analysis of the indicated proteins in control and RNF31-silenced A431 cells after treatment with TNF-α (10 ng/ml).

I further confirmed this activation pattern in RNF31-silenced HeLa cells. Although degradation of IκB-α and translocation of p65 were more significantly suppressed with the low dose of TNF-α (2 ng/ml), the high dose of TNF-α (10 ng/ml), which was similar to the concentration used for apoptosis induction, induced robust activation of NF-κB in RNF31-silenced cells (Figure 16A, B and C). Because dissociation of the p65 complex from IκB-α is the critical process for NF-κB complex to enter into the nucleus, I monitored the interaction between p65 and IκB-α. Similar to activation of the signaling cascade, the profile of degradation was comparable and the associated p65 with IκB-α was consistent with the level of IκB-α in both control and RNF31 silenced HeLa cells, which indicated that dissociation of the p65 from IκB-α was intact in the RNF31-silenced cells (Figure 17). The upstream indicators of NF-κB activation such as the translocation and DNA binding ability of p65 (Figures 18 A and B), and the translocated NF-κB family members, including p65, p50, p52, and c-Rel (Figure 19), demonstrated intact activation of NF-κB signaling upon TNF-α stimulation in RNF31-silenced cells.

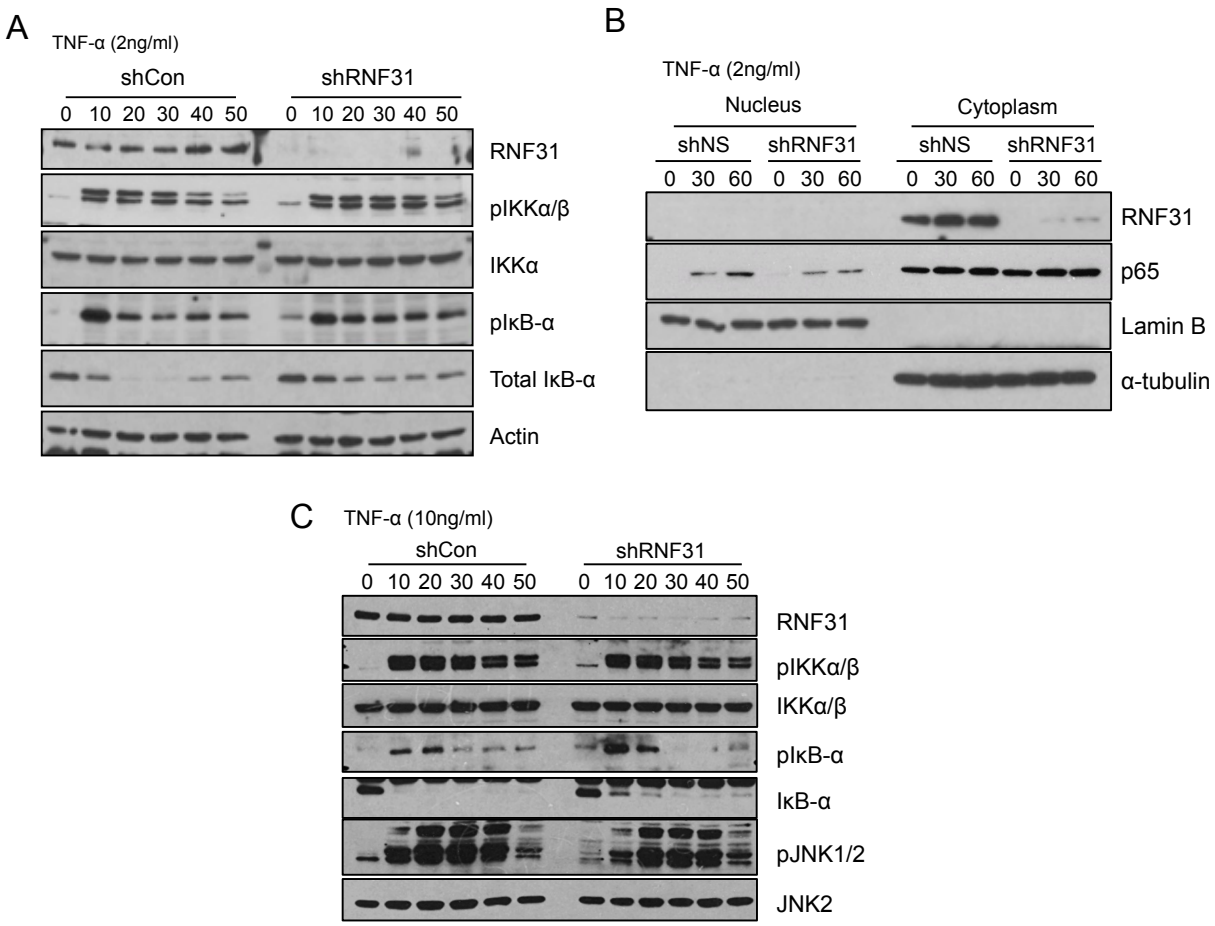


Figure 16 Activation of NF-κB by TNF-α stimulation in RNF31-silenced HeLa cells.

Control and RNF31-silenced HeLa cells were treated with TNF-α at different concentrations (2 ng/ml [A and B] and 10 ng/ml [C]) for the indicated times, and total cell lysates (A and C) and nuclear and cytoplasmic extracts (B) were subjected to WB analysis.

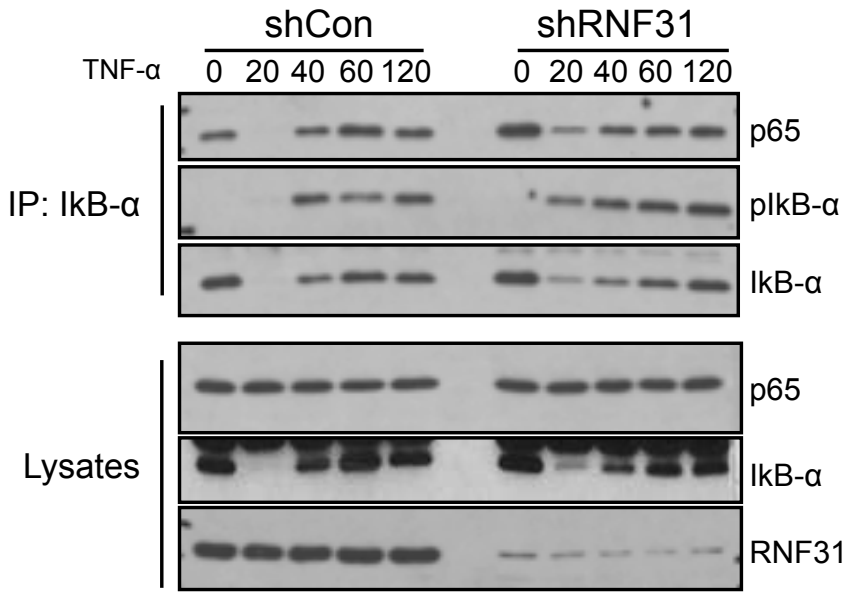


Figure 17 Dissociation of the p65 from IκB-α was intact in the RNF31-silenced cells.

WB analysis of immunoprecipitates from control and RNF31 silenced HeLa cells treated with TNF-α (10 ng/ml) using an anti-IκB-α antibody.

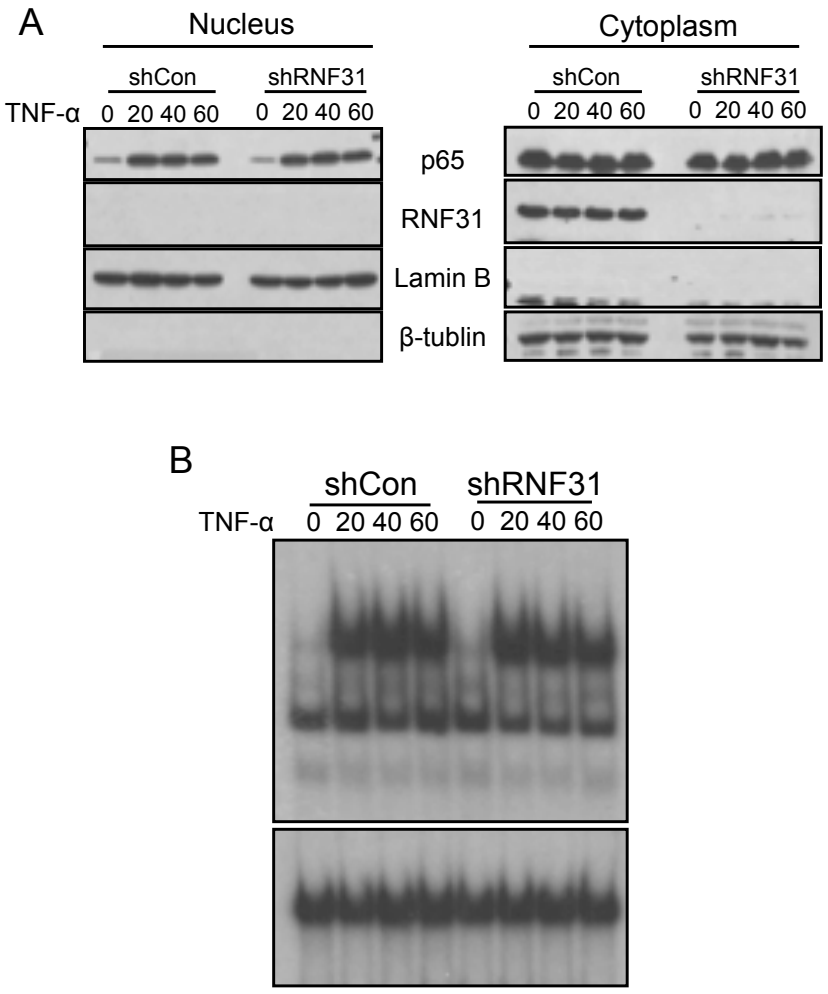


Figure 18 Translocation of p65 and its DNA binding activity were not imparied in RNF31 silenced cells upon activation of TNF-α pathway.

(A) WB analysis of the indicated proteins in nuclear and cytoplasmic extracts from TNF-α–treated (10 ng/ml) control and RNF31 silenced A431 cells. (B) Electrophoretic mobility shift assay of nuclear extracts from TNF-α–treated (10 ng/ml) control and RNF31 silenced A431 cells using NF-κB and OCT1 probes.

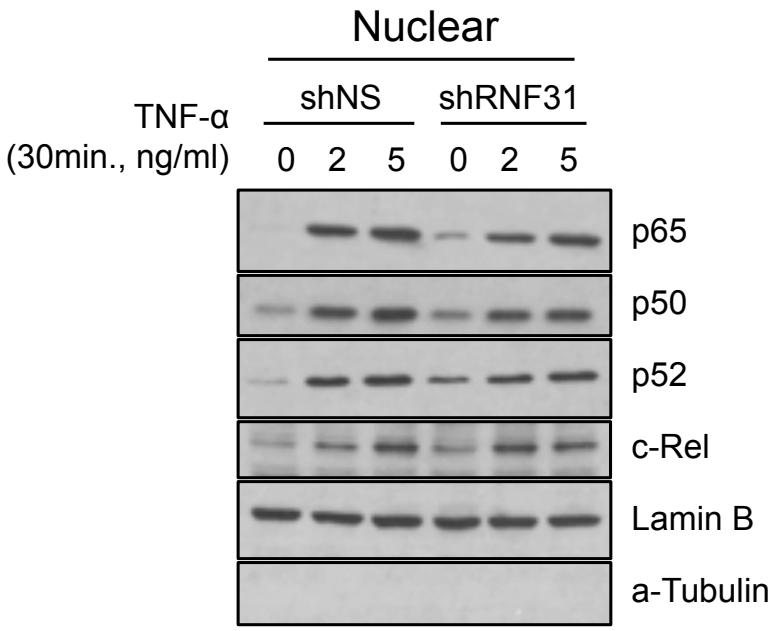


Figure 19 Translocation of NF-κB Family Members into the Nucleus in TNF-α–Stimulated, RNF31-Silenced HeLa cells.

Control and RNF31-silenced HeLa cells were stimulated with TNF-α at the indicated concentrations for 30 min, and WB analysis of nuclear extracts was performed to detect the indicated proteins.

Next, I specifically blocked the NF-κB pathway in HeLa cells to demonstrate that sensitization to TNF-α/CHX-induced apoptosis by RNF31 silencing is independent of NF-κB signaling. To induce apoptosis in HeLa cells, I treated them with CHX to block the expression of TNF-α target genes. However, leaking of NF-κB activation may play a role in apoptosis induction, as CHX is a chemical inhibitor of protein synthesis that does not completely eliminate synthesis. Therefore, delayed NF-κB activation may affect the apoptosis pathway enough to suppress the expression of antiapoptotic molecules in RNF31-silenced cells, leading to sensitization of the cells to apoptosis. To test this possibility, I inhibited translocation of the NF-κB complex in HeLa cells via pretreatment with SN50, a peptide that inhibits p50 translocation [191], because upstream molecules such as IKKs are also involved in the various pathways that regulate apoptosis, so targeting upstream events could bring broad effects not just on the NF-κB signaling pathway Although translocation of p65 was completely blocked by pretreatment with this peptide (Figure 20B), apoptosis was induced by stimulation with TNF-α and CHX at identical time points in both SN50-pretreated and untreated HeLa cells (Figure 20A). In addition, introduction of an IκB-α super repressor into HeLa cells completely blocked the degradation of IκB-α but did not alter the pattern of PARP cleavage upon treatment with TNF-α and CHX (Figure 21). This set of data demonstrates that RNF31 governs apoptosis in an NF-κB signaling-independent manner.

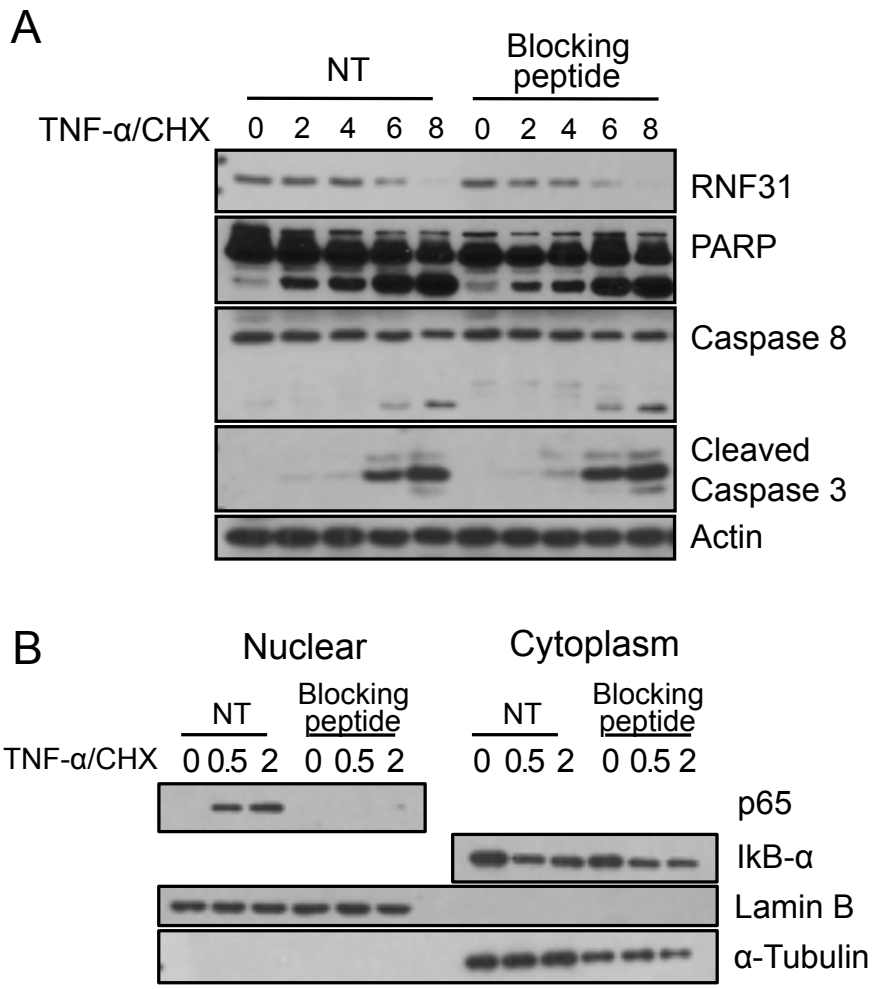


Figure 20 Blocking of p65 translocation did not change the sensitivity of HeLa cells to TNF-α/CHX- induced apoptosis.

WB analysis of lysates (A) and nuclear and cytoplasmic extracts (B) from HeLa cells pretreated with or without an NF-κB–blocking peptide (100 μg/ml) followed by stimulation with TNF-α/CHX (10 ng/ml and 10 μg/ml, respectively).

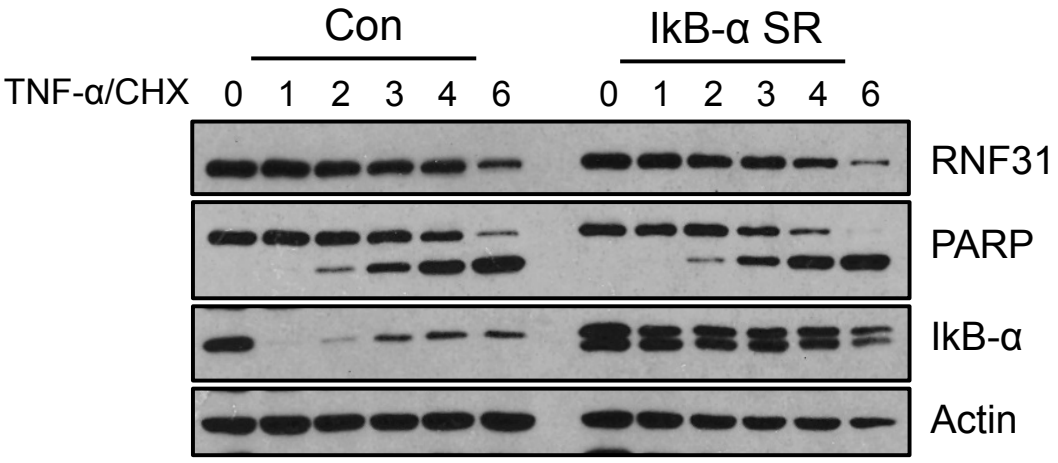


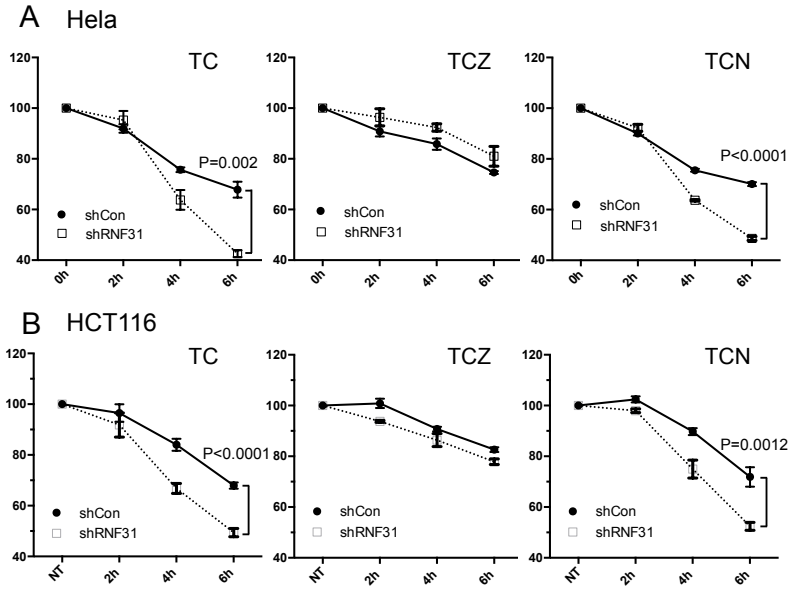
Figure 21 Introduction of IκB-α super repressor did not alter the sensitivity of HeLa cells to TNF-α/CHX- induced apoptosis.

WB analysis of control and IκB-α super repressor-introduced HeLa cells after treatment with TNF-α and CHX (10 ng/ml and 10 μg/ml, respectively).

4.2.3 Silencing of RNF31 mainly sensitizes cells to extrinsic apoptosis

Authors reported that TNF-α is capable of inducing not only apoptosis but also necroptosis, which is programmed necrosis [198]. Therefore, I examined whether RNF31 is involved in TNF-α–induced necroptosis. Active caspase 8 inhibits the induction of necroptosis via cleavage of RIP1 and RIP3. Therefore, I treated HeLa, HCT116, HT29 and HepG2 cells with the pan-caspase inhibitor, Z-VAD-FMK to block apoptosis and activate necroptosis under TNF-α– and CHX-based treatment conditions. I then monitored the cell viability using an MTT assay. Silencing of RNF31 sensitized HeLa and HCT116 cells to TNF-α/CHX-induced cell death, and cotreatment with Z-VAD-FMK inhibited cell death of control and RNF31 silenced cells and abolished this sensitization. However, treatment with Necrostatin-1 (Nec-1), the RIPK 1 inhibitor, which blocks necroptosis, did not significantly alter the sensitivity of RNF31-silenced cells to TNF-α/CHX-induced cell death (Figures 22A and B). Authors also reported that HT29 and HepG2 cells are sensitive to necroptosis upon TNF-α–based treatment [199-201]. Therefore, I treated HT29 and HepG2 cells with TNF-α and CHX together with Z-VAD-FMK and/or Nec-1 to determine the function of RNF31 in the cell death pathways of these cells. I did not observe a significant difference in cell viability between control and RNF31-silenced HT29 and HepG2 cells with and without cotreatment with Z-VAD-FMK and Nec-1 (Figures 23A and B). RNF31 knockdown efficiency in each cell was tested with western blot assay (Figure 24). Next, I examined whether RNF31 regulates the intrinsic apoptosis process, an apoptosis pathway that is activated by intrinsic stimuli, including DNA damage and Endoplasmic reticulum (ER) stress inducers [144]. To activate intrinsic apoptosis, I treated control and RNF31-silenced HeLa and A431 cells with the DNA damage inducer doxorubicin. In both groups of cells, cleavage of PARP was initiated at similar time points irrespective of

RNF31 expression (Figure 25). Taken together, these data demonstrated that RNF31 dominantly regulates extrinsic apoptosis activation.



Relative OD value (± SD)			
		shCon	shRNF31
	NT	100.00	100.00
TNF-a/CHX	2h	91.96 (±1.66)	95.29(±3.64)
	4h	75.71(±0.93)	63.79(±3.88)
	6h	**67.86(±3.14)**	**42.58(±1.33)**
TNF-a/CHX +zVAD	2h	90.82 (±2.02)	96.40(±3.40)
	4h	85.81(±2.24)	92.36(±1.44)
	6h	**74.60(±0.55)**	**81.01(±3.88)**
TNF-a/CHX +Nec	2h	90.01(±0.77)	92.24(±1.37)
	4h	75.53(±0.57)	63.67(±0.30)
	6h	**70.07(±0.78)**	**48.63(±0.79)**

Relative OD Value (± SD)			
		shCon	shRNF31
	NT	100.00	100.00
TNF-a/CHX	2h	96.48 (±3.46)	91.81(±4.78)
	4h	84.00(±2.38)	66.82(±1.98)
	6h	**67.93(±1.26)**	**49.42(±1.62)**
TNF-a/CHX +zVAD	2h	100.86 (±1.83)	93.78(±0.40)
	4h	90.75(±0.90)	86.39(±2.57)
	6h	**82.64(±0.94)**	**77.85(±1.09)**
TNF-a/CHX +Nec-1	2h	102.44 (±1.20)	97.98(±0.72)
	4h	89.74(±1.34)	74.96(±3.55)
	6h	**71.88(±3.83)**	**52.40(±1.58)**

Figure 22 RNF31 silencing altered the sensitivity of HeLa and HCT116 cells to TNF-α/CHX- induced apoptosis, not necroptosis.

MTT analysis of HeLa (A), and HCT116 (B) cells treated with and without TNF-α and CHX (10 ng/ml and 10 μg/ml, respectively); TNF-α, CHX, and Z-VAD-FMK (10 ng/ml, 10 μg/ml, and 10 μM, respectively); TNF-α, CHX, and Nec-1 (10 ng/ml, 10 μg/ml, and 50 μM, respectively); or TNF-α, CHX, Z-VAD-FMK, and Nec-1 (10 ng/ml, 10 μg/ml, 10 μM, and 50 μM, respectively) for the indicated times.

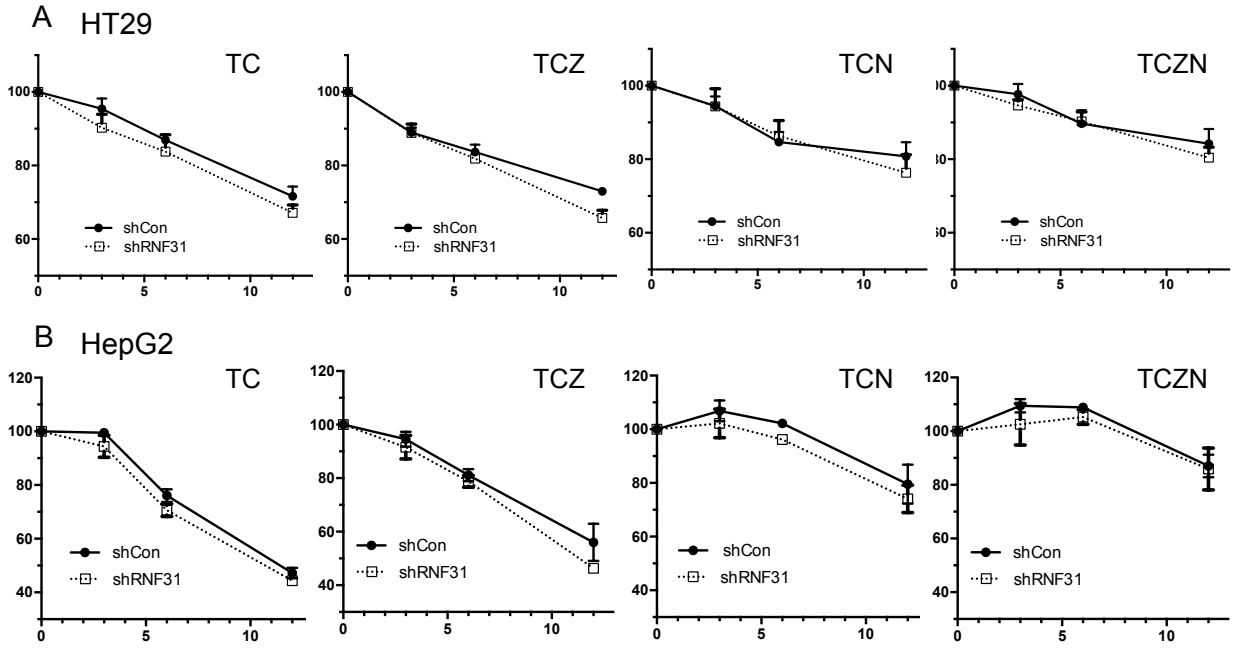


Figure 23 RNF31 silencing did not alter the sensitivity of HT29 and HepG2 cells to TNF-α/CHX- induced cell death.

MTT analysis of HT29 (A), and HepG2 (B) cells treated with and without TNF-α and CHX (10 ng/ml and 10 μg/ml, respectively); TNF-α, CHX, and Z-VAD-FMK (10 ng/ml, 10 μg/ml, and 10 μM, respectively); TNF-α, CHX, and Nec-1 (10 ng/ml, 10 μg/ml, and 50 μM, respectively); or TNF-α, CHX, Z-VAD-FMK, and Nec-1 (10 ng/ml, 10 μg/ml, 10 μM, and 50 μM, respectively) for the indicated times.

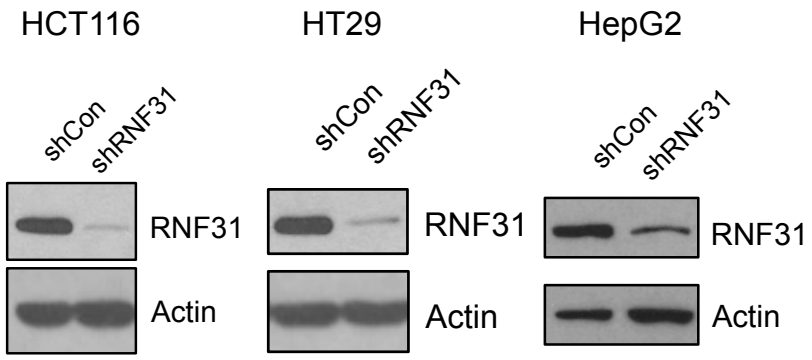


Figure 24 The efficiency of RNF31 knock-down in HCT116, HT29 and HepG2 cells.

WB analysis of the indicated proteins in lysates of control and RNF31-silenced HCT116, HT29 and HepG2 cells.

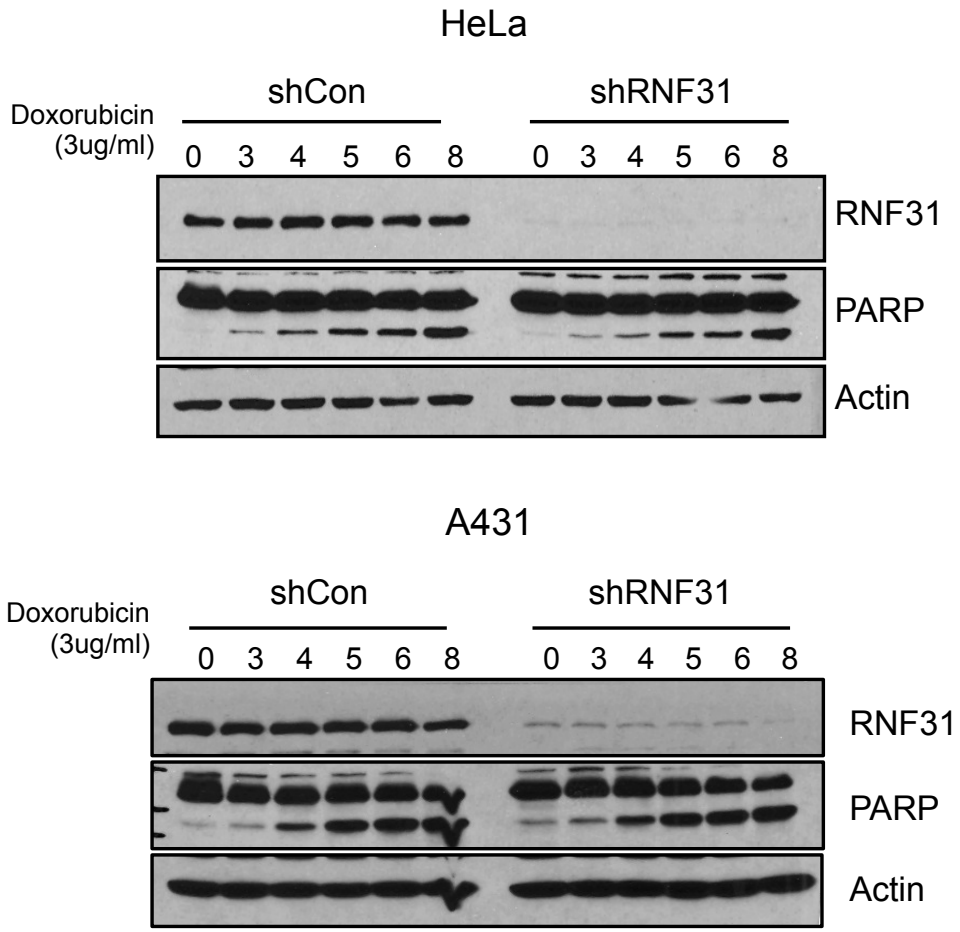


Figure 25 RNF31 silencing did not change the sensitivity to DNA damage induced apoptosis.

WB analysis of lysates of control and RNF31 silenced HeLa and A431 cells treated with doxorubicin (3 μg/ml) for the indicated periods.

4.2.4 LUBAC inhibits apoptosis via stabilization of cFLIP in a linear ubiquitination-dependent manner

I then questioned how RNF31 controls the apoptosis pathway. To elucidate the mechanism of apoptosis regulation, I studied the levels of apoptosis-related molecules in TNF-α treated HeLa cells. Since the protein synthesis was blocked by treatment with CHX, I excluded the possibility that induction of proapoptotic proteins is the reason for this phenomenon. Furthermore, authors reported that treatment with the proteasome inhibitor bortezomib reduced the severity of skin problems in Sharpin-deficient mice [202]. Therefore, I profiled the diminution of antiapoptotic molecules in TNF-α/CHX-treated HeLa cells. Although RNF31 silencing did not alter the level of these molecules, including BCL2 family proteins (BCL_{xL}, BCL2, and NOXA) and cIAP1/2 (Figure 26), I found that the level of cFLIP decreased markedly in RNF31-silenced HeLa cells upon treatment with TNF-α and CHX (Figure 27A). To determine whether the rapid decrease in cFLIP in RNF31-silenced cells was dependent on activation of TNF-α signaling, I treated control and RNF31-silenced HeLa cells with CHX only. Additionally, I pretreated them with Z-VAD-FMK or the proteasome inhibitor MG132 to determine whether the decrease in cFLIP resulted from caspase activity or occurred in a degradation-dependent manner. I observed a similar pattern of decreased cFLIP in control and RNF31-silenced cells upon CHX-based treatment, indicating that RNF31 silencing did not alter the basal turnover rate for cFLIP (Figure 27B). Moreover, the pretreatment with Z-VAD-FMK inhibited cleavage of PARP but did not restore level of cFLIP. However, MG132-based pretreatment completely blocked the decrease in cFLIP, cleavage of PARP, and degradation of IκB-α (Figure 28A), suggesting that the TNF-α/CHX-induced decrease in cFLIP depended on proteasome-dependent degradation. To confirm the role of degradation process in the increased sensitivity of RNF31-silenced cells to apoptosis, I pretreated control and RNF31-silenced HeLa

with MG132 and then induced apoptosis with treatment with TNF-α and CHX. As observed in Figure 28A, pretreatment of control HeLa with MG132 completely blocked decrease of cFLIP and cleavage of cFLIP. Moreover, I observed that silencing of RNF31 sensitized HeLa cells to TNF-α/CHX-induced apoptosis. However, this sensitized responses and even apoptosis induction of RNF31-silenced cells were completely abolished by pretreatment with MG132 (Figure 28B), indicating that sensitization of cells to apoptosis by RNF31 silencing is mediated by the proteasome-dependent degradation pathway.

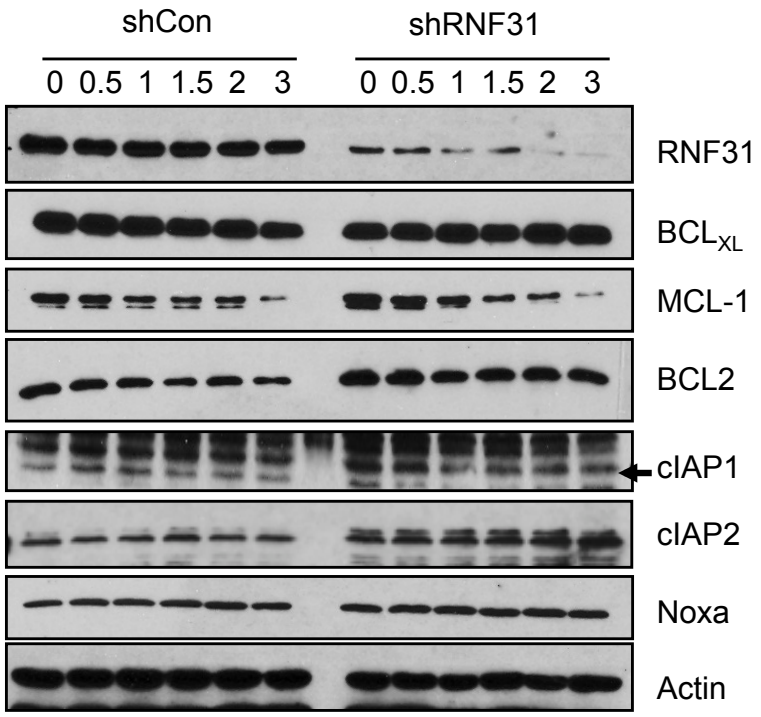


Figure 26 Patterns of BCL2 family proteins and cIAP1/2 in RNF31-silenced HeLa cells.

Control and RNF31-silenced HeLa cells were stimulated with TNF-α and CHX (10 ng/ml and 10 μg/ml, respectively), and the indicated proteins in the cells were analyzed using Western blotting.

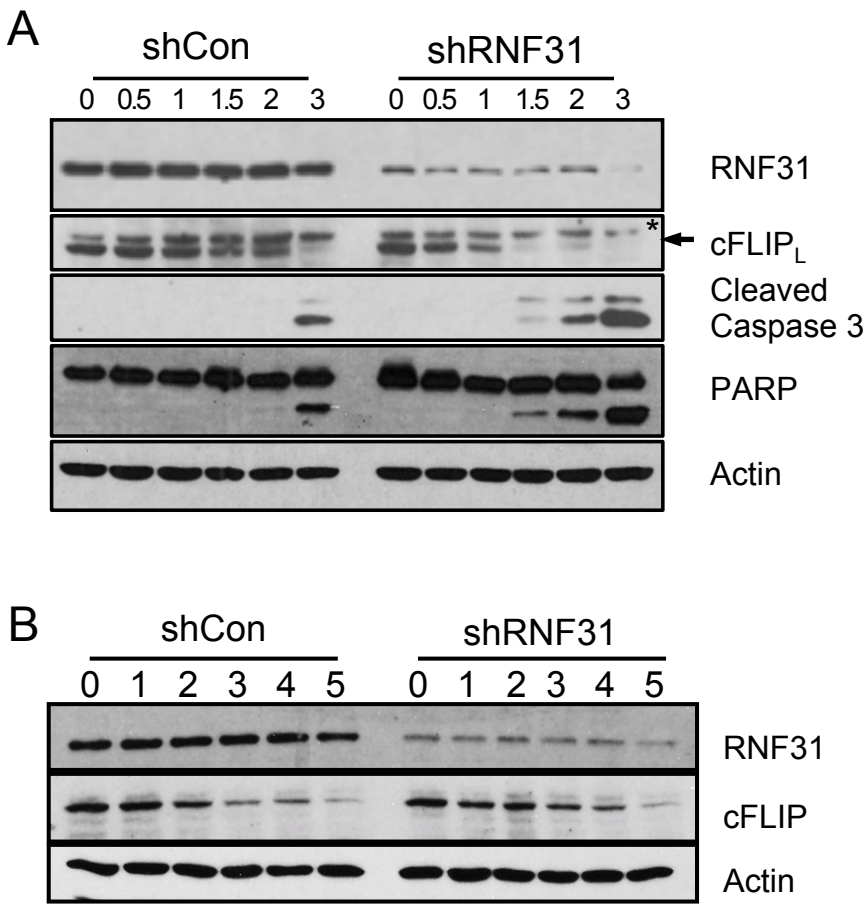


Figure 27 Silencing of RNF31 accelerates cFLIP degradation upon TNF-α/CHX treatment but did not alter the basal turnover of cFLIP.

(A) WB analysis of control and RNF31 silenced HeLa cells after treatment with TNF-α and CHX (10 ng/ml and 10 μg/ml, respectively) for the indicated times. *Nonspecific band. (B) WB analysis of lysates of CHX-treated (20 μg/ml) control and RNF31 silenced HeLa cells.

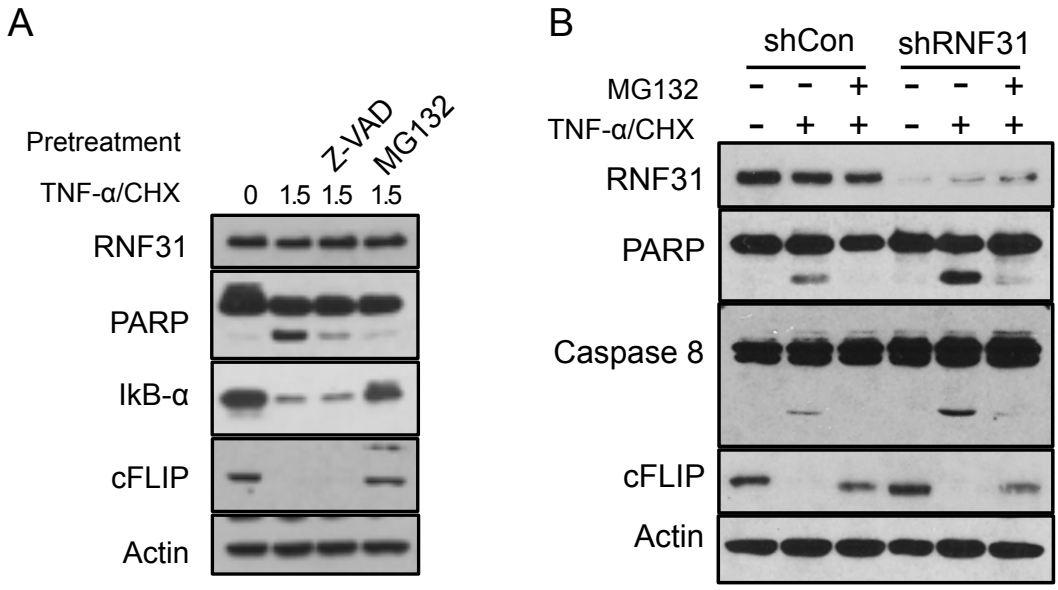


Figure 28 Pretreatment with MG132 prevents the decrease of cFLIP upon TNF-α/CHX treatment and induction of apoptosis in control and RNF31 silenced HeLa cells.

(A) WB analysis of HeLa cells pretreated with Z-VAD-FMK (10 μM) or MG132 (20 μM) and then stimulated with TNF-α and CHX (40 ng/ml and 10 μg/ml, respectively) for the indicated periods. (B) WB analysis of control and RNF31-silenced HeLa cells pretreated with Z-VAD-FMK (10 μM) and then stimulated with TNF-α and CHX (40 ng/ml and 10 μg/ml, respectively) for the indicated periods.

cFLIP is an antiapoptotic molecule that forms a complex with caspase 8 and suppresses its protease activity [203]. Because authors reported that the TNF-α/c-Jun N-terminal kinase (JNK)/ITCH signaling axis regulates cFLIP turnover [204], I sought to determine whether this axis explains our observation of sensitization by RNF31 silencing. Pretreatment of HeLa cells with the JNK inhibitor SP600125 markedly blocked the phosphorylation of c-Jun induced by treatment with TNF-α and CHX, but cFLIP still degraded in 75 min, indicating that JNK activity is not critical for cFLIP stability (Figure 29). Moreover, the level of ITCH was remained unchanged upon treatment with TNF-α and CHX in both control and RNF31-silenced HeLa cells for up to 3 hr, although PARP cleavage and decreased cFLIP were triggered earlier in RNF31-silenced cells than in control cells (Figure 30A). Additionally, knockdown of ITCH expression in HeLa cells did not change the cFLIP level upon treatment with TNF-α and CHX (Figure 30B), implying that TNF-α/JNK/ITCH is not a mechanism of RNF31 dependent sensitization to apoptosis.

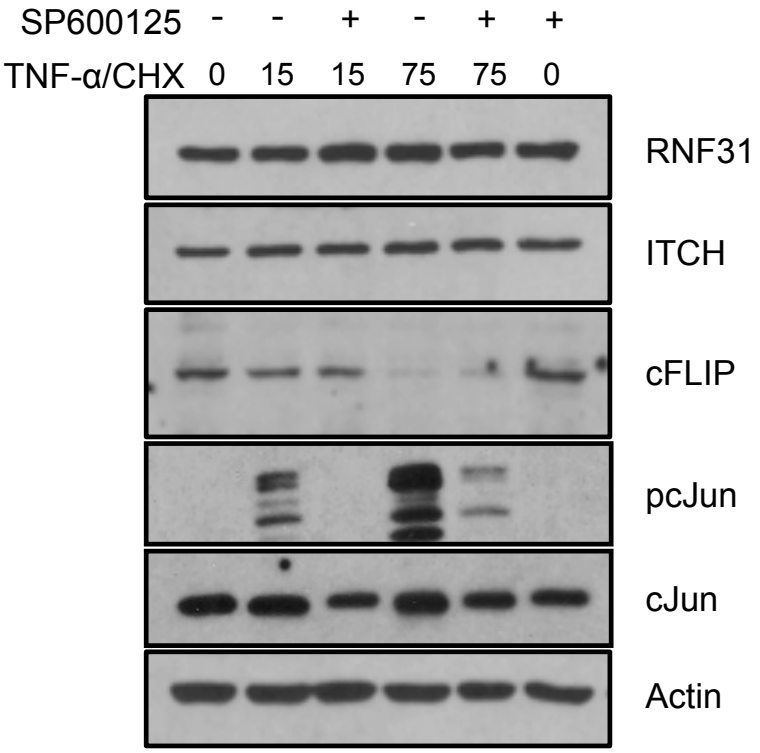


Figure 29 Levels of cFLIP in TNF-α and CHX-Treated HeLa Cells with and without Pretreatment with the JNK Inhibitor SP600125.

HeLa cells were pretreated with SP600125 (10 μM) for 1 hr and stimulated with TNF-α and CHX (10 ng/ml and 10 μg/ml, respectively) for the indicated times (min). The level of each protein was measured using Western blotting with the indicated antibodies.

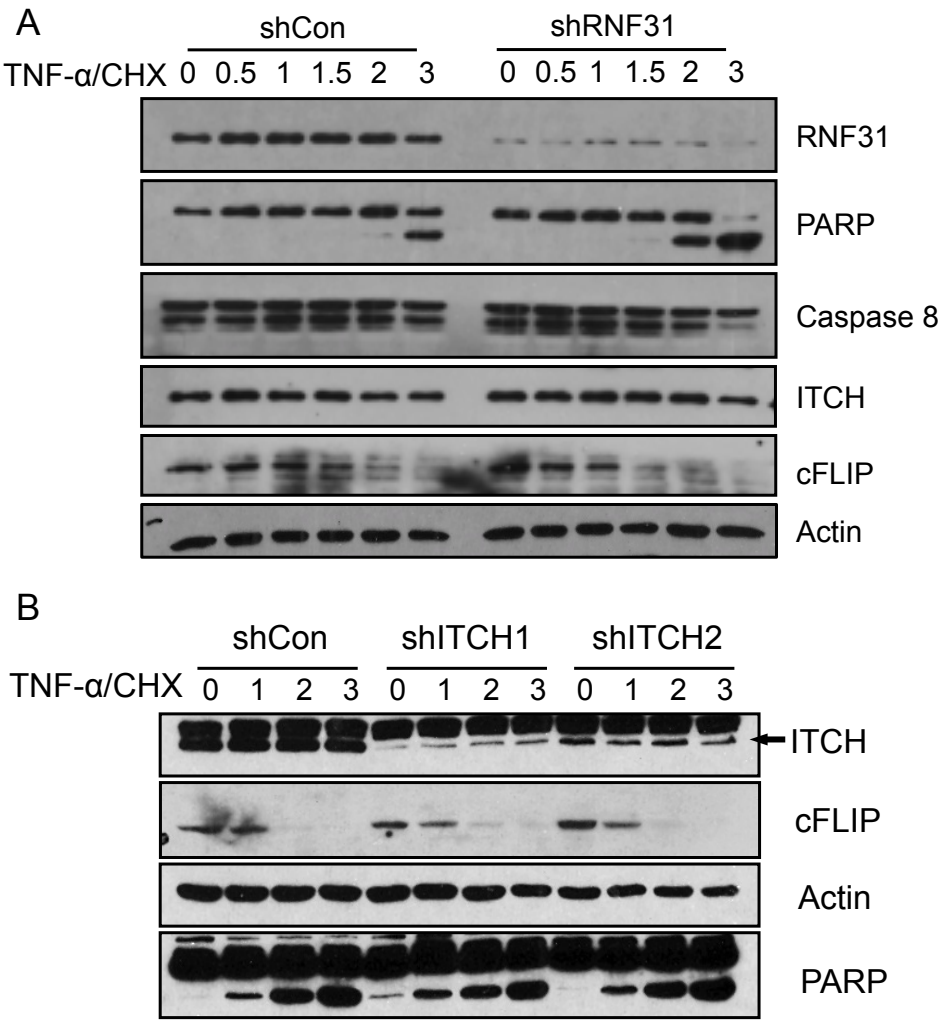


Figure 30 Levels of ITCH in TNF-α and CHX-treated HeLa cells and comparable induction of apoptosis in ITCH-silenced HeLa cells.

(A) Control and RNF31-silenced HeLa cells were stimulated with TNF-α and CHX for the indicated times, and WB analysis was performed to monitor the level of the indicated proteins. (B) Control and ITCH-silenced HeLa cells (using shRNA constructs targeting different sequences in cFLIP) were treated with TNF-α and CHX (10 ng/ml and 10 μg/ml, respectively) for the indicated time points, and total lysates were subjected to a WB assay to monitor the indicated proteins.

Next, I examined whether RNF31 directly regulates the stability of cFLIP. I induced the expression of Myc-tagged RNF31 together with FLAG-tagged cFLIP in 293T cells and FLAG-tagged cFLIP was immunoprecipitated. This coimmunoprecipitation experiment demonstrated that RNF31 binds with cFLIP (Figure 31A). Moreover, stimulation of HeLa cells stably expressing FLAG-tagged cFLIP with TNF-α led to inducible interaction between cFLIP and LUBAC components, including RNF31 and Sharpin (Figure 31). I then performed an *in vitro* ubiquitination assay to determine whether cFLIP is a novel substrate of LUBAC. I incubated recombinant cFLIP with and without recombinant LUBAC, E1, E2, and lysine KO ubiquitin (all lysines are mutated to arginine) and detected linear ubiquitination of cFLIP with antibodies against cFLIP and linear ubiquitination. Although LUBAC generated linear ubiquitination regardless of the substrate, immunoblotting with a cFLIP-specific antibody illustrated that cFLIP is specifically ubiquitinated in a linear linkage manner (Figure 32). In addition, expression of LUBAC particularly promoted linear ubiquitination of cFLIP as well as the known substrate RIP1 in 293T cells (Figure 33).

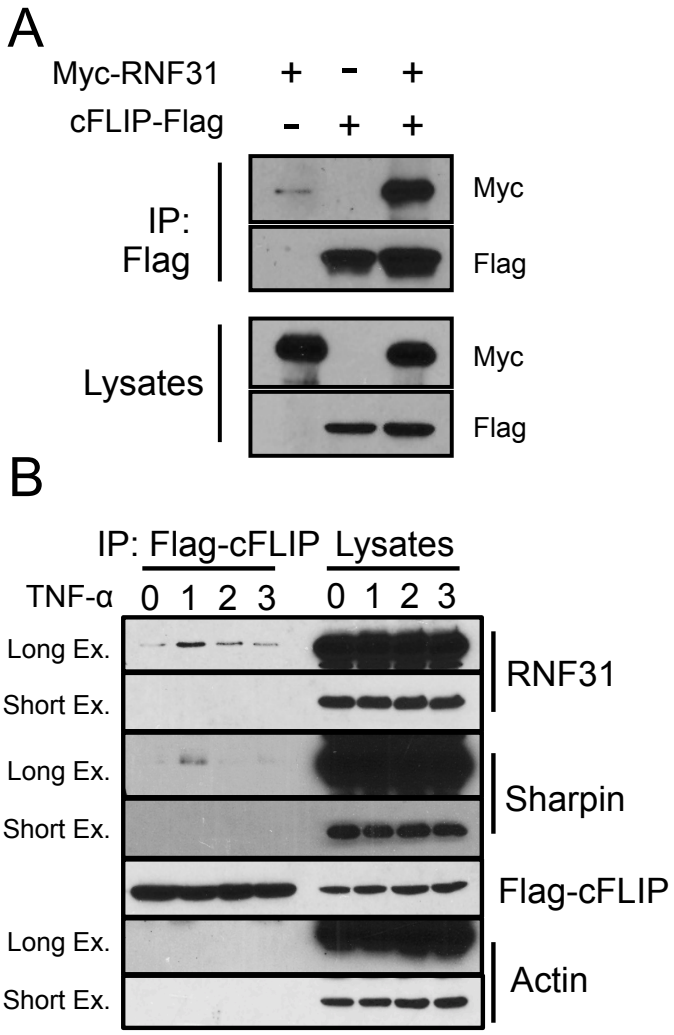


Figure 31 LUBAC binds to cFLIP under both overexpresion and endogenous conditions.

(A) WB analysis of immunoprecipitates from 293T cells transiently transfected with the indicated plasmids using anti-FLAG beads. (B) WB analysis of immunoprecipitates from HeLa cells stably expressing FLAG-cFLIP after stimulation with TNF-α (20 ng/ml) for the indicated times using anti-FLAG beads.

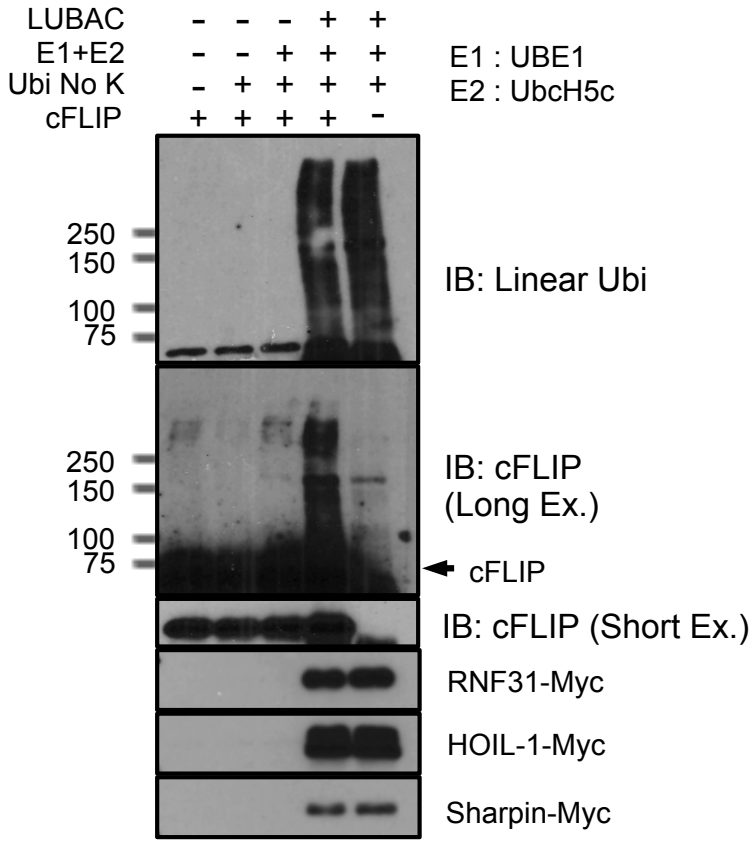


Figure 32 cFLIP is conjugated with linear ubiquitination chains by LUBAC *in vitro*.

Results of an *in vitro* ubiquitination assay in which FLAG-cFLIP was incubated with purified myc-LUBAC, E1, E2, and lysine KO ubiquitin followed by immunoprecipitation with anti-FLAG beads and WB analysis.

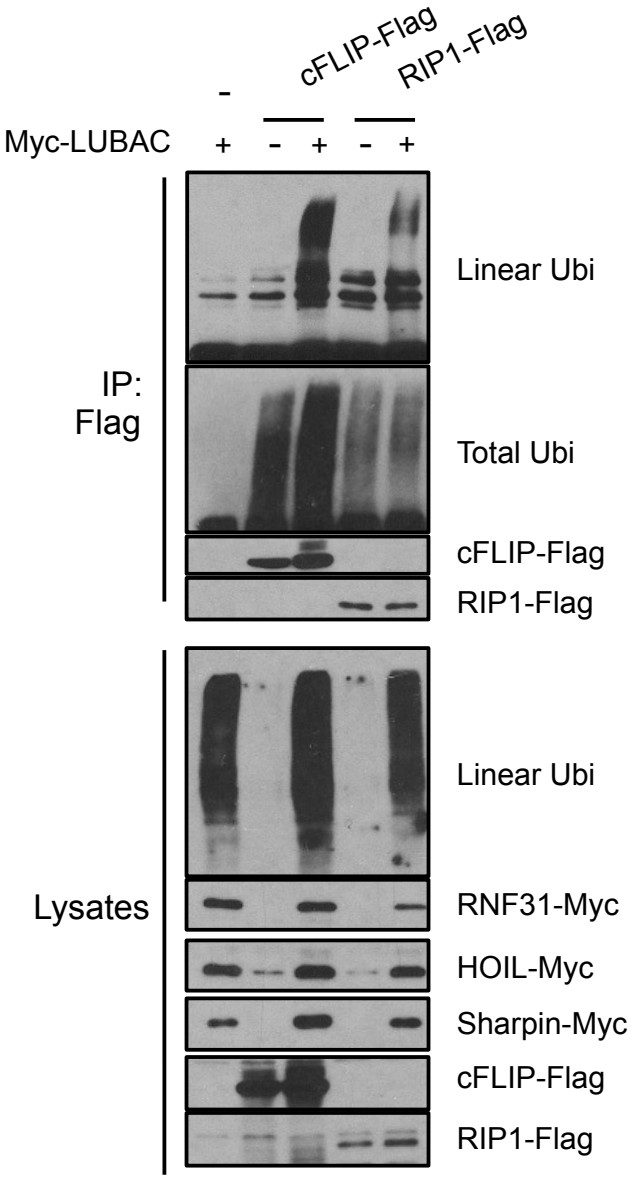


Figure 33 cFLIP is conjugated with linear ubiquitination chains by LUBAC.

WB analysis of immunoprecipitates from 293T cells transiently transfected with the indicated constructs using anti-FLAG beads.

Because cFLIP degraded rapidly in RNF31-silenced cells, I hypothesized that linear ubiquitination stabilizes cFLIP by competing with K-48 ubiquitination, which suppresses proteasome-dependent degradation. To test this hypothesis, I introduced FLAG tagged cFLIP with and without the three LUBAC components into 293T cells and analyzed the pattern of cFLIP ubiquitination. Expression of all three components specifically generated linear ubiquitination of cFLIP. However, their expression led to lower K-48 ubiquitination of cFLIP than did expression of only HOIL-1 and Sharpin. Additionally, expression of RNF31 with containing the catalytically dead mutation C885S along with HOIL-1 and Sharpin, which failed to generate the linear forms of ubiquitin linkage, restored K-48 ubiquitination of cFLIP (Figure 34). To confirm regulation of cFLIP ubiquitination by LUBAC, I examined the K-48 ubiquitination of cFLIP in control and RNF31-silenced HeLa cells introduced with FLAG-cFLIP and observed that silencing of RNF31 promoted K-48 ubiquitination of cFLIP at the endogenous level upon treatment with TNF-α and CHX (Figure 35).

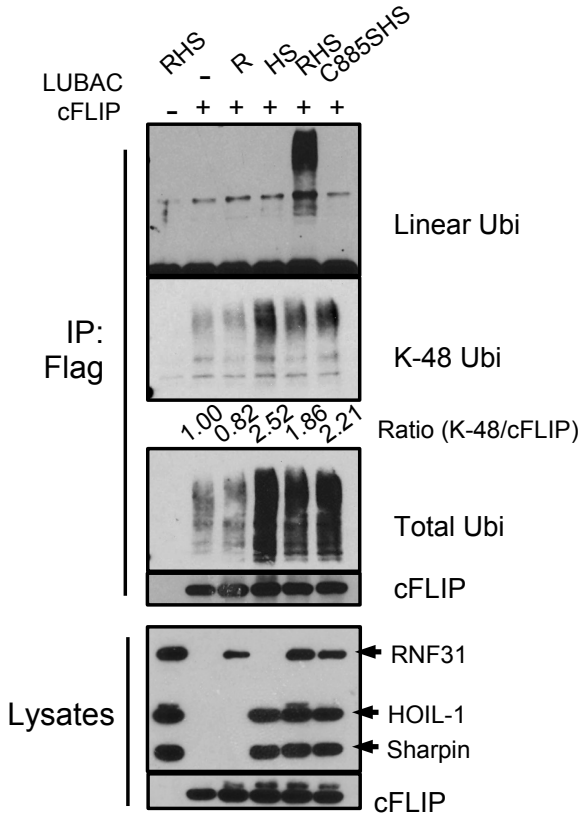


Figure 34 Induction of linear ubiqutination inhibits K-48 ubiquitianion of cFLIP.

WB analysis of immunoprecipitates from 293T cells transfected with FLAG-cFLIP and the indicated LUBAC components using anti-FLAG beads. RHS: RNF31, HOIL-1, and Sharpin; R: RNF31, HS: HOIL-1 and Sharpin; C885SHS: C885S RNF31, HOIL-1, and Sharpin.

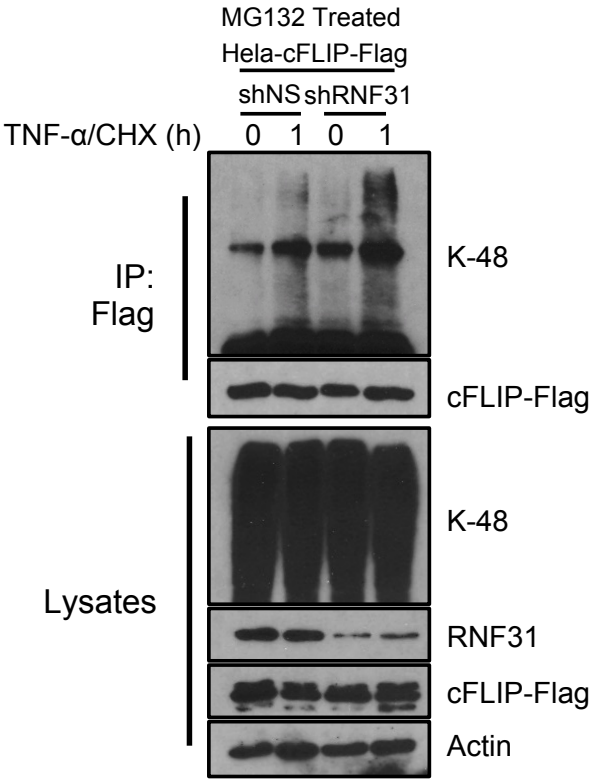


Figure 35 RNF31 silencing increases K-48 ubiquitination of cFLIP upon TNF-α and CHX.

WB analysis of immunoprecipitates from lysates of control and RNF31 silenced HeLa cells stably expressing FLAG-cFLIP after pretreatment with MG132 followed by treatment with TNF-α and CHX (40 ng/ml and 10 μg/ml, respectively) for 1 hr using anti-FLAG beads.

4.2.5 Lysine 49 in Death Effector Domain 1 of cFLIP Is a Target of Linear Ubiquitination

RNF31 and cFLIP are composed of multiple domains (Figure 36A). Therefore, I next examined which domains in cFLIP and RNF31 are responsible for their interaction with each other. The expression of full-length cFLIP along with RNF31 domains indicated that the RING1-in between ring-RING2 (RBR) domain of RNF31 is essential for binding with cFLIP (Figure 36B). Furthermore, a coimmunoprecipitation experiment with full-length RNF31 and constructs encoding cFLIP domains demonstrated that death effector domain 1 (DED1) of cFLIP is critical for the interaction between RNF31 and cFLIP (Figure 36C), suggesting that the RBR domains of RNF31 and DED1 of cFLIP are responsible for their binding. DED1 of cFLIP contains lysines 18, 49, 69, 73, and 77 (Figure 37A). Hence, I generated cFLIP mutants in which each lysine is mutated to arginine, and I monitored their ability to be conjugated with the linear form of ubiquitin linkage. I induced expression of each mutant together with LUBAC components in 293T cells and immunoprecipitated them. Western blotting of the precipitated with a linear ubiquitination-specific antibody demonstrated that lysine 49 of cFLIP is the target residue for linear ubiquitination (Figure 37B). Next, I genetically deleted cFLIP from HeLa cells using the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system (Figure 38) [187] and reconstituted deleted cFLIP with WT or K49R cFLIP. I then monitored the level of K-48 ubiquitination and sensitivity of the cells to TNF-α– and CHX-induced apoptosis to confirm the biological significance of this target site. First, I observed greater K-48 ubiquitination of K49R cFLIP than of WT cFLIP in HeLa cells upon treatment with TNF-α (Figure 39). Moreover, I observed cleaved bands of apoptosis indicators, including PARP, caspase 8, and caspase 3, earlier in K49R cFLIP-rescued HeLa cells than in WT cFLIP-rescued cells upon stimulation with TNF-α and CHX. Consistent with the hypothesis, the level of K49R cFLIP decreased faster than did WT cFLIP upon treatment with TNF-α and CHX (Figure 40), indicating that

lysine 49 plays a role in degradation of cFLIP and sensitivity to TNF-α– and CHX-induced apoptosis.

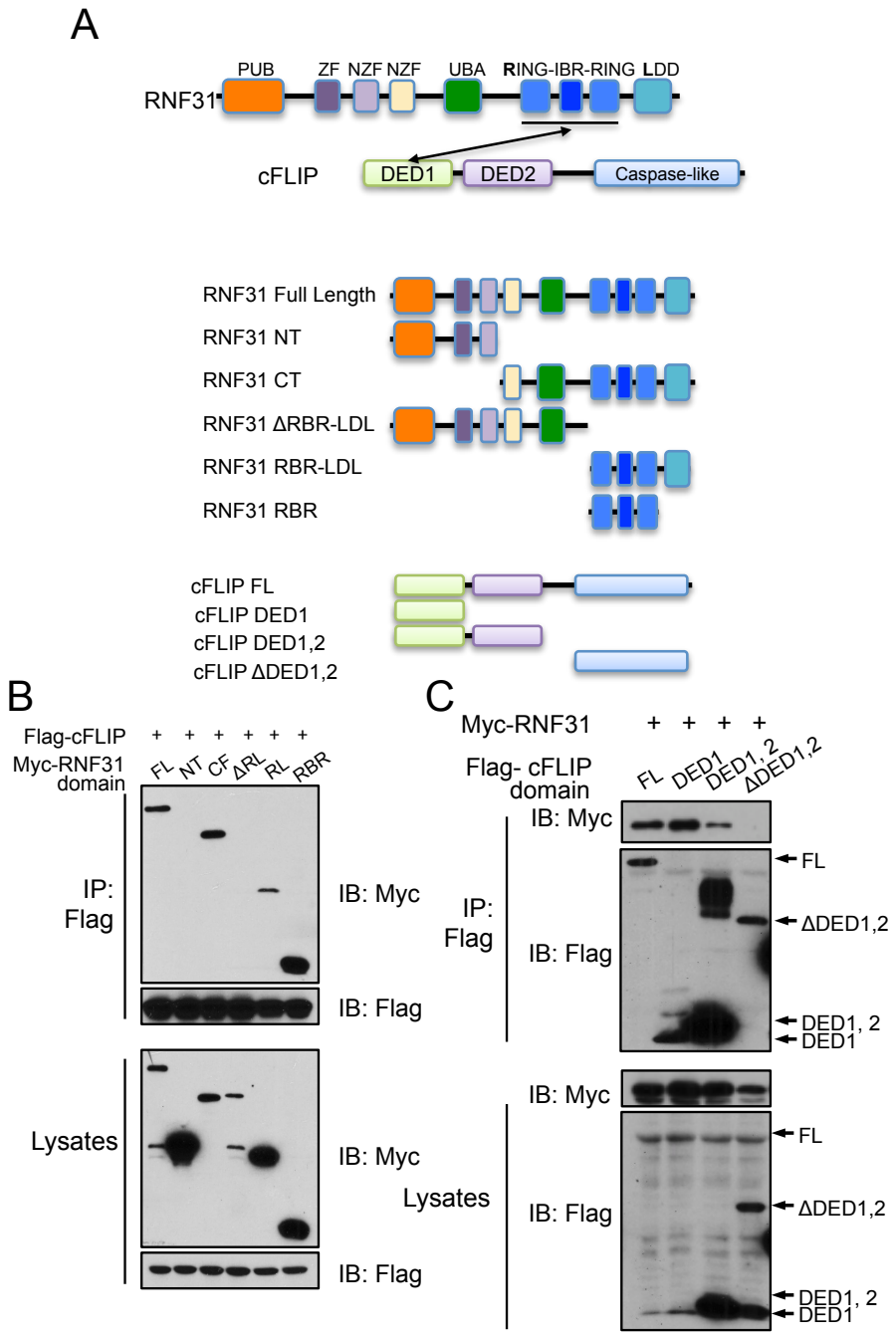


Figure 36 RNF31 and cFLIP binds through RBR and DED1 interaction.

(A) Schematic of domains of RNF31 and cFLIP and their truncated mutants. (B and C) WB analysis of immunoprecipitates using anti-FLAG beads and total lysates of 293T cells transfected with the plasmids encoding the indicated domains. FL: full length, NT: N-terminal fragment, CT: C-terminal fragment, ΔRL: RBR-LDL domain-deleted fragment, RL: RBR-LDL domain, RBR: RBR domain.

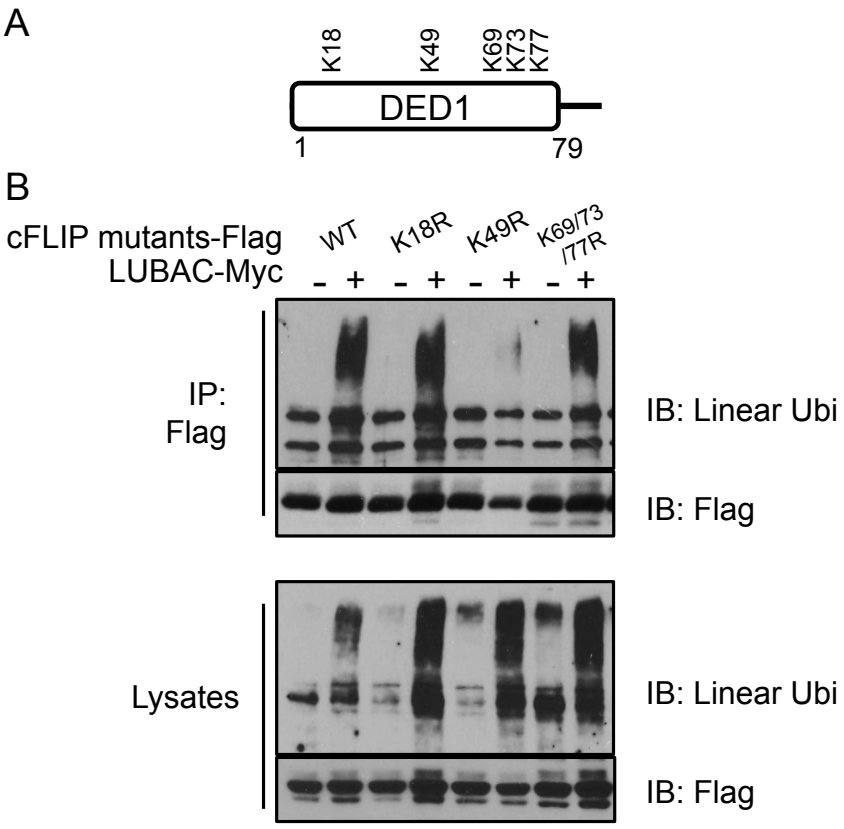


Figure 37 Lysine 49 of cFLIP is the residue for linear ubiquitination.

(A) Lysines in the DED1 of cFLIP. (B) WB analysis of immunoprecipitates using anti-FLAG beads and lysates of 293T cells transfected with the indicated constructs.

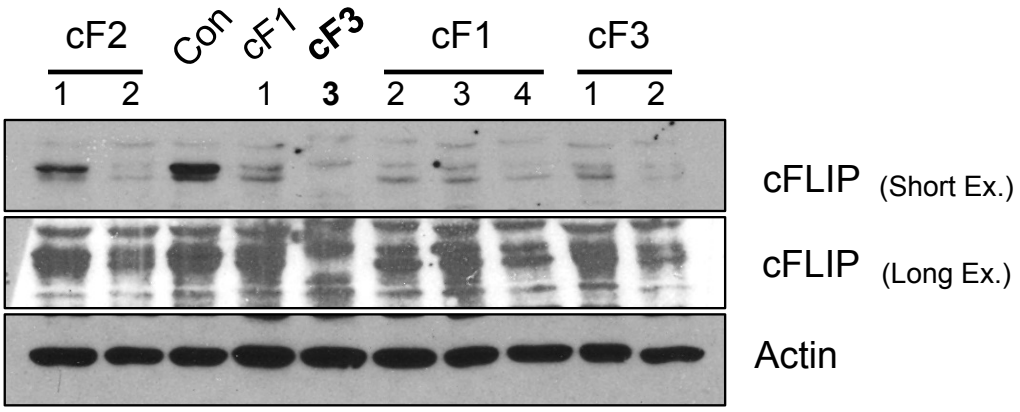


Figure 38 Deletion of cFLIP in HeLa cells using the CRISPR system.

HeLa cells were subjected to deletion of the *cFLIP* gene using the CRISPR system, and infected cells with CRISPR virus were selected using puromycin (2 μg/ml). After isolation of single cells via serial dilution, colonies that arose from single cells were subjected to WB analysis to monitor the expression of cFLIP.

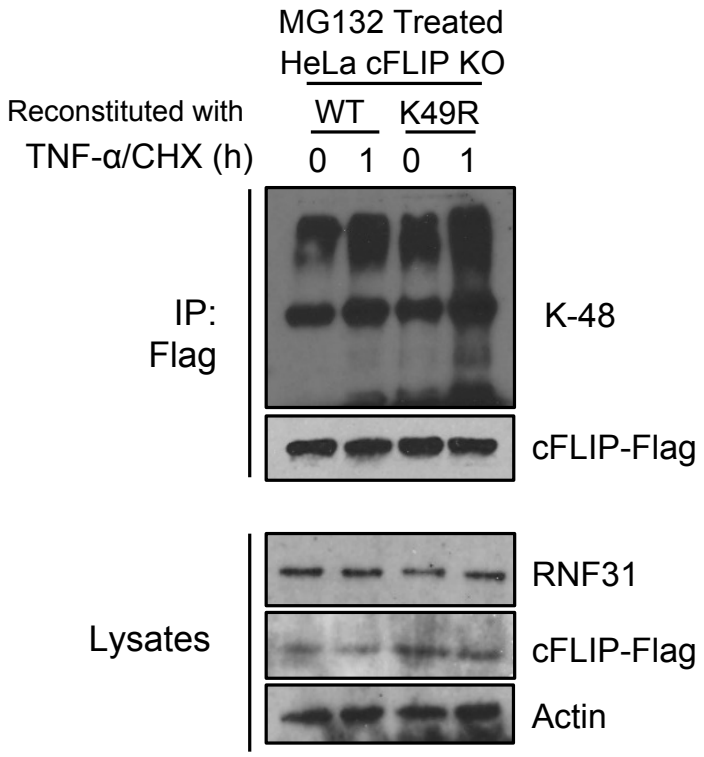


Figure 39 Recontitution with K49R cFLIP increases K-48 ubiquitination of cFLIP upon TNF-α and CHX.

WB analysis of immunoprecipitates using anti-FLAG beads and lysates of HeLa stably expressing FLAG-WT cFLIP or FLAG-K49R cFLIP after treatment with TNF-α and CHX (40 ng/ml and 10 μg/ml, respectively) for 1 hour.

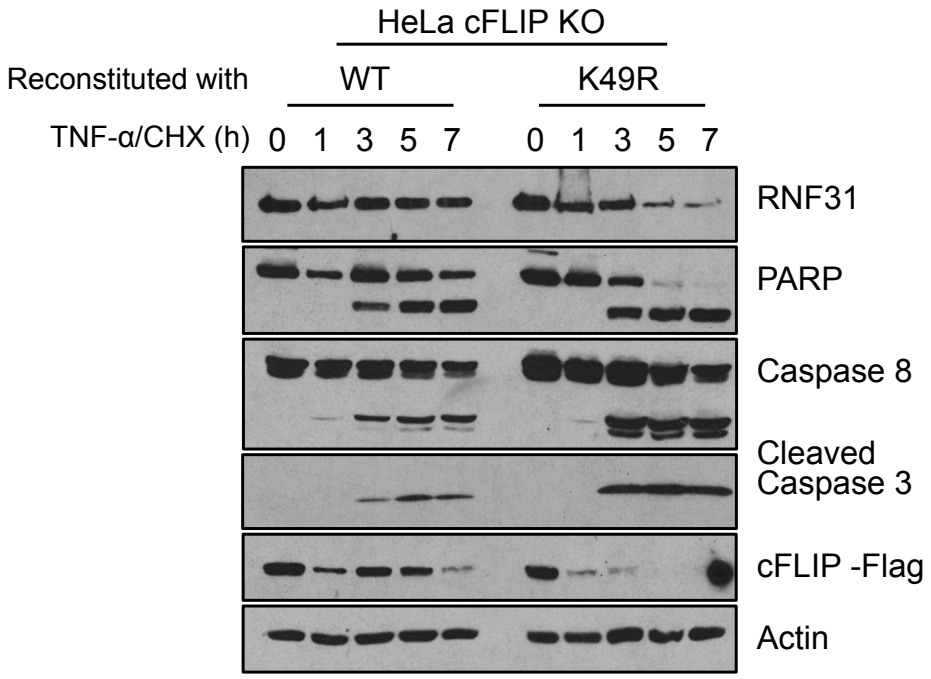


Figure 40 Reconstitution with K49R cFLIP sensitizes cells to TNF-α and CHX induced apoptosis compared to reconstitution with WT cFLIP.

WB assay of the indicated proteins in cell lysates of HeLa stably expressing FLAG-WT cFLIP or FLAG-K49R cFLIP after stimulation with or without TNF-α and CHX (100 ng/ml and 20 μg/ml, respectively).

4.2.6 Deletion of RNF31 using the CRISPR system leads to sensitization of Jurkat cells to TNF-α–induced apoptosis via destabilization of cFLIP.

Although our knockdown system markedly decreased the expression of RNF31 in HeLa cells at the protein level, I used the CRISPR system to genetically delete *RNF31* from the Jurkat cells. This is an advanced model of knockdown system used to demonstrate the physiological function of RNF31. After introduction of guide RNA targeting *RNF31* and CRISPR-associated protein 9 into Jurkat cells, followed by puromycin selection, I isolated two cells in which RNF31 expression was completely abolished (Figure 41). To confirm the physiological role of RNF31, I treated RNF31-deleted Jurkat cells with TNF-α and CHX and analyzed the initiation of apoptosis in them. I clearly observed indicators of apoptosis, including cleavage of PARP, caspase 8, caspase 9, caspase 3, and RIP1, in RNF31-deleted cells 1 hr after stimulation but at later time points in control Jurkat cells (Figure 42A). Also, cFLIP was degraded markedly faster in RNF31-KO cells than in control cells 1 hr after treatment with TNF-α and CHX, but it remained unchanged up to 3 hrs in control Jurkat cells (Figure 43). Additionally, single stimulation with TNF-α highly activated caspases in RNF31-KO Jurkat cells, although I observed markedly less cleavage of caspases and PARP in control cells (Figure 42B). Next, I examined NF-κB activation by TNF-α in RNF31-KO Jurkat cells. Phosphorylation of IKK α/β and degradation of IκB-α was slightly suppressed and phosphorylation of IκB-α was maintained slightly longer in RNF31-KO Jurkat cells than in control Jurkat cells. Additionally, JNK activation in KO cells was defective upon stimulation with TNF-α (Figure 44A). Translocation of p65 was delayed, as well, and I observed a significant defect in activation of NF-κB 10 min after stimulation (Figure 44B). Consistent with this, the TNF-α–dependent DNA-binding activity of the NF-κB complex in RNF31-KO Jurkat cells was delayed 10 min after stimulation but was not markedly defective 30 min after it (Figure 44C), indicating that RNF31 played a partial role in TNF-α–induced NF-κB activation in Jurkat cells.

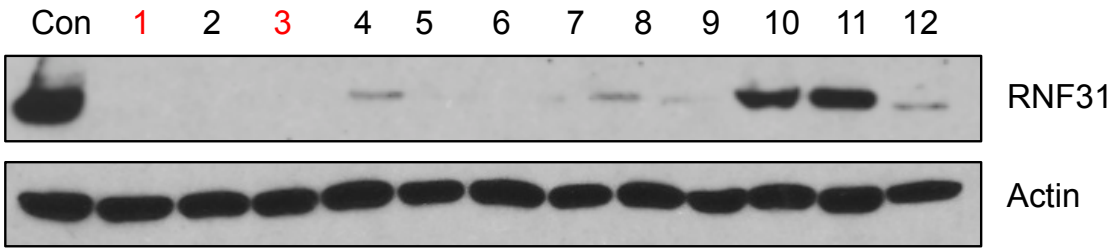


Figure 41 Deletion of RNF31 in Jurkat cells using the CRISPR system.

Jurkat cells were subjected to deletion of the *RNF31* gene using the CRISPR system, and infected cells with CRISPR virus were selected using puromycin (2 μg/ml). After isolation of single cells via serial dilution, colonies that arose from single cells were subjected to WB analysis to monitor the expression of RNF31.

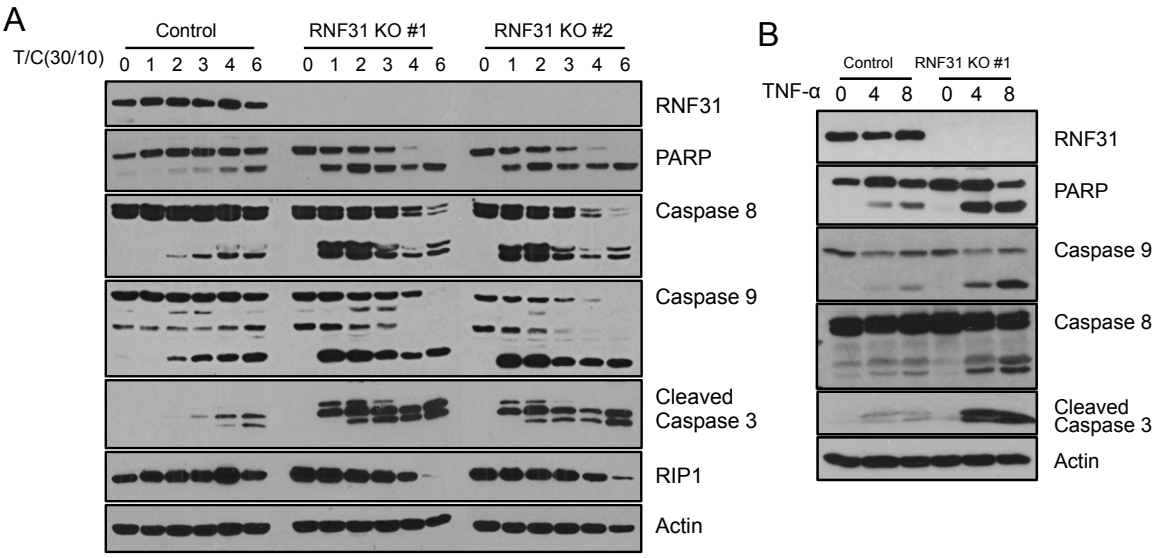


Figure 42 Deletion of RNF31 sensitized cells to TNF-α/CHX- or TNF-α-induced apoptosis.

WB analysis of the indicated proteins in control and RNF31-deleted Jurkat cells (#1 and #2) after treatment with TNF-α and CHX (30 ng/ml and 10 μg/ml, respectively, A) or TNF-α (50 ng/ml, B) for the indicated times.

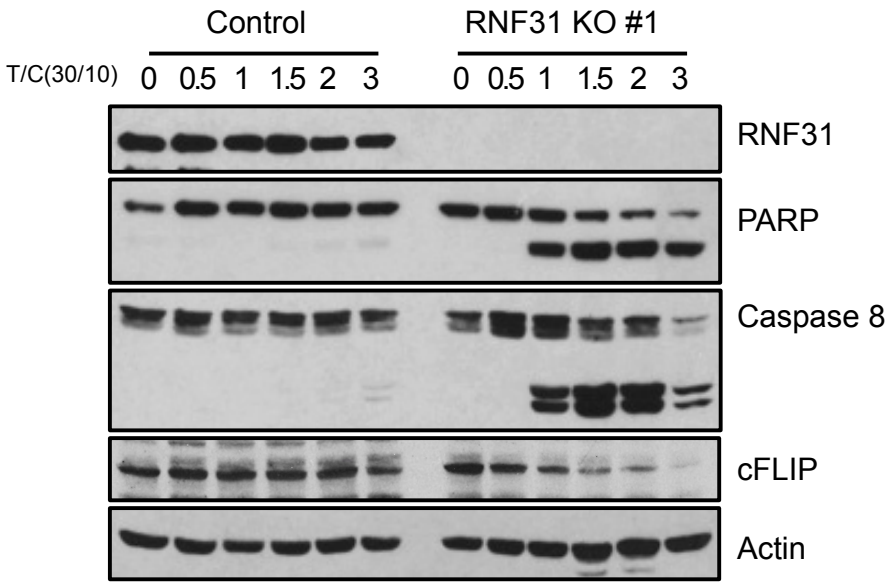


Figure 43 Degardation of cFLIP is accelerated in RNF31 deleted Jurkat cells compared to control cells.

WB analysis of the indicated proteins in control and RNF31-deleted Jurkat cells (#1) after treatment with TNF-α and CHX (30 ng/ml and 10 μg/ml, respectively) for the indicated times.

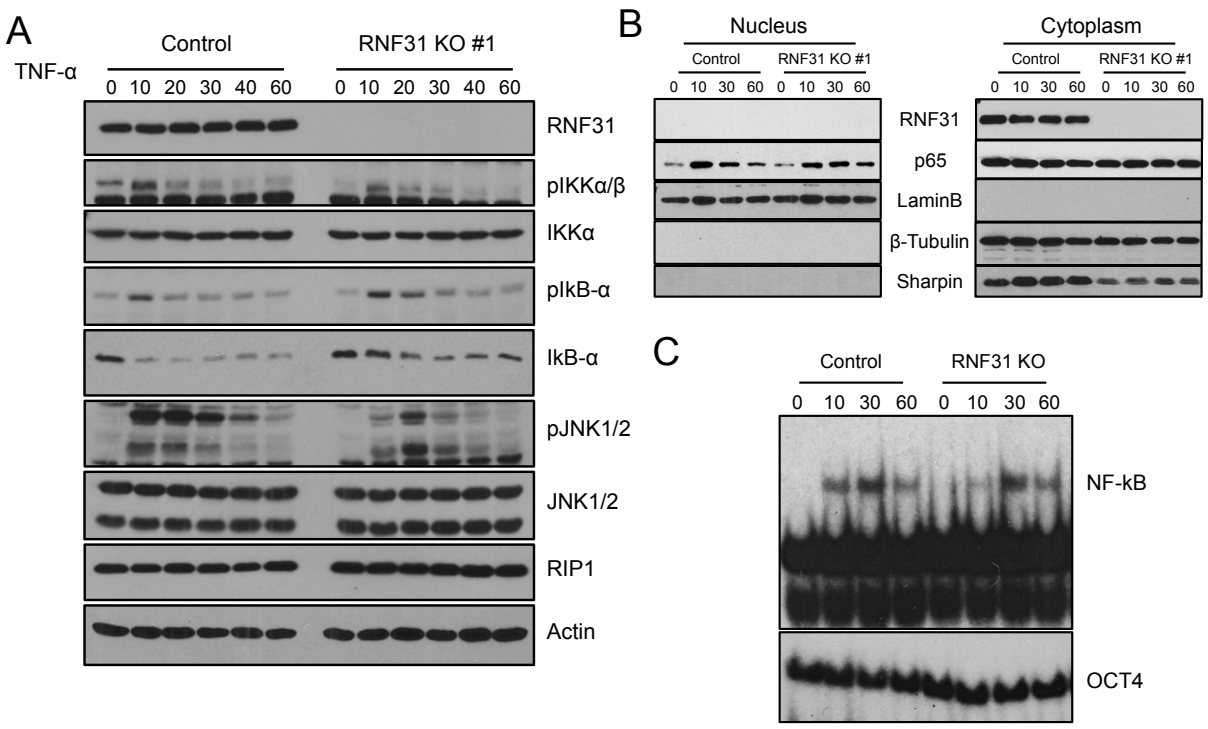


Figure 44 Deletion of RNF31 delays the early activation of NF-κB signaling by TNF-α.

(A) WB analysis of the indicated proteins from total lysates (B) and nuclear and cytoplasmic extracts (E) of control and RNF31-deleted Jurkat cells (#1) after stimulation with TNF-α (20 ng/ml) for the indicated times. (C) Electrophoretic mobility shift assay of nuclear extracts from TNF-α–treated (20 ng/ml) control and RNF31-deleted Jurkat cells using NF-κB and OCT1 probes.

4.3 Summary

LUBAC regulates the NF-κB signaling pathway by conjugating linear ubiquitination chains, further recruiting downstream molecules to TNF receptor complex. However, a deficiency in the components of LUBAC results in severe deregulation of mouse development, which cannot be explained with LUBAC's role in the NF-κB signaling pathway. As described herein, I proposed that LUBAC directly regulates apoptosis and tested our hypothesis (Figure 44). I found that silencing of RNF31 sensitized cells to TNF-α–induced apoptosis in an NF-κB–independent but ligase activity-dependent manner. To protect cells against death, LUBAC targeted cFLIP as a substrate and stabilized it by preventing K-48 ubiquitination. In addition, I genetically deleted *RNF31* from Jurkat cells using the CRISPR system and confirmed the role of RNF31 in the apoptosis and NF-κB signaling pathways.

In summary, our data established RNF31 and linear ubiquitination as key mediators of TNF-α–meditated apoptosis, revealing a novel function of RNF31 in signaling pathways other than NF-κB signaling. Moreover, I found that cFLIP is a novel substrate of linear ubiquitination and identified the target site of this modification, clearly identifying the mechanism of how LUBAC and linear ubiquitination regulate apoptosis. Our findings also demonstrated that linear ubiquitination stabilizes its substrates by competing with the K-48 ubiquitination chain, suggesting dynamic regulation of posttranslational modifications by linear ubiquitination. Finally, I propose a new genetic model that can be used to determine the function of RNF31using the CRISPR system. The present study is the first to provide insight into the physiological roles of RNF31 and linear ubiquitination in the apoptosis pathway and to demonstrate that disruption of this machinery would be a novel therapeutic strategy for apoptosis-related diseases.

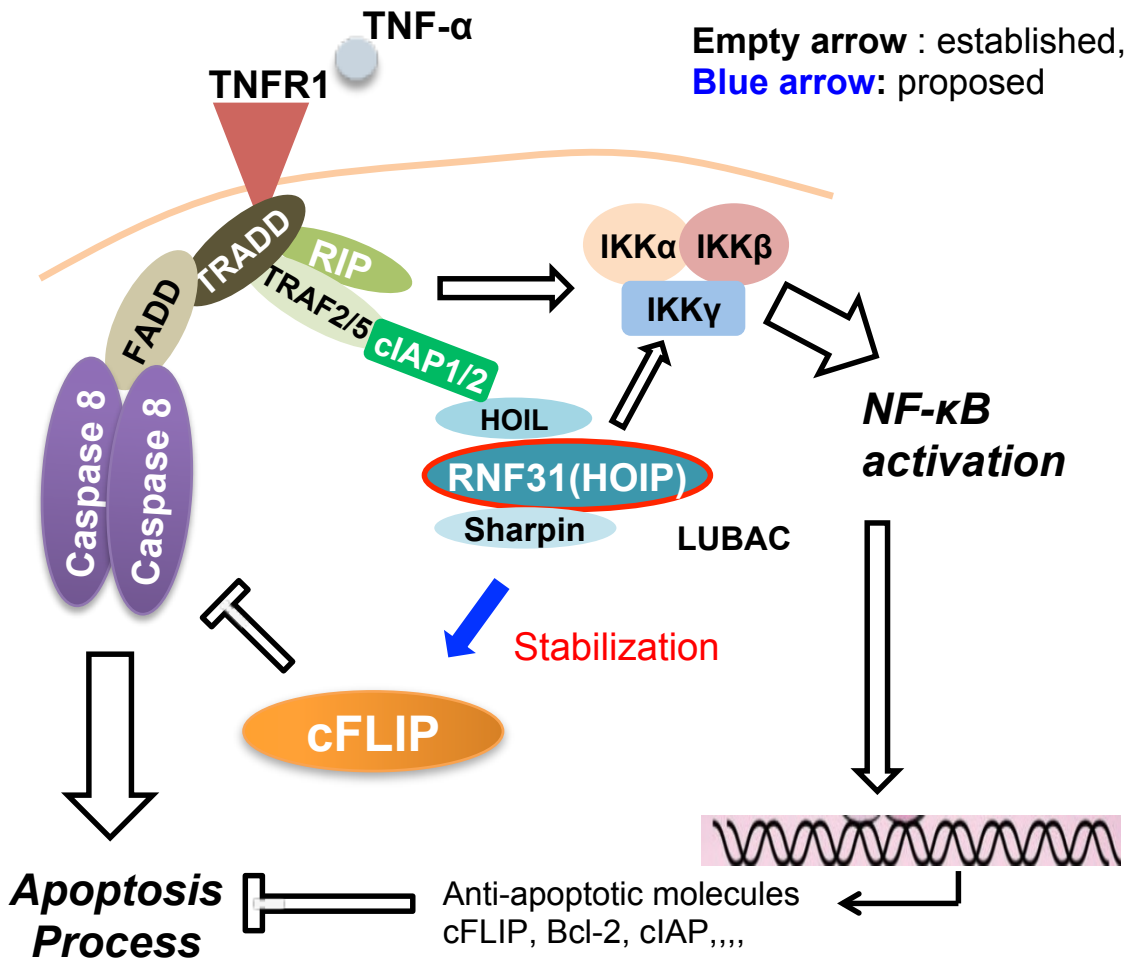


Figure 45 Proposed model of RNF31 function in apoptosis.

Upon TNF-α stimulation, the TNF-α/FADD/caspase 8 axis activates apoptosis, which is inhibited by cFLIP. Simultaneously, TRAF 2/5 and the RIP complex trigger NF-κB signaling activation, which promotes cell survival by regulating gene expression. In this model, RNF31 suppresses the apoptosis pathway by stabilizing cFLIP, which directly inhibits activation of the apoptosis pathway.

CHAPTER V: Regulation of linear ubiquitin chain assembly complex by Caspase-mediated cleavage of RNF31

5.1 Introduction

The nuclear factor-κB (NF-κB) signaling pathway plays a critical role in various cellular processes, including proliferation, differentiation, survival, and death. In the resting status, inhibitor of κB-α (IκB-α) sequesters the NF-κB complex in the cytoplasm through interaction with it. Through the activation of IκB kinase (IKK) complex (composed of IKKα/β/γ), followed by phosphorylation of IκB-α, which leads to the degradation of IκB-α, free NF-κB complex acquires the capacity to enter the nucleus and induce target gene expression [72]. Previous studies have revealed that K63-linked polyubiquitination of IKKγ (also called NEMO) is critical for NF-κB activation [205]. Recently, linear ubiquitination was identified as a novel type of ubiquitination that is the ubiquitin linkage between the N-terminal Met of one ubiquitin and C-terminal Gly of another [91, 92, 97]. To date, the linear ubiquitin chain assembly complex (LUBAC), which is composed of one main E3 ligase, ring finger protein 31 (RNF31 and also known as HOIP), and two associated proteins, HOIL-1 and Sharpin, is the only E3 ligase complex for linear ubiquitination. Upstream activation leads to the linear ubiquitination of NEMO. Then, these modified molecules function as a bridge between the receptor complex and downstream IKK complex to activate NF-κB signaling [93]. Genetic studies have shown that defects in HOIL-1 or Sharpin result in reduced phosphorylation and degradation of IκB-α, impaired and delayed nuclear translocation of the NF-κB subunit p65, diminished overall gene induction, and increased tumor necrosis factor (TNF)-induced cell death [91, 92, 97].

The activation of NF-κB signaling not only directly prompts cell growth and proliferation but also suppresses cell death by up regulating anti-apoptotic molecules that inhibit the function of caspases. Caspases are regulatory proteases that are essential for apoptosis activation. Briefly, diverse extrinsic factors (death receptor activators such as TNF-α and TNF-related apoptosis-inducing ligand [TRAIL]) or intrinsic factors (DNA damage inducers and UV)

promote the cleavage of initiator caspases (caspase 8 or 9), thereby activating them. Then, these active initiators further process effector caspases (caspase 3, 6, and 7), which in turn execute cell death by processing cellular proteins [142]. To balance the cell death and survival, diverse inhibitory mechanisms that are regulated by survival signaling participate in the suppression of caspase cascade. For example, the activation of NF-κB promotes the expression of inhibitors of apoptosis proteins (IAPs), cellular FLICE-inhibitory protein (cFLIP) and B-cell lymphoma 2 (BCL2) family members and these molecules suppress the function of caspases [172]. Reversely, active apoptosis signaling dampens the survival signaling by the cleavage of components in survival signaling. Critical components such as p65, receptor-interacting protein 1 (RIP1) and NEMO in NF-κB singling are the substrates of caspases and the cleavage of these molecules results in the suppression of survival signaling [175, 176, 178]. Although NF-κB and apoptosis pathways actively interplay to balance the death and survival, the reciprocal regulation between these two are not completely established.

Here, I present a novel crosstalk between cell death signaling and survival pathway. RNF31, a major E3 ligase in LUBAC for linear ubiquitination, is cleaved in an effector caspase-dependent manner in apoptotic conditions. This cleavage event attenuates the capability of RNF31 to activate NF-κB signaling, thereby leading to the sensitization of the resistant cells to TNF-α-induced apoptosis.

5.2 Results

5.2.1 RNF31 is cleaved under apoptotic condition.

To determine whether LUBAC components are regulated during apoptosis, I monitored the level of RNF31 in apoptotic condition. Upon TNF-α and cycloheximide (CHX) treatment, apoptosis signaling was activated, as evidenced by cleavage of caspases 3 and 9 and PARP. Along with these indicators, I also observed a significant decrease in full-length RNF31 (about 110~20 kDa) and inducible bands (70~75 kDa) (Figure 46A). Since TNF-α receptor (TNFR) and other death receptors share signaling components to promote cell death, I then induced apoptosis by using TRAIL, which activates death receptors 4 and 5 (DR4 and DR5). The cleavage of RNF31 in TRAIL-stimulated cells indicated that RNF31 is cleaved under apoptosis induced by other DR activation (Figure 46B). To generalize this phenomenon, I treated different cancer cells with TNF-α and CHX. The cleaved RNF31 could be seen in every examined cell line (Figure 47), indicating that the cleavage of RNF31 is a general process in wide range of cells.

The apoptosis pathway is triggered by two major sources, extrinsic and intrinsic inducers [142]. To determine whether RNF31 is cleaved under both conditions, I treated cells with one of two DNA damage inducers, doxorubicin (Dox) or camptothecin (CPT). Since caspase 9 is the initiator caspase in the intrinsic pathway and caspase 3 is the executioner caspase for both pathways, I monitored the cleavage of caspases 3 and 9. Consistent with the observation under extrinsic activation, cleaved bands of RNF31 were detected upon treatment with DOX and CPT (Figure 48A). Additionally, I observed the cleavage of RNF31 under apoptosis induced by Smac mimetic, which directly activates initiator and effector caspases (Figure 48B). Taken together, these findings suggest that RNF31 is cleaved upon the activation of extrinsic and intrinsic apoptosis pathways.

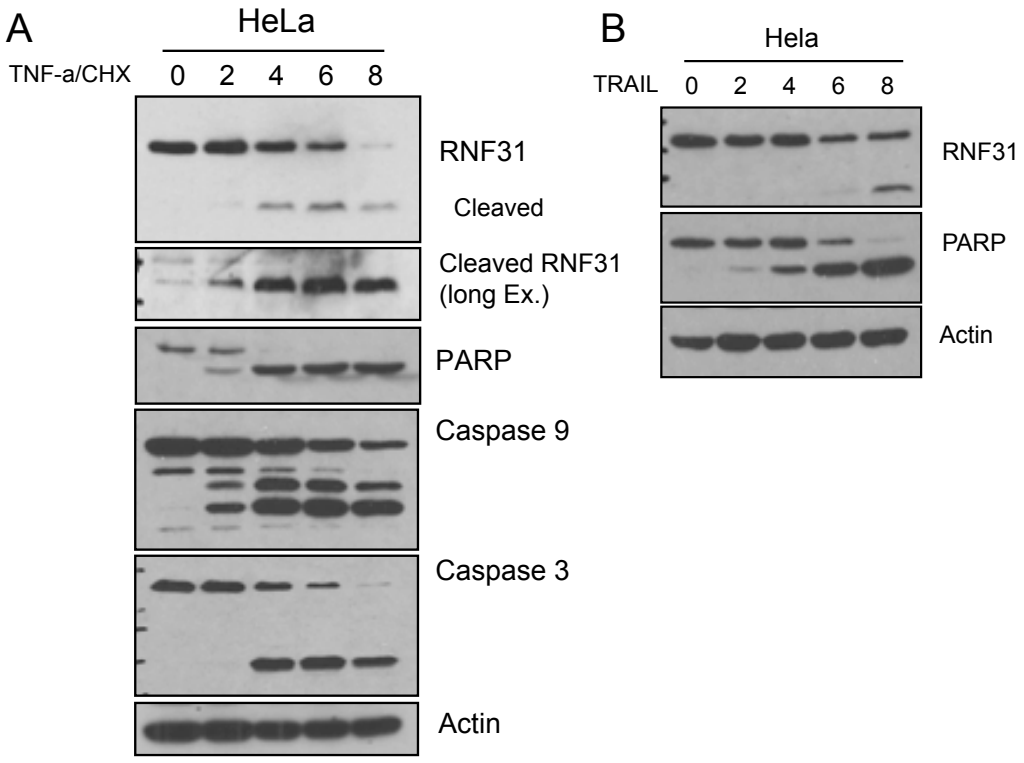


Figure 46 RNF31 is cleaved by TNF-α or TRAIL treatments.

(A) HeLa cells were stimulated with/without TNF-α and CHX (40ng/ml, 10ug/ml). Lysates were analyzed by Western blot (WB) analysis. (B) The experiments were performed as in (A) with HeLa cells treated with TRAIL (100ng/ml).

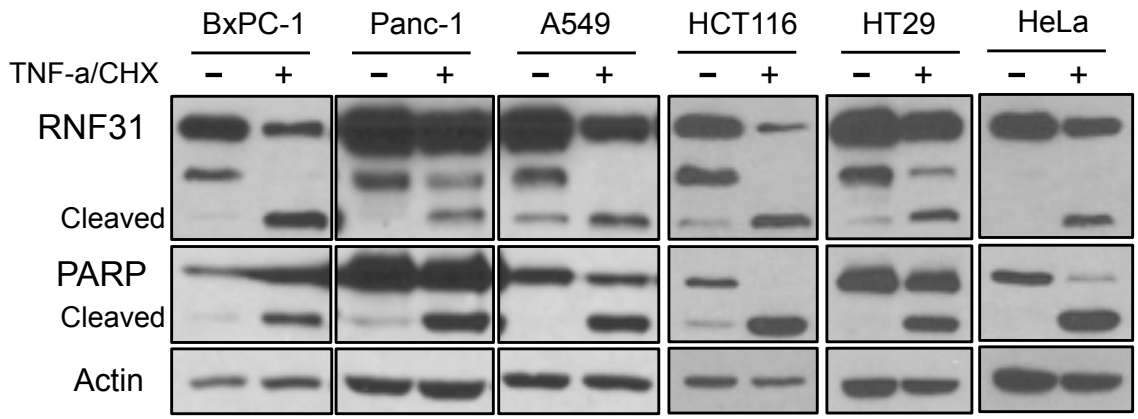


Figure 47 RNF31 is cleaved upon TNF-α-induced apoptosis condition in various cell lines.

Pancreatic adenocarcinoma (BxPC-1, Panc-1), lung adenocarcinoma (A549), colorectal adenocarcinoma (HCT116 and HT29) and cervical adenocarcinoma (HeLa) were stimulated with TNF-α/CHX (20ng/ml,10ug/ml) for 6 hours and the indicated proteins were monitored by WB.

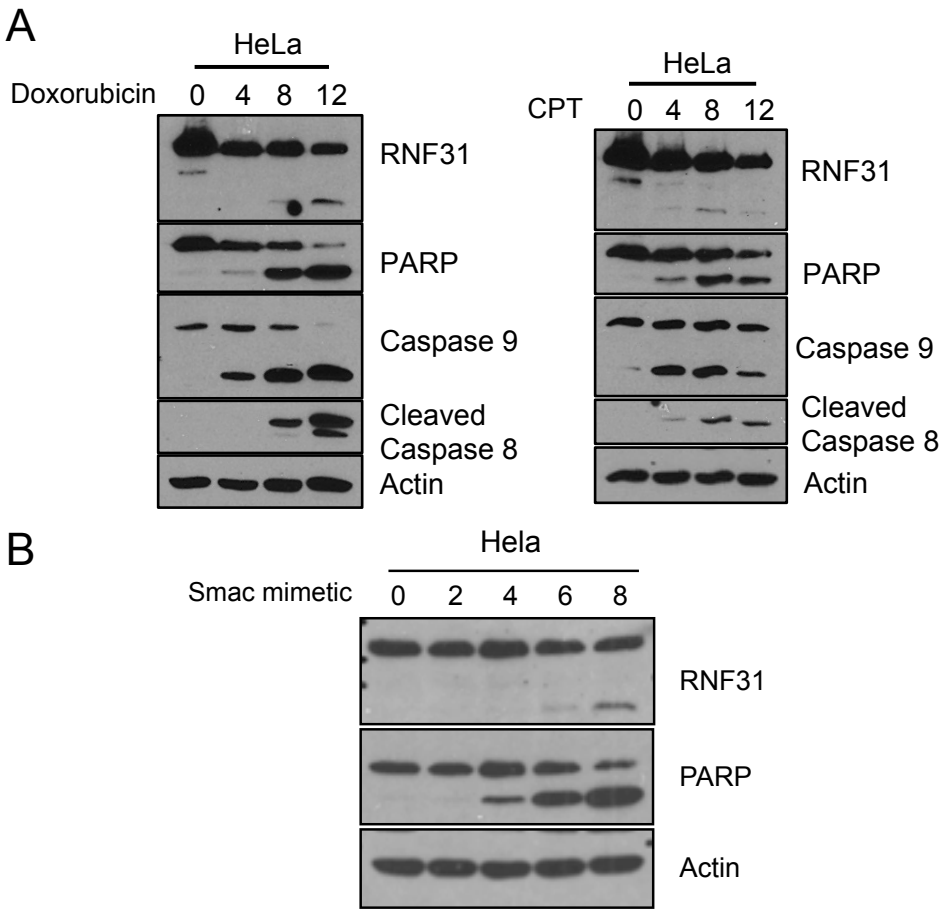


Figure 48 DNA damage inducer and Smac mimetic induce the cleavage of RNF31.

(A) The experiments were performed as in Figure 45 with HeLa cells exposed to Dox (3ug/ml) or CPT (20uM). (B) The experiments were performed as in Figure 45 with HeLa cells treated with Smac mimetic (20uM).

5.2.2 Effector caspases are responsible for RNF31 cleavage during apoptosis, not necroptosis process

Although caspases are proteases that are dominantly activated in apoptosis, other proteases may be responsible for the cleavage of RNF31. To test this hypothesis, I applied pan-caspase inhibitor Z-VAD-FMK before TNF-α and CHX stimulation to block general caspase function. Treatment with *de novo* protein synthesis inhibitor CHX did not induce cleavage of either PARP or RNF31 and I could not observe any cleaved bands of PARP and RNF31 in TNF-α-treated cells 8 hours after treatment because HeLa cells are resistant to TNF-α-induced apoptosis. However, TNF-α/CHX treatment activated apoptosis (cleavage of PARP) and induced the cleavage of RNF31 4 hours after stimulation. Notably, the cleavage of RNF31 was completely blocked by 1 hour of Z-VAD pretreatment (Figure 49). The degradation of IκB-α in TNF-α/CHX treated cells with or without Z-VAD indicated that stimulation was intact.

Since FADD and caspase 8 are critical for triggering cell death under TNFR activation, I next examined the function of FADD and Caspase-8 in RNF31 cleavage upon TNF-α/CHX treatment by using FADD- or Caspase-8-deficient Jurkat cells. Moreover, treatment of these modified cells with TNF-α/CHX activated necroptosis. Therefore, the observation in these deficient cells would reveal whether necroptosis enable to induce the cleavage of RNF31. Upon TNF-α/CHX treatment, the cleaved RNF31 was observed 4 hours after stimulation, and this cleavage event was completely blocked by treatment with Z-VAD in wild-type (WT) Jurkat cells. However, the cleaved band was not detected in FADD- or Caspase-8-deficient Jurkat cells (Figure 50), implying that FADD and Caspase-8 are vital for the cleavage of RNF31 in TNF-α/CHX induced apoptosis, and RNF31 is not cleaved in the necroptosis.

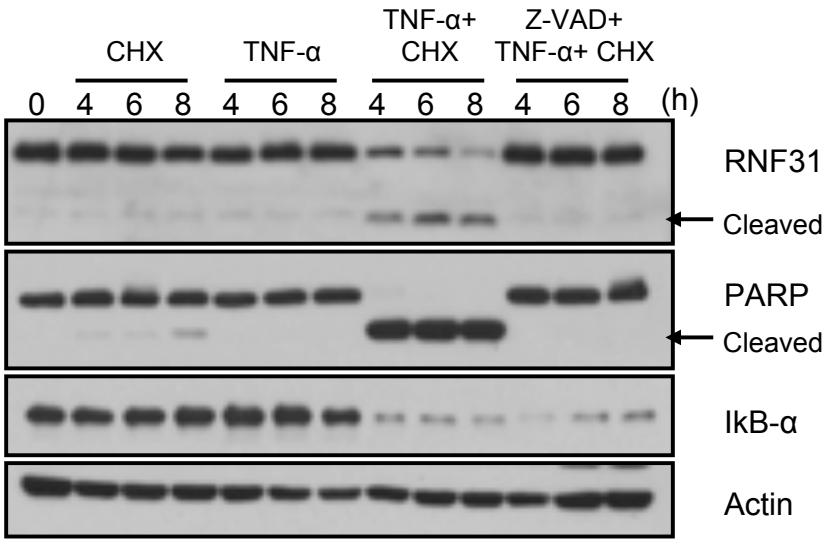


Figure 49 Pancaspase inhibitor, Z-VAD blocks the cleavage of RNF31.

HeLa were pretreated with/without zVAD (20uM), followed by TNF-α (20ng/ml), CHX(10ug/ml) or TNF-α/CHX treatment and the proteins were analyzed by WB.

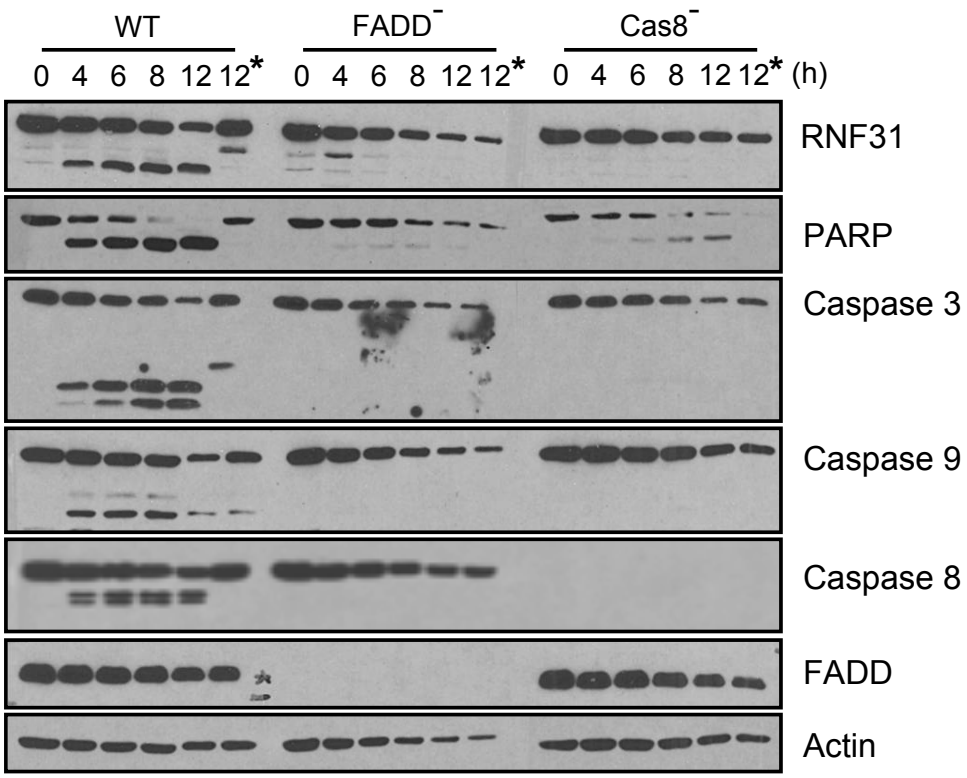


Figure 50 The cleavage of RNF31 is inhibited in FADD and Caspase 8 deficient cells.

WT, FADD deficient and Caspase 8 deficient Jurkat were stimulated with TNF-α/CHX (20ng/ml, 10ug/ml) for indicated times and the proteins were analyzed with WB. The asterisk indicated the pretreatment of pan-caspase inhibitor; Z-VAD.

Each caspase recognizes a specific sequence of its targets, and this specificity allows them to have a different role in cellular processes [206]. Therefore, I sought to determine which caspase is responsible for the RNF31 cleavage. Since previous results indicated that Caspase-8 is essential for the cleavage of RNF31 under TNF-α stimulation, apoptosis was induced in A431 epidermal carcinoma cells, which have undetectable levels of Caspase-8 owing to the mutation. Because the deficiency of Caspase-8 leads to resistance to extrinsic inducers, I treated the cells with Dox or CPT to activate apoptosis, and these agents induced the cleavage of RNF31 as well as PARP and Caspase-9 (Figure 51). Cleavage of RNF31 in Caspase-8-deficient Jurkat cells treated with intrinsic inducers (Dox, CPT, or 5-Fu) further supported the notion that Caspase-8 is dispensable for RNF31 cleavage (Figure 52). Thus, I performed an *in vitro* cleavage assay to identify the caspase responsible for the cleavage of RNF31. Incubation of immunoprecipitated RNF31 with various recombinant caspases indicated that caspase 3 or caspase 6 is able to process RNF31. The identical level of heavy chain in each sample suggested that equivalent levels of recombinant RNF31 were presented before the reaction (Figure 53). These data indicate that apoptosis and, specifically, caspase activation are fundamental for RNF31 cleavage, and effector caspases 3 and 6 are responsible for this process.

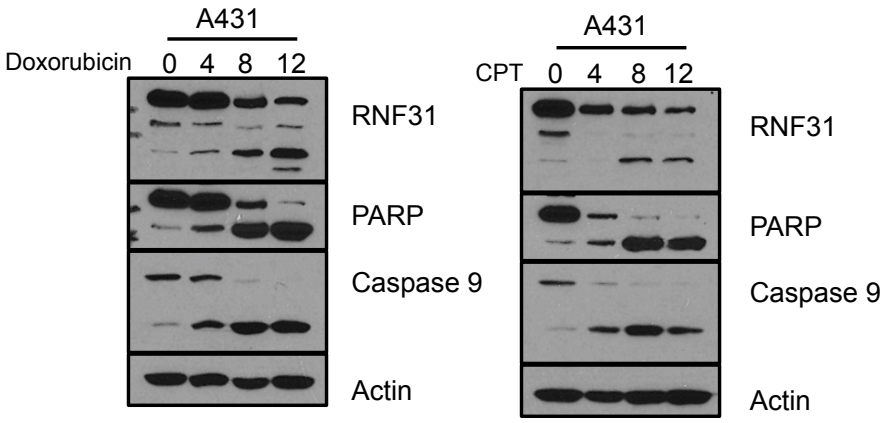


Figure 51 DNA damage inducer cleaves RNF31 in A431.

A431 cells were treated with Dox (3ug/ml) or CPT (20uM) and the indicated proteins were analyzed by WB.

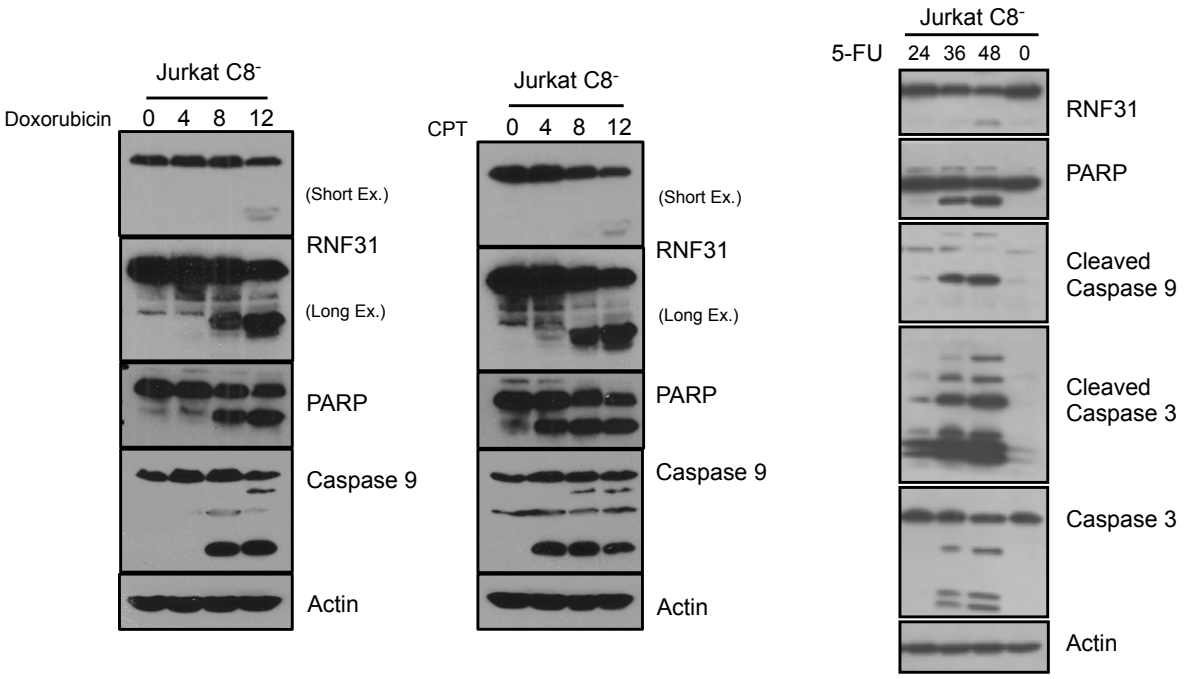


Figure 52 DNA damage inducer cleaves RNF31 in Caspase 8-deficient Jurkat.

Caspase 8-deficient Jurkat cells were stimulated with Dox (3ug/ml), CPT (20uM) or 5-Fu (20ug/ml). The lysates were subjected to WB.

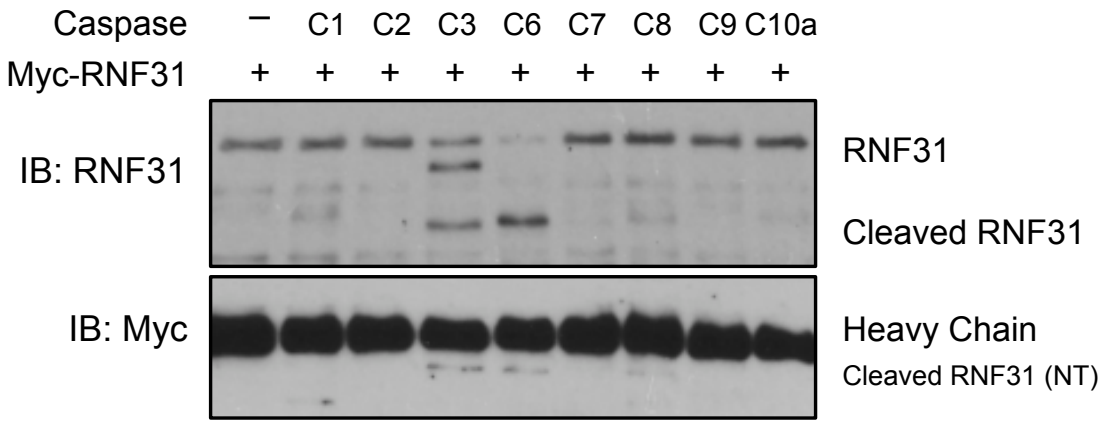
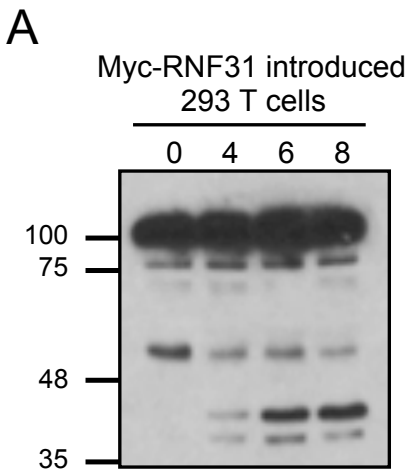


Figure 53 Caspase 3 and 6 are the responsible protease for RNF31 cleavage.

Myc-tagged RNF31 proteins were incubated with/without the indicated recombinant caspases for 2 hours. Cleaved RNF31 bands were analyzed via WB.

5.2.3 Cleavage of RNF31 is dependent on Asp348, Asp387, and Asp390, and suppresses its function in the NF-κB pathway.

The apoptosis pathway actively communicates with a survival signaling pathway (NF-κB) to govern physiological features of cells. Therefore, I hypothesized that the apoptosis pathway suppresses the function of LUBAC in survival signaling via the cleavage of RNF31. To test this hypothesis, I first identified the cleavage sites in RNF31. Treatment of 293T cells expressing N-terminus Myc-tagged RNF31 with TNF-α/CHX generated cleaved bands of RNF31 around 40 kDa in a time-dependent manner (Figure 54A). Then, I found that aspartate 348, 387, and 390 are potential cleavage sites based on data generated using the web-based prediction software Cascleave [207] (Figure 54B). Since the prediction suggested that aspartate 390 had the highest probability score, I first generated a D390A RNF31 mutant. However, the cleavage of RNF31 was still observed under apoptotic conditions (through TNF-α/CHX treatment or cFLIP expression). Finally, I found that the triple mutant, D348/387/390A, completely blocked the cleavage (Figure 55A). Since the expression of caspases in 293T cells rapidly promoted cell death, I alternatively introduced cFLIP expression that initiated moderate levels of apoptosis [208]. Furthermore, an *in vitro* cleavage assay with recombinant WT and mutant RNF31 proteins showed that caspase 6 was not able to process D348/387/390A RNF31 (Figure 55B), demonstrating that aspartate 348, 387, and 390 are sites at which cleavage is initiated by effector caspases.



B

Position	Cleavage site
52	LQLD*AARL
110	STVD*AVQG
130	EQPD*GLSF
348	**LEPD*LARG**
387	**LVVD*SRDA**
390	**DSRD*AGIC**
636	PSWD*GPDK
639	DGPD*KQSL
754	DLTD*DTQL
776	LEPD*AYAL

Figure 54 The cleavage of RNF31 occurs at Aspartate 348, 387 and 390.

(A) 293T cells were transfected with RNF31 conjugating Myc at N-terminus and then, treated with TNF-α/CHX (40ng/ml, 10ug/ml). The cleaved RNF31 was detected by WB. (B) Estimated cleavage sites of RNF31 by caspases

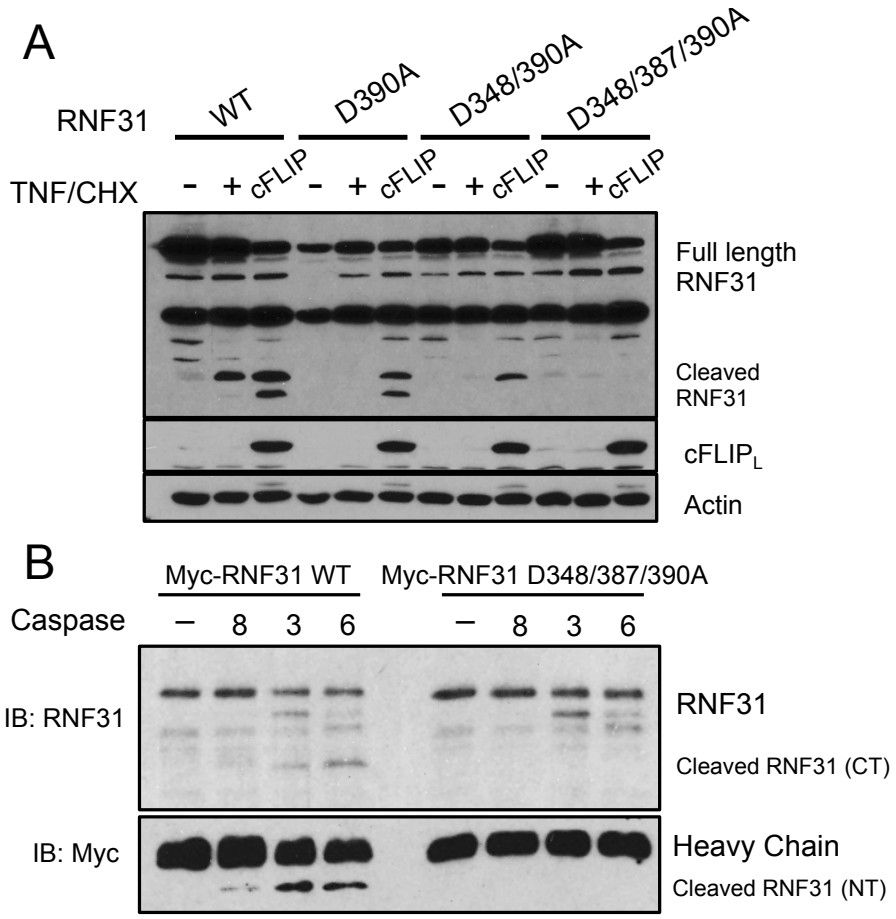
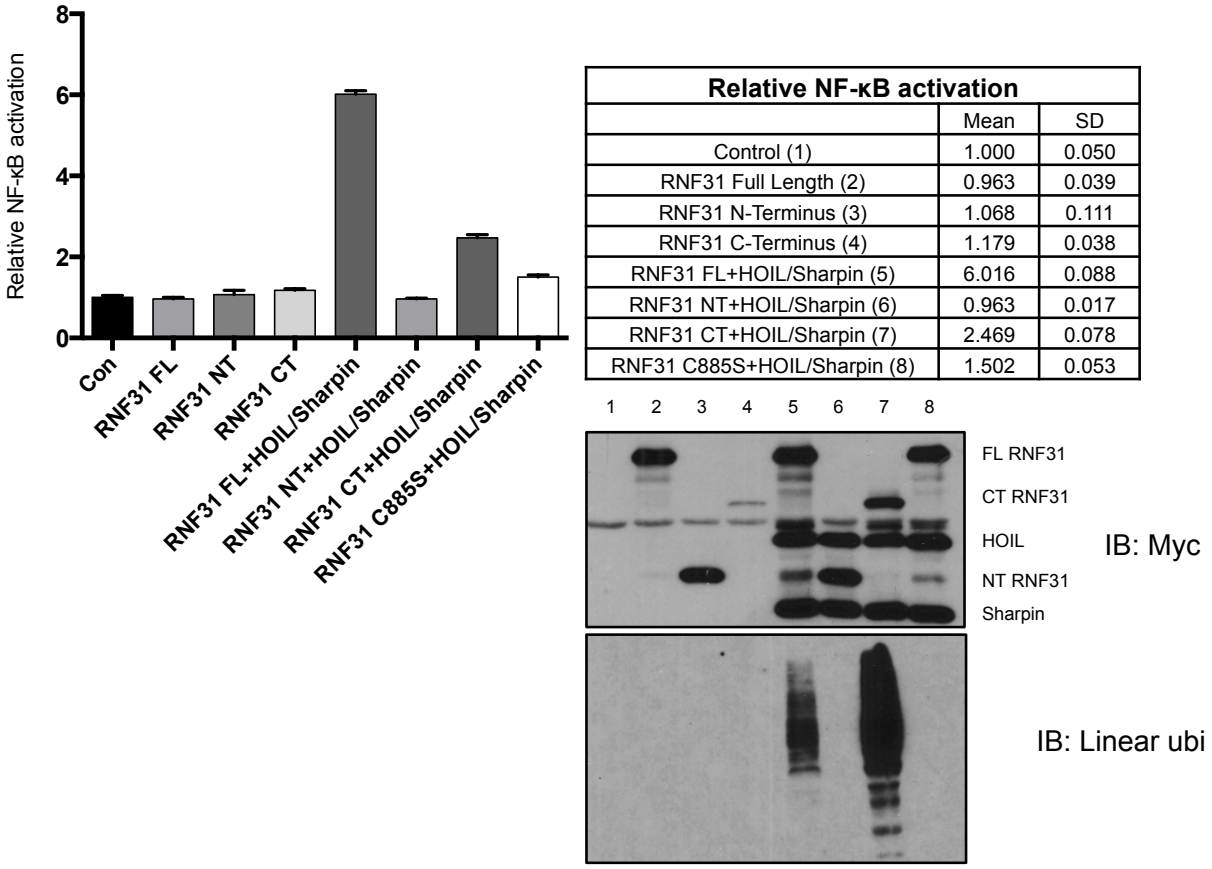


Figure 55 Mutation of aspartate 348, 387 and 390 to arginine prevents the cleavage of RNF31.

(A) 293T were transfected with the plasmid encoding Myc-conjugated WT, D390A, D348/390D or D348/387/390A RNF31 and the cleaved fragment was examined by WB. (B) Recombinant WT or D348/387/390A mutant RNF31 were incubated with/without caspase 8, caspase 3 or caspase 6 for 1 hour and cleaved RNF31 fragment was examined by WB.

Next, I examined the role of this cleavage in NF-κB activation. Previous studies have shown that full-length RNF31 (together with HOIL-1/Sharpin) can activate NF-κB while the deletion of ZF domain resulted in the partial defect of NF-κB activation [92]. Therefore, I hypothesized that this cleavage represses the capacity of RNF31 to activate the NF-κB pathway. The luciferase assay with the full-length RNF31 and cleaved fragments of RNF31 demonstrated that neither of the cleaved fragments could fully activate NF-κB, even when they expressed together with HOIL-1/Sharpin. Specifically, the C-terminal RNF31 fragment (hereafter "CT RNF31") only partially induced NF-κB activation, although CT RNF31 generated linear ubiquitination (Figure 56). The C885S RNF31 mutant that lost its catalytic activity had defective NF-κB activation, confirming that NF-κB activation by LUBAC depends on the catalytic activity of RNF31 [197]. Then, I tested the functional capacity of each fragment to bind with NEMO because this interaction is vital for NF-κB activation. Consistent with previous reports [189], NEMO was able to interact with full-length RNF31 (FL RNF31) and N-terminal RNF31 fragment (NT RNF31) (containing the ZF domain), but not with CT RNF31. The defective binding ability was recovered when CT RNF31 was expressed together with HOIL/Sharpin (Figure 57). Then, the ubiquitination assay to assess the capability of the cleaved fragments to conjugate ubiquitin chains showed that CT RNF31 still initiated the linear ubiquitination of NEMO in the presence of HOIL-1/Sharpin (Figure 58). Consistent with the findings for NEMO, not only FL RNF31 but also CT RNF31 together with HOIL-1/Sharpin generated the linear ubiquitination of RIP1, another known substrate of LUBAC for linear ubiquitination (Figure 59), although no interaction between RIP1 and any form of RNF31 was observed, even in the presence of HOIL-1/Sharpin (DATA NOT SHOWN). This series of data suggests that cleaved fragments of RNF31 are less potent NF-κB activators than full-length fragment, but the CT fragment can still generate the linear ubiquitination of NEMO and RIP in

the presence of HOIL-1/Sharpin Further studies are required to determine how linear ubiquitination control NF-κB signaling and how the cleavage of RNF31 is involved in the activation of this pathway.



Relative NF-κB activation		
	Mean	SD
Control (1)	1.000	0.050
RNF31 Full Length (2)	0.963	0.039
RNF31 N-Terminus (3)	1.068	0.111
RNF31 C-Terminus (4)	1.179	0.038
RNF31 FL+HOIL/Sharpin (5)	6.016	0.088
RNF31 NT+HOIL/Sharpin (6)	0.963	0.017
RNF31 CT+HOIL/Sharpin (7)	2.469	0.078
RNF31 C885S+HOIL/Sharpin (8)	1.502	0.053

Figure 56 The cleavage of RNF31 restricts its function on NF-κB activation.

293T were transfected with NF-κB luciferase reporter genes and the indicated RNF31 mutants with/without HOIL-1 and Sharpin. Luciferase reporter assay was performed with the lysates. The expression of transfected mutants and generation of linear ubiquitination were confirmed by WB.

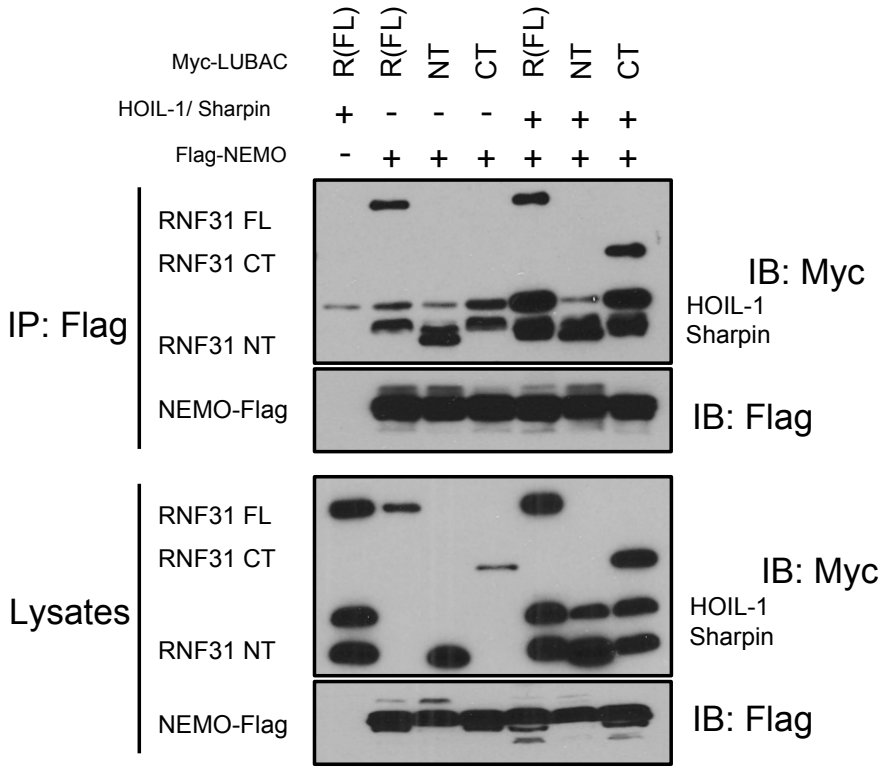


Figure 57 CT RNF31 did not bind with NEMO, but did when expressed with HOIL-1 and Sharpin.

Full-length or cleaved fragments of RNF31 were introduced into 293T cells with/without HOIL/Sharpin and NEMO. Flag-tagged NEMO was precipitated with M2 flag bead and expressed LUBAC components and the interacting proteins were analyzed with WB.

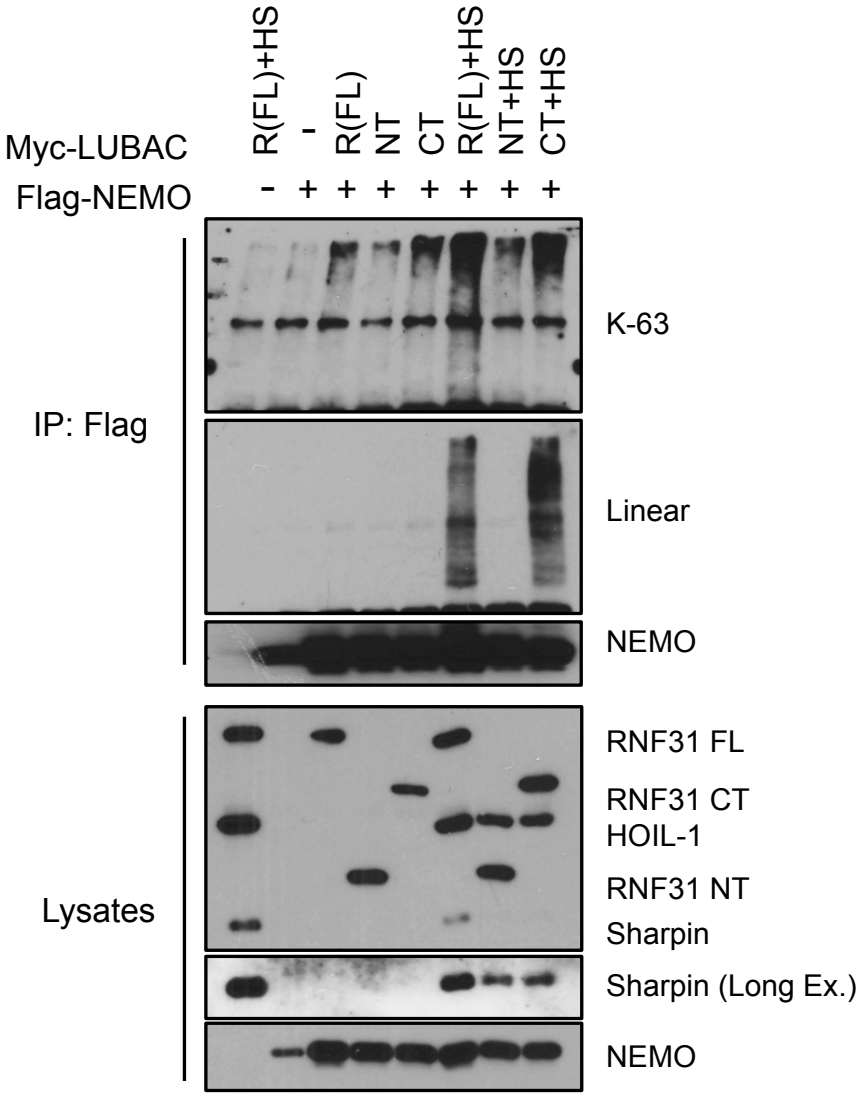


Figure 58 CT RNF31 fragment is able to induce linear ubiquitination of NEMO.

Flag-tagged NEMO were introduced into 293T cells with/without LUBAC components. After being prepared with 2% SDS lysis buffer and boiling, lysates were incubated with flag bead. Linear ubiquitination of precipitated NEMO and the expression of introduced molecules were detected with WB. were confirmed by WB.

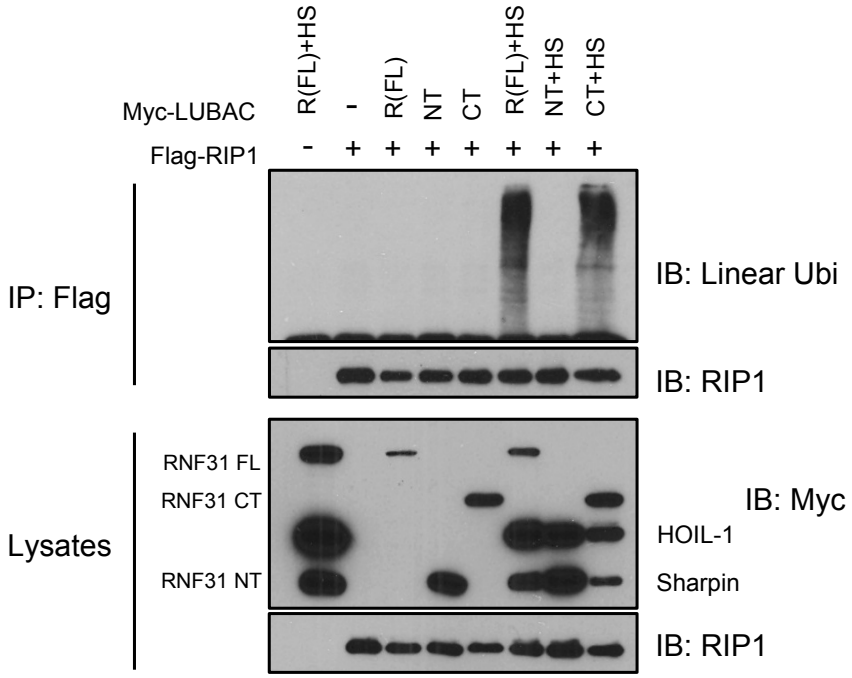


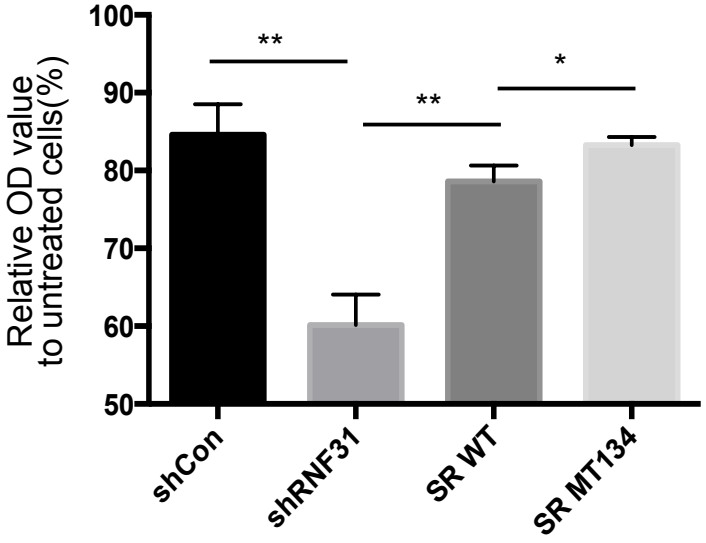
Figure 59 CT RNF31 fragment is able to induce linear ubiquitination of RIP1.

Flag-tagged RIP1 were introduced into 293T cells with/without LUBAC components. After being prepared with 2% SDS lysis buffer and boiling, lysates were incubated with flag bead. Linear ubiquitination of precipitated RIP1 and the expression of introduced molecules were detected with WB. were confirmed by WB

5.2.4 Mutation of cleavage sites leads to partial resistance to apoptosis

To determine the physiological role of RNF31 cleavage in the cell death process, I generated RNF31 KD HeLa cells, in which silenced RNF31 is reconstituted with WT or D348/387/390A mutant RNF31 (hereby called SR WT and SR MT134, respectively). The MTT assay to test the sensitivity of these modified cells to TNF-α induced apoptosis revealed that the silencing of RNF31 significantly decreased the cell viability upon TNF-α treatment. And the reconstitution of KD cells with WT RNF31 rescued its resistance to apoptosis and D348/387/390A mutant expression further protected cells from apoptosis (Figure 60). I then sought to determine the role of the RNF31 cleavage in sensitivity to apoptosis with TRAIL, which dominantly activates apoptosis signaling with minor activation of the NF-κB pathway. SR MT134 cells were more resistant than SR WT cells to TRAIL-induced apoptosis in low dose of TRAIL treatment, while higher doses of TRAIL induced comparable levels of apoptosis in both cell lines (Figure 61). Next, SR WT and SR MT134 cells were sequentially treated with TRAIL and TNF-α to further demonstrate the function of cleavage. First, RNF31 had been cleaved in advance by pretreatment with TRAIL; TNF-α was then administered to further promote cell death. Since TRAIL dominantly activates caspases that lead to the cleavage of RNF31, and TNF-α activates survival signaling as well as the apoptosis pathway, I expected this set of treatments to demonstrate the effect of RNF31 cleavage on TNF-α-induced cell death. In SR WT cells, pretreatment with TRAIL generated synergetic effects on the induction of apoptosis by TNF-α such as increased amount of cleaved PARP and caspase 8. However, the sequential treatment had no synergetic effects on apoptosis induction in MT134 rescued cells (Figure 62A). The role of cleavage was further confirmed with annexin V staining after single or sequential treatment. Sequential treatment with TRAIL and TNF-α enhanced the number of apoptotic cells in SR WT cells compared with TRAIL treatment while similar number of

apoptotic SR MT134 cells was induced by TRAIL and the sequential treatment. (Figure 62B). Since the effector caspases that are activated by intrinsic inducers are responsible for the cleavage of RNF31 as well, it is possible that not only TRAIL but also intrinsic death signals sensitize cells to death receptor-induced cell death. To test this hypothesis, I pretreated SR WT and SR MT134 cells with the intrinsic inducer doxorubicin to trigger the cleavage of RNF31. Then, cells were treated with TNF-α to further activate the apoptosis pathway. Since the apoptosis induced by intrinsic factors is a more prolonged process than DR-mediated cell death, the effect of cleavage resistant mutation is less potent. However, it is clear that the additional treatment with TNF-α disrupted the viability of only SR WT cells, not SR MT134 cells (Figure 63), implying the role of RNF31 cleavage by an intrinsic inducer in TNF-α-induced apoptosis. These results demonstrate that the cleavage of RNF31 sensitizes cells to TNF-α-induced death by suppressing survival signaling.



Group	Realtive OD values (%)
shCon	84.59±2.269
shRNF31	60.14±2.260
SR WT	78.58±1.188
SR MT134	83.29±0.5969

Figure 60 Reconstitution with MT134 enhances the resistance to TNF-α-induced apoptosis.

shCon, shRNF31, WT RNF31 rescued shRNF31 (SR WT) and MT134 mutant RNF31 rescued shRNF31 (SR MT134) HeLa cells were stimulated with/without TNF-α (100 ug/ml, 24 hours) and cell viability was measured by MTT assay.

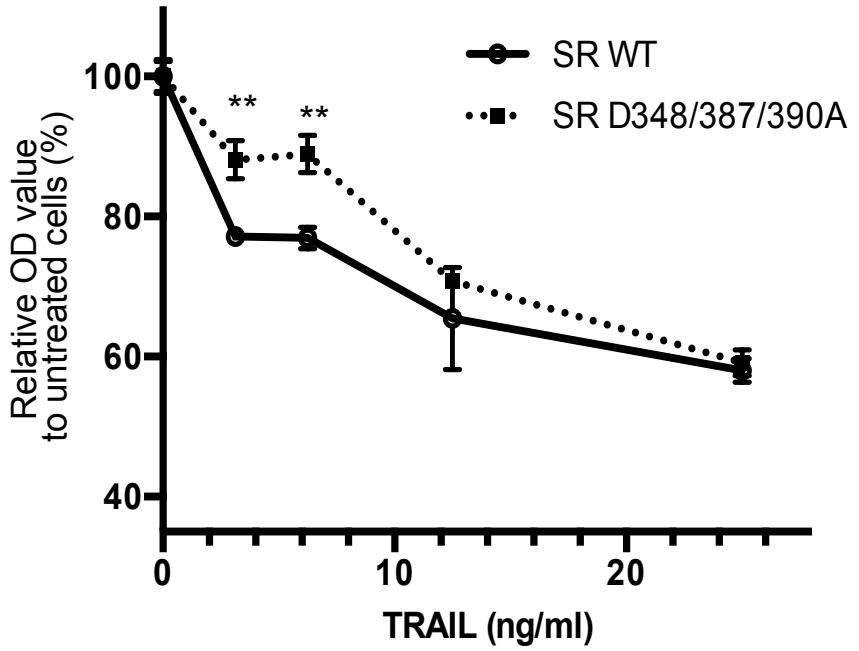
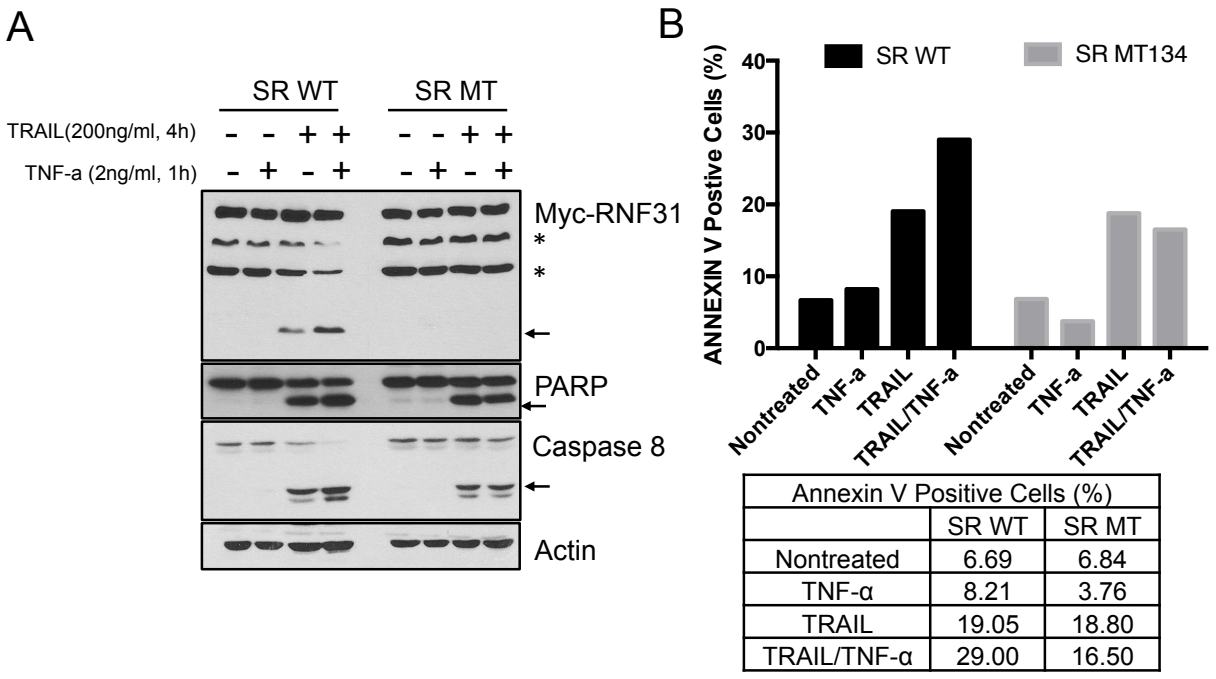


Figure 61 Reconstitution with MT134 enhances the resistance to low does of TRAIL-induced apoptosis.

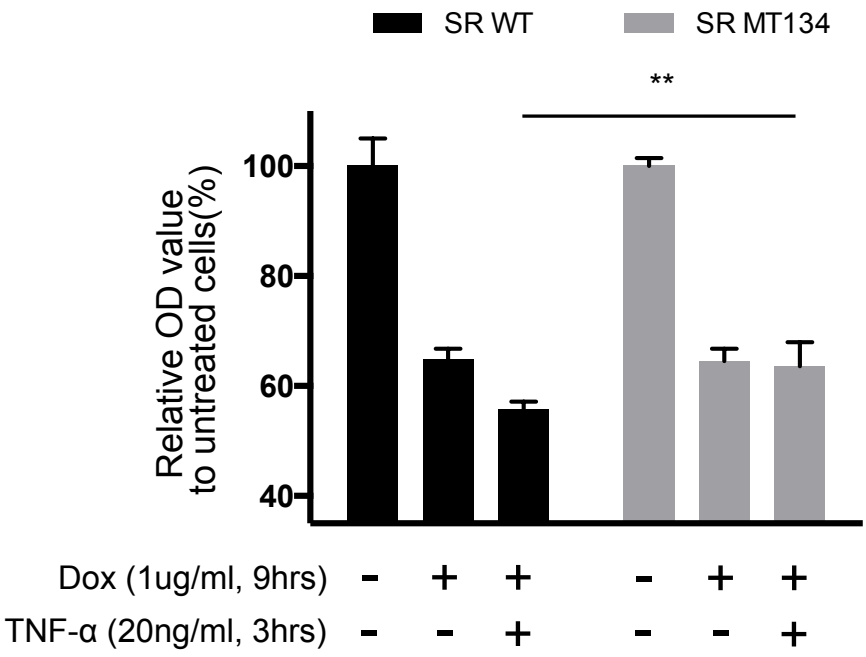
SR WT and SR MT134 HeLa cells were treated with TRAIL for 24 hours and the cell viability was detected by MTT assay.



Annexin V Positive Cells (%)		
	SR WT	SR MT
Nontreated	6.69	6.84
TNF-α	8.21	3.76
TRAIL	19.05	18.80
TRAIL/TNF-α	29.00	16.50

Figure 62 Recontitution with MT134 prevents the sensitization of HeLa by pretreatment with TRAIL.

(A) SR WT and SR MT134 HeLa cells were pretreated with/without TRAIL (200 ng/ml, 4 hours) and then, stimulated with/without TNF-α (2 ng/ml, 1h). The indicated proteins were analyzed by WB. (B) SR WT and SR MT134 cells were stimulated with/without TRAIL (100 ug/ml, 5 hours) and treated with/without TNF-α (10 ug/ml, 2 hours). After Annexin V staining, apoptotic cells were analyzed with flow cytometry.



Relative OD value to untreated cells (%)		
	SR WT	SR MT134
Dox	64.852±1.933	64.553±2.231
Dox + TNF-α	55.690±1.473	63.617±4.364

Figure 63 Pretreatment with Doxorubicin sensitizes WT RNF31 recontituted HeLa cell to TNF-α induced apoptosis, but not MT134 reconstituted HeLa.

Cells were treated with Dox (1 ug/ml for 9 hours) and the indicated concentration of TNF-α was applied. Cell viability was measured by MTT assay. The asterisk indicated non-specific Band.

5.3 Summary

Caspases directly execute cell death by destroying fundamental proteins and indirectly restrict survival signaling by cleaving the cell survival mediators. Here, I present a novel mechanism whereby caspases regulate the NF-κB pathway. Once effector caspases are activated by intrinsic or extrinsic signals, they suppress the function of RNF31 and LUBAC in NF-κB signaling by the cleavage process. This negative regulation attenuates the inhibitory role of NF-κB in death signaling and finally accelerates cell death or sensitizes resistant cells to cell death (Figure 64). Although it has been reported that LUBAC play a critical role in the activation of NF-κB signaling, previous studies have suggested that LUBAC plays a role in the apoptosis process. Sharpin-deficient cpdm MEFs have increased sensitivity to TNF-α-induced apoptosis [91, 97], and RNF31-silenced ovarian cancer cells are more sensitive to cisplatin-induced death [209]. Although the mechanism of apoptosis regulation is not completely demonstrated, these previous studies support our finding that inhibition of LUBAC by caspase-dependent RNF31 cleavage sensitizes cancer cell to apoptosis.

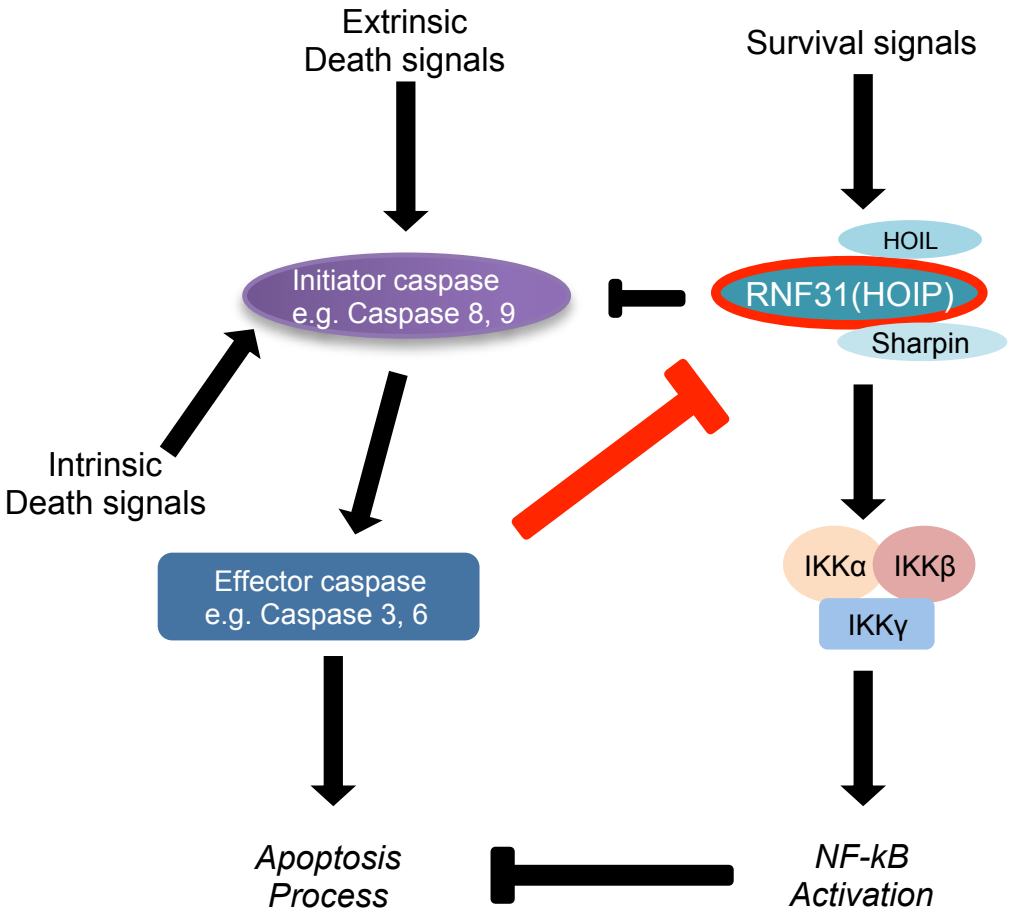


Figure 64 Proposed model of interaction between Caspases and LUBAC.

Activation of effector caspases from extrinsic and intrinsic death inducer leads to cleavage of RNF31 that inhibit LUBAC function in NF-κB pathway. Suppression of NF-κB by this process further dampens inhibitory function on apoptosis that eventually accelerate apoptosis process.

Chapter VI: Discussion

6.1 Significance of this study

Tumor necrosis factor family (TNFs) members induce both apoptotic and survival pathways that are crucial for tumors to create favorable conditions. Indeed, many tumors abuse the TNF-α signaling pathway for tumor formation and progression. Therefore, a clear understanding of the TNF-α pathway is important not only to suppress the formation of tumors but also to stop the progression of cancer. The regulation of apoptosis by RNF31 and its feedback loop could be important mechanisms for tumor cells to survive in unfavorable conditions. Thus, disruption of this machinery would provide a novel therapeutic strategy for tumors. Moreover, deregulation of NF-κB and/or apoptosis signaling results in immune related diseases, since both pathways are critical for normal development of immune system. Therefore, clear understanding of these pathways will improve approaches to control these diseases. In addition, this study will present the first insight into the physiological role of RNF31 and linear ubiquitination in the apoptosis pathway.

6.2 RNF31 in Mouse development

Genetically modified mouse is a valuable method to investigate the role of a target molecule in cellular events, especially signaling involved in mouse development. With the RNF31 knockout mouse, I found the pivotal function of RNF31 in mouse development. Deletion of RNF31 resulted in embryonic lethality of mouse between 7 and 9 days of pregnancy. And this lethality of RNF31 knockout mouse was not rescued by breeding with TNFR1 knockout mouse, indicating that RNF31 has a TNFR1-independent function in mouse development. Moreover, this mouse model demonstrated that RNF31 has a NF-κB independent role in mouse development since deficiency of NF-κB component dose not result in embryonic lethality (Table 1). Therefore, my study proposed the TNFR1- and NF-κB- independent function in mouse development.

Recently, another independent group reported the role of RNF31 in mouse development. The authors generated RNF31 knockout mouse and found the lethality of this mouse. And they crossed RNF31 knockout mouse with TNFR1 and TNFA knockout mouse to rescue the lethality. However, these crossing did not rescue the lethality but did delay the embryonic death. Additionally, endothelial specific deletion of RNF31 in mouse showed the embryonic lethality at same days as straight RNF31 knockout mouse, indicating that endothelial defect of RNF31 is the main cause of RNF31 knockout mouse's lethality. Finally, the authors concluded that enormous apoptosis of endothelial cells in straight RNF31 knockout or endothelial deletion of RNF31 could be accountable for defects of vascularization and consequently promote the lethality.

Genotype	Lethality
nfkb1$^{-/-}$	No
nfkb2$^{-/-}$	No
c-Rel$^{-/-}$	No
rela$^{-/-}$	Yes (~E15)
relb$^{-/-}$	No
ikka$^{-/-}$	Die at birth
ikkb$^{-/-}$	Yes (~E13)
nemo$^{+/-}$	Females are viable
nemo$^{-/-}$	Yes (~E10-11)

Table 1 The phenotype of NF-κB signaling related molecule knockout mouse.

6.3 RNF31 in Apoptosis

LUBAC is proposed to regulate the TNF-α–induced NF-κB signaling pathway. However, previously reported data demonstrated that LUBAC and linear ubiquitination play roles in cell death processes. For example, Sharpin-deficient MEF cells are more sensitive than WT cells to FADD-mediated cell death [91]. Moreover, RNF31 depletion leads to sensitization of cancer cells to cisplatin-induced cell death [209]. Recently, authors reported that RNF31 deficiency led to mouse embryonic lethality and that TNF receptor deletion prolonged this lethality [196]. Other investigators suggested a role for LUBAC in apoptosis with the finding that sensitization of epidermal keratinocytes to TNF-α–mediated cell death resulted in skin inflammation in Sharpin-deficient mice [194, 195]. However, the precise mechanism by which LUBAC regulates apoptosis remains to be elucidated. In particular, the physiological role of RNF31 is unclear owing to the lethality of RNF31 deficiency in mice. The present study is the first to provide genetic evidence of the molecular mechanism of how RNF31 and LUBAC regulate the apoptosis signaling pathways.

As described above, LUBAC is composed of three components: HOIL-1, Sharpin, and RNF31. RNF31 is a key molecule containing a crucial catalytic domain and requires either HOIL-1 or Sharpin as an associated molecule to generate linear ubiquitination. Biochemical evidence suggests that both HOIL-1 and Sharpin alone can induce linear ubiquitination and activate NF-κB signaling [91, 92, 97]. However, whether they have identical functions at the endogenous level remains in question. Sharpin-deficient cells are sensitive to FADD-mediated apoptosis, and KO mice exhibited hyperactivation of caspases in keratinocytes [202]. Also, our data on Sharpin-silenced HeLa cells demonstrated that Sharpin negatively regulates the cell death pathway. However, authors reported that HOIL-1–deficient mice had normal phenotypes [91] and even resistance to lipopolysaccharides (LPS)-induced lethality [124]. Moreover,

Sharpin and HOIL-1 have played roles in different signaling pathways in a LUBAC-independent manner [210-213]. Therefore, RNF31 may target different substrates depending on its associated molecules and regulate different signaling pathways. Further studies are required to clarify this. Necroptosis is the other type of cell death process that can be activated by TNF-α. Generally, upon TNF-α stimulation, activated caspase 8 suppresses necroptosis via cleavage of RIP1 and RIP3, which are key molecules for necroptosis. However, when caspase 8 or its function and/or activity is lost, RIP1 and RIP3 form a complex (necrosome) that promotes necroptosis [198]. Therefore, TNF-α stimulation dominantly promotes apoptosis in the majority of cells, as necroptosis is inhibited via this mechanism. Some cells are more susceptible than others to TNF-α–induced necroptosis, though, including HT29, HepG2, and L929 cells [199, 214, 215]. Our data demonstrated that RNF31 silencing sensitized HeLa and HCT116 cells (in which apoptosis is dominantly activated by TNF-α–based treatment; HeLa cells do not respond to TNF-α–induced necroptosis because RIP3 is defective in them) but did not significantly alter the sensitivity of HT29 or HepG2 cells (in which necroptosis is sensitive to TNF-α) to cell death, indicating that RNF31 primarily regulates the apoptosis pathway. Consistent with this observation, deletion of TRADD in keratinocytes completely prevented skin lesion development in *cpdm* mice [194]. Also, deficiency in RIP3 and MLKL (a molecule downstream of the RIP1/RIP3 complex to activate necroptosis) only mildly delayed the phenotype [195]. These findings indicated that LUBAC primarily regulates apoptosis, as TRADD-deficient MEFs are resistant to apoptosis, not necroptosis [194]. However, necroptosis still may be regulated by LUBAC in a cell- or tissue-specific manner, because some phenotypes of *cpdm* mice are rescued by deficiency in RIP3 or MLKL, such as the liver and splenic phenotypes [195]. Therefore, further investigation of cell death and linear ubiquitination in mouse development is required to answer these questions.

Although cFLIP is cleaved by activated caspase 8 during apoptosis [203], it is also actively modified by posttranslational modifications such as ubiquitination that lead to proteasome-dependent degradation. Thus, proteasome inhibitors such as MG132 and *clasto*-lactacystin protect cells from death receptor-mediated apoptosis via stabilization of cFLIP [216]. Thus far, researchers have identified two different ubiquitination sites—lysine 167 and lysine 192/195 [217, 218]—which are the targets of K-48 ubiquitination. Our present results suggest the existence of a novel ubiquitination site in cFLIP that is the target residue for linear ubiquitination. Linear ubiquitination of this residue regulates cFLIP stability by competing with K-48 ubiquitination. Previous studies supported these findings by suggesting a critical role for cFLIP in skin inflammation. First, cFLIP is highly expressed in the basal layer of the epidermis, indicating a pivotal role for it in the epidermis [219]. Second, mice with conditional deletion of cFLIP in the epidermis harbor severe inflammation of the skin originating from TNF-α–mediated keratinocyte apoptosis [220], which is similar to the phenotype in *cpdm* mice described above. Third, authors reported that treatment with the proteasome inhibitor bortezomib actually alleviated dermatitis in *cpdm* mice [202], indicating that proteasome-dependent degradation is critical for development of the skin phenotype in those mice. These studies suggested that degradation of cFLIP is the key process explaining the cause of the *cpdm* skin phenotype. However, I do not know whether both K-48 and linear ubiquitinations have identical cFLIP sites or which modification is dominant. I studied the role of ITCH in cFLIP degradation upon TNF-α–treatment but found that ITCH did not regulate the stability of cFLIP in our experimental model. Because identifying the E3 ligase that competes with LUBAC for destabilization of cFLIP is pivotal to answering this question, further investigations of E3 ligase targeting cFLIP are required. Furthermore, phosphorylation of cFLIP is a prerequisite for K-48 ubiquitination; phosphorylation of threonine 166 is a prerequisite for ubiquitination of lysine

167 [218], and phosphorylation of serine 193 is a prerequisite for ubiquitination of lysine 192/195 [217]. Therefore, phosphorylation of cFLIP possibly regulates its linear ubiquitination and stability. Further studies are required to validate this regulation.

Although data on Sharpin-defective MEFs and from biochemical experiments indicated that linear ubiquitination regulates activation of NF-κB signaling, others reported that these defects are not significant [221] and that Sharpin deficiency even leads to hyperactivation of NF-κB signaling [202]. Our data also indicated that silencing of RNF31 expression and deletion of *RNF31* did not significantly alter activation of the NF-κB pathway upon TNF-α stimulation. Recently, authors reported that HOIL-1 regulates NF-κB signaling in a cell-specific manner. HOIL-1 deficiency has a critical effect on NLRP3 inflammasome activation rather than NF-κB regulation in macrophages and primarily controls the NF-κB pathway in MEFs [124]. Therefore, the function of LUBAC in NF-κB signaling likely depends on the cellular context.

6.4 Cleavage of RNF31

RNF31 contains two functional domains to activate the NF-κB pathway, catalytic domain, RING between RING (RBR) and interacting domain, zing finger (ZF) [93]. Of note, the cleavage site that I discovered in this study is located between NZF1 and NZF2. Based on previous studies showing that ΔZF or NZF1 mutants have decreased ability to activate NF-κB signaling [92, 222], I speculated that the cleavage of RNF31 results to the separation of two functional domains (RBR catalytic domain and NZF1 domain), and therefore, cleaved fragments are not able to fully induce NF-κB activation. However, I questioned this model on the basis of ubiquitination data with NEMO and RIP1. Previous studies [189] and our data showed that a ΔZF or CT fragment could bind with NEMO in the presence of HOIL-a/Sharpin but the ΔZF or CT fragment alone was not able to. Moreover, CT is capable of conjugating linear ubiquitination chains to its substrates, NEMO and RIP1. Since the expression of linearly ubiquitinated NEMO is adequate to activate NF-κB signaling [189], these data suggest that RNF31 has an additional function in the downstream of ubiquitinated NEMO to regulate the NF-κB pathway. Thus, the cleavage RNF31 inhibits this additional function, not linear ubiquitination to suppress NF-κB activation. To determine the mechanism how RNF31 regulates NF-κB activation, further investigations are required

The stimulation of reconstituted cells with either TNF-α or TRAIL demonstrated the importance of balance between death and survival signaling. Since HeLa cells are resistant to TNF-α-induced death due to the dominant activation of NF-κB signaling [223], low doses of TNF-α were not able to activate caspases, and the cleavage of RNF31 was not enough to inhibit survival signaling. However, high doses triggered RNF31 cleavage, which blocks the activation of survival signaling. This observation was reversed in cells treated with TRAIL since TRAIL dominantly activates apoptosis in HeLa cells [223]. While high doses of TRAIL induced a high

degree of apoptosis that overwhelm inhibitory feedback from NF-κB activation, low doses activated NF-κB signaling and induced a lower degree of apoptosis. Therefore, restoration of KD cells with MT134 RNF31 enhanced the resistance to DR-mediated cell death when stimulated by high doses of TNF-α or low doses of TRAIL. Furthermore, the role of cleavage was further demonstrated in sequential treatment experiments. Since different factors that independently trigger survival and death govern the physiological outcomes in a combinational manner, this would be a good model to study the crosstalk between cell death and survival.

Many death-inducing agents such as TRAIL, FasL, and DNA damage inducers simultaneously activate NF-κB signaling that often leads to resistance to treatment [224]. Thus, the disruption of LUBAC or RNF31 activity could destroy the balance, which can be a promising target for treating deregulated cell death-oriented diseases. Our study not only expands our knowledge on the crosstalk between cell death and survival and but also provides a possible target mechanism to treat diseases resulted from the unbalance between death and survival. Specifically, the presented model; the sensitization of cells by RNF31 cleavage might represent a therapeutic strategy to increase the efficacy of drug by providing preferable condition in the combination therapy

CHAPTER VI: Future directions

7.1 Regulation of cFLIP by CK2, LUBAC and unknown E3 ligase

During the investigation of linear ubiquitination site on cFLIP, I found that phosphorylation of cFLIP might be a prerequisite for linear ubiquitination. First, I found that lysine 49 of human cFLIP is a potent target of ubiquitination based on the database of PhosphoSitePlus® (www.phosphosite.org) [225] which is the initiation of our approach to find out the linear ubiquitination site of cFLIP. At the same time, I found the serine phosphorylation site next to this potent ubiquitination site, serine 51. Since phosphorylation is often required to be conjugated with ubiquitin chains, I hypothesized that phosphorylation of Serine 61 is required for linear ubiquitination of cFLIP at lysine 49. Immunoprecipitation assay was performed to test whether the mutation of serine 51 in cFLIP affects the linear ubiquitination of cFLIP. I transfected flag tagged S51E (constitutively active form) and S51A (dominant negative form) mutant cFLIP into 293 T cell together with and without LUBAC. After 24 hours, flag tagged mutant cFLIP was immunoprecipitated with anti-Flag M2 bead, and the precipitates were analyzed using a WB assay. As shown in Figure 37, WT cFLIP was conjugated with linear ubiquitin chains. However, when serine 51 was mutated to glutamate, cFLIP did not get linearly ubiquitinated while mutation to alanine did not change the capacity of cFLIP to be conjugated with linear ubiquitin chains (Figure 65). Based on this data, I modified the hypothesis to "Phosphorylation of cFLIP at S51 suppresses linear ubiquitination of cFLIP at Lysine 49". To further demonstrate that Serine 51 is critical for linear ubiquitination and the stability of cFLIP, degradation pattern of WT, S51E and S51A mutants after treatment with TNF-a and CHX and sensitivity of reconstituted HeLa cell with each mutant to TNF-a induced apoptosis would be tested. Interestingly, the database from MyHits indicated that Serine 51 of cFLIP is a predicted phosphorylation site of cFLIP and this sites is possibly regulated by Casein Kinase II (CK2) [226]. Additionally, D. Llobet *et. al.* reported that CK2 regulates cFLIP stability and sensitivity of cells to apoptosis [227]. Thus, I hypothesized that phosphorylation of Serine 51 by CK2

(which is constitutively activated in normal cells [228]) inhibits linear ubiquitination of cFLIP. The critical experiment to test this hypothesis is a linear ubiquitination assay with CK2 inhibitors. Since I observed increased linear ubiquitination of S51E mutant compared with WT cFLIP in Figure 65, I speculate that pretreatment with CK2 inhibitor will enhance linear ubiquitination of cFLIP leading to stabilization of it. Additionally, information on E3 ligase for cFLIP ubiquitination is largely unknown. Therefore, identifying E3 ligase that competes with LUBAC to regulate cFLIP stability is important to complete the puzzle of RNF31 biology. These further studies will provide the first evidence that linear ubiquitination is regulated by phosphorylation and elucidate the regulatory mechanism of cFLIP stability with PTM such as phosphorylation and ubiquitination.

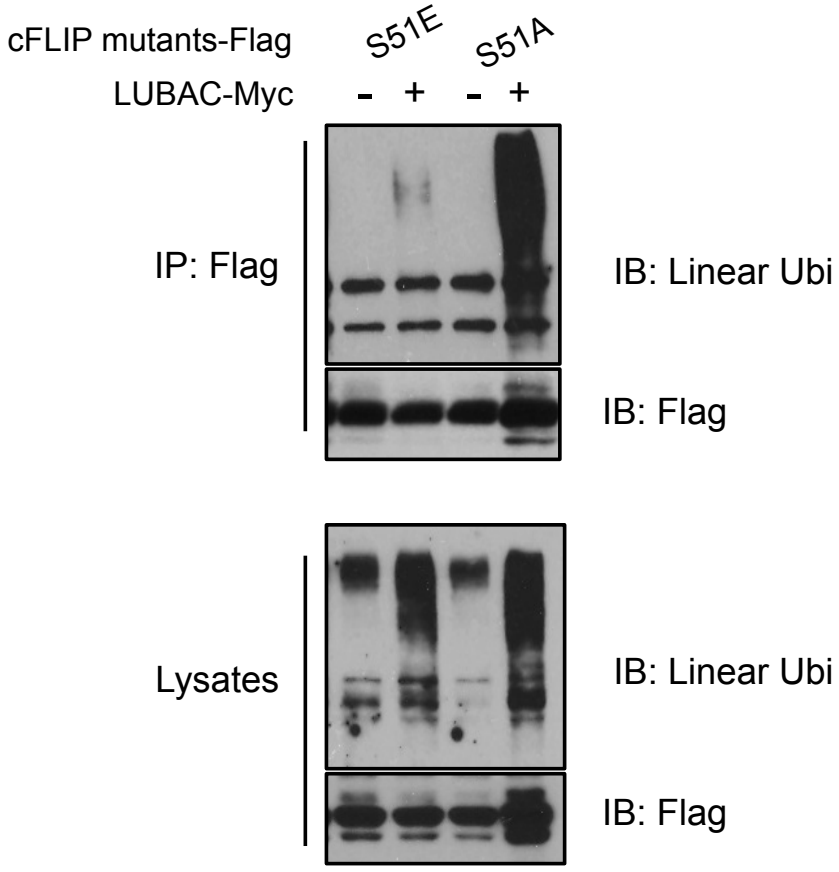


Figure 65 Mutation of Serine 51 alters the linear ubiquitination of cFLIP.

WB analysis of immunoprecipitates using anti-FLAG beads and lysates of 293T cells transfected with the indicated constructs.

7.2 Novel substrates of LUBAC

To date, known substrates of linear ubiquitination are limited: RIP1, NEMO and ASC. Additionally, I reported that cFLIP is a novel target of LUBAC for linear ubiquitination. However, LUBAC components deleted mouse shows dramatic phenotypes including embryonic death, severe skin inflammation and immune cell infiltration [194-196] indicating that there should be more substrates of linear ubiquitination. Therefore, it is important to screen a novel substrate of linear ubiquitination to describe the function of RNF31 and LUBAC. Although linear ubiquitination specific antibody is developed, the level of endogenous form is too weak to identify associated proteins through immunoprecipitation assay. Alternatively, identifying LUBAC associated proteins will lead to the discovery of novel substrates for linear ubiquitination. First, RNF31 will be introduced to Jurkat using retroviral system to generate stable cells expressing flag tagged RNF31. After stimulation with TNF-a, RNF31 will be precipitated with Flag M2 bead and associated proteins will be isolated after silver staining, followed by mass spectrometry analysis. The obstacle of this approach is that identified proteins are associated with single RNF31 and not LUBAC, which is not a target of linear ubiquitination. In order to exclude this possibility, Jurkat expressing flag tagged HOIL-1 or Sharpin will be generated and the identical experiments will be performed. Then, the candidate that binds with RNF31 and HOIL-1 or Sharpin will be selected for further molecular approach to confirm its ability to be conjugated with linear ubiquitin chains by LUBAC.

However, my data demonstrated that RIP1 did not bind with RNF31 or LUBAC although LUBAC is able to conjugate linear ubiquitin chains on RIP1. This mass spectrometry approach will omit some essential substrates. As an alternative, mutant ubiquitin in which all lysines are mutated to arginines will be introduced to Jurkat. Since the attachment of tagging proteins such as flag or His at N-terminus or C-terminus will prevent them from forming linear ubiquitination, the introduced ubiquitin will contain tagging protein in the middle of protein in

order to maintain its capability to generate linear ubiquitination. Using specific antibody to tagging protein, all linearly ubiquitinated proteins will be co-precipitated with mutated ubiquitins and associated molecules will be analyzed by mass spectrometry. Identifying a novel substrate for linear ubiquitination will be critical in expanding our knowledge on the biology of linear ubiquitination.

7.3 Targeting RNF31 in diseases such as cancers and immune diseases

As described in Chapter 2, RNF31 regulates apoptosis through the stabilization of cFLIP. Apoptosis is one of the hallmarks of cancer and a promising target for cancer therapy [229]. Therefore, targeting RNF31 would be a therapeutic approach to control cancers. In compared to normal bone marrow cells, I found that Smoldering Myeloma expresses the elevated level of RNF31 (Figure 66, [230]). Therefore, targeting RNF31 will sensitize myeloma cells to apoptosis by destabilization of cFLIP and suppress the NF-κB signaling, involved in the proliferation and survival of myeloma cells [231], which would make those cells vulnerable to conventional therapies. Targeting RNF31 in tumors in which cFLIP expression is elevated could be an effective way to control those cancers. For example, lymphomas [232-235] and colon cancers [236, 237] express high level of cFLIP and they are resistant to apoptosis. To evaluate the potential of RNF31 as a target of cancer therapy, expression of RNF31 will be silenced in myeloma, lymphomas, or colon cancer in which RNF31 or cFLIP are highly expressed and the RNF31 silenced cells will be injected into the xenograft mouse model. By monitoring the tumor growth and size, the effectiveness of targeting RNF31 on cancer treatment will be evaluated.

As described in the introduction, apoptosis and NF-κB signaling pathways play essential roles in immune responses. Hence, deregulation of these signaling often leads to autoimmune disease [238]. Since RNF31 and LUBAC are key mediators in these signaling, it is important to examine the function of these molecules in these auto-immune diseases. For example, to evaluate the role of RNF31 in rheumatoid arthritis (RA), conventional RA mouse models like collagen-induced arthritis (CIA) or human TNF-a transgenic mouse model can be utilized [239]. Since straight knockout of RNF31 results in embryonic lethality, cell specific KO (T cell or B cell specific) mouse, which is the C57BL/6 strain (susceptible to CIA induction [240]) mouse will be generated to study the function of these molecules. Inoculation of type II heterologous collagen with Freund's adjuvant to this mouse model will induce acute symptoms that are

similar to human RA. To study its function in chronic RA model, this conditional knockout (CKO) mouse will breed with human TNF-a transgenic mouse [239], which spontaneously develops chronic RA symptoms. By comparing initiation and symptoms of RA between WT and CKO mice, the role of RNF31 in acute and chronic RA will be demonstrated. The roles of immune related disease and the function of RNF31 in immune response are poorly understood. The function in immune responses could be investigated using genetically modified mouse model as well. First, the characterization of immune cell specific knockout mouse will help to illuminate its role in the immune system such as differentiation, population and development of immune cells. Moreover, microbe infection such as virus, bacteria and fungi in these conditional knockout mice will illustrate the immune regulating function of RNF31.

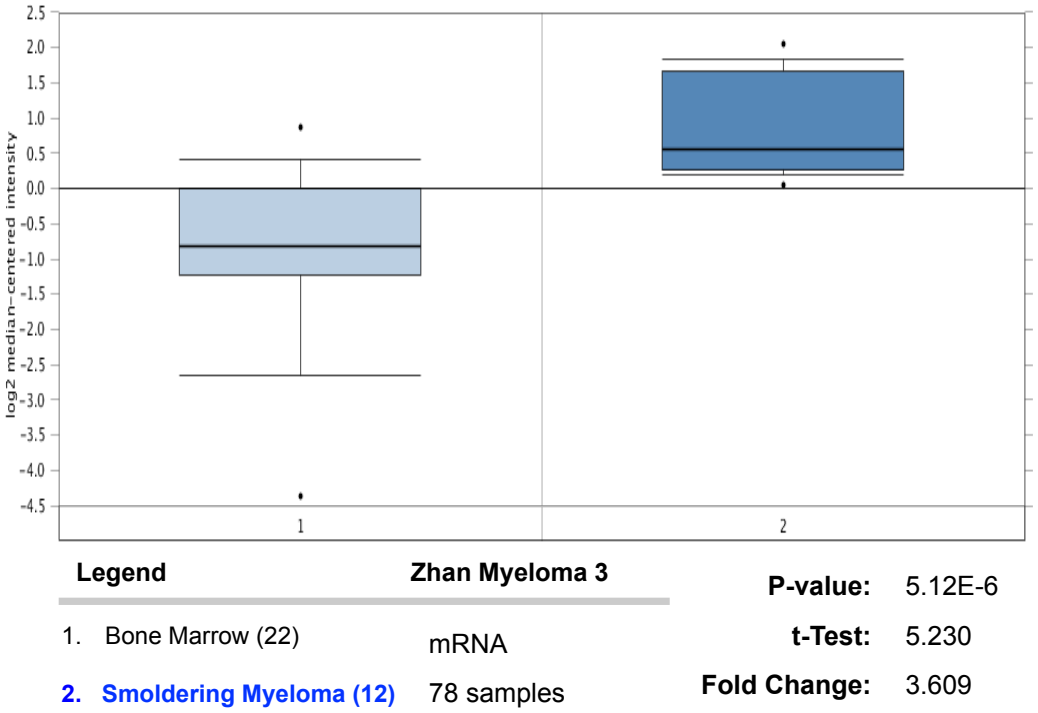


Figure 66 The mRNA expression level of RNF31 is elevated in Smoldering Myeloma.

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