

# Structural basis of cell apoptosis and necrosis in TNFR signaling

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**Abstract** The tumor necrosis factor receptors (TNFRs) play essential roles in innate and adaptive immunity. Depending on conditions, TNFR induces multiple cell fates including cell survival, cell apoptosis, and cell programmed necrosis. Here, we review recent progress in structural studies of the TNFR signaling pathway. The structural basis for the high order signal complexes, including the DISC, ripoptosome, necrosome, and RIP3/MLKL complex, may provide novel insights for understanding the biophysical principles of cell signaling cascades.

**Keywords** TNFR · Apoptosis · Necrosis · RIP1 · RIP3 · MLKL

## Introduction

Tumor necrosis factor (TNF) plays critical roles in diverse cellular processes and many pathological conditions by binding and activating its receptor TNFR [1]. The TNFR superfamily contains 29 members, which are transmembrane receptors composed of an extracellular domain and an intracellular domain. There are two groups of TNFRs, one is activating receptors that trigger multiple signaling pathways, such as nuclear factor  $\kappa$ B (NF- $\kappa$ B) and mitogen-activated protein kinase (MAPK) pathways [2, 3]. The other is death receptors (DRs), like TNFR1 and Fas, which

have a specific death domain (DD) that mediates cell apoptosis and necrosis. Among TNFR superfamily members, TNFR1 and CD40 can activate the NF- $\kappa$ B signaling pathway. Upon binding TNF $\alpha$ , the trimeric TNFR associates with the intracellular effector TNF receptor associated DD protein (TRADD), forms a large complex (complex I) by recruiting other factors including receptor-interacting protein kinase 1 (RIP1), cellular inhibitor of apoptosis protein 1 and 2 (cIAP1/2), and TNFR associated factors 2 (TRAF2) (Fig. 1). In complex I, the E3 ligases TRAF2/cIAP in addition with LUBAC (linear ubiquitin chain assembly complex) form K63 or K11 polyubiquitin chains on RIP1 [4]. The polyubiquitinated RIP1 engages downstream adaptors such as TGF $\beta$  activating kinase 1 (TAK1) and NEMO to activate the IKK complex, which in turn activating NF- $\kappa$ B transcription, promoting cell survival, proliferation and differentiation [5, 6]. Similar to the TNFR1 pathway, the trimeric TRAF6 directly interacts with the intracellular region of CD40, acting as the ubiquitin ligase to induced K63-linked polyubiquitination [7]. The polyubiquitin chains recruit downstream proteins, activating NF- $\kappa$ B translocation and transcription [8]. In addition to NF- $\kappa$ B activation, TNFR can also induce cell apoptosis and cell programmed necrosis upon binding with TNF $\alpha$  [9]. In this review, we will focus on and illustrates the structural basis for cell apoptosis and necrosis induced by TNFR signaling.

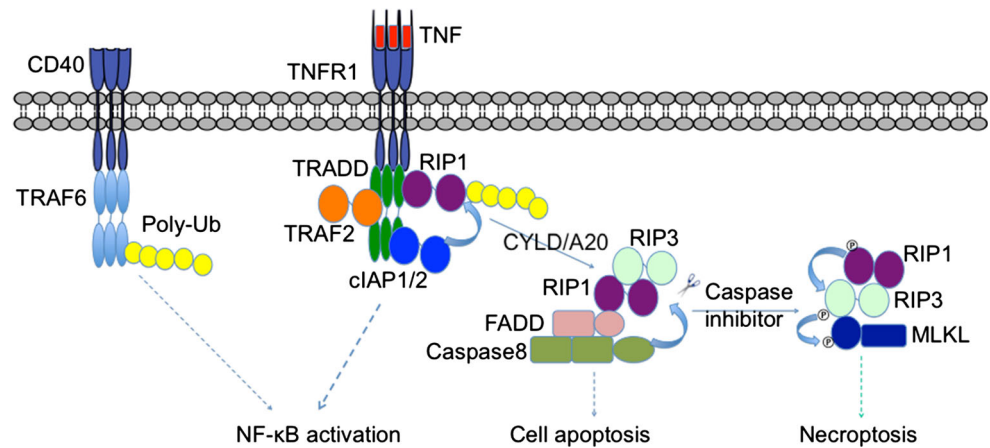
## Cell apoptosis induced by TNFR1 and Fas

In complex I, if the K63-linked polyubiquitin chain of RIP1 is removed by the deubiquitinases CYLD or A20, or blocked by knocking out of E3 ligases cIAPs, RIP1 and the related protein RIP3, together with FADD, TRADD, and

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**Fig. 1** Overview of the signaling pathways induced by TNFR. CD40 triggers NF- $\kappa$ B translocation and transcription via the adaptor protein TRAF6. TNFR1 is a pleiotropic receptor that induces activating and death signaling pathways including NF- $\kappa$ B, cell apoptosis, and cell necroptosis

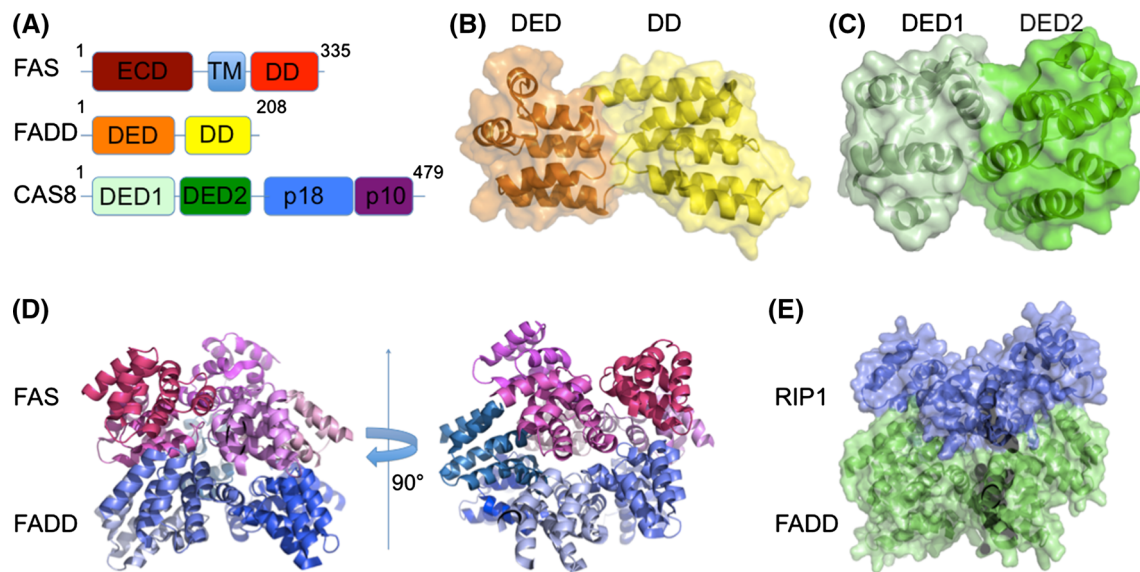


caspase-8, form a second complex (complex II). When caspase-8 is activated, it can cleave RIP1 or RIP3 at residues D324 or D328 respectively, and in turn inducing cell apoptosis [10, 11]. RIP1, FADD, and caspase-8 are the key components of this complex, and collectively referred to as the ripoptosome [12].

### Structural basis of the ripoptosome

FADD consists of a DD and a death effector domain (DED) (Fig. 2a). DD, DED, in addition with CARD and PYRIN (PYD) belong to the DD superfamily, which can form oligomeric complexes involved in immunity and the

inflammasome [13–15]. FADD interacts with RIP1 via the DD interaction, whereas recruits caspase-8 and -10 via the interactions with the tandem DEDs in the prodomain of the caspases [16, 17]. The typical DD is composed of a six alpha helices bundle [18]. The DD, DED, and full-length FADD structures have been determined by NMR and X-ray crystallography (Fig. 2b) [16, 19–21]. Although no high resolution structures are available for RIP1 DD or the DD complex between RIP1 and FADD, the MALS, electron microscopy (EM) average study and mutation analysis reveal that RIP1 and FADD form a large complex, probably in a 5:5 ratio [22]. This complex is very similar to the previous reported DD oligomers in the PIDDosome and Myddosome [18, 23]. Mutations on the interface between RIP1



**Fig. 2** Structures of the DISC and ripoptosome. **a** Domain schematics of human Fas, FADD, and procaspase (CAS8). *ECD* extracellular domain, *TM* transmembrane domain, *DD* death domain, *DED* death effector domain, *p18*, *p10* subunits of procaspase-8. **b** Solution structure of FADD (PDB: 2GF5). **c** Structure of the tandem DED

domain of a viral caspase 8/10 inhibitory protein vFLIP (PDB: 2BBR). **d** The crystal structure of Fas DD with FADD DD, which forms a 5:5 asymmetric architecture (PDB: 3OQ9). **e** Structural model of RIP1 DD:FADD DD complex based on the Fas/FADD complex structure

and FADD affect complex formation [22]. The catalytic domain of caspase-8 forms a dimeric structure shared by all caspases [24, 25]. As the tandem DED of caspase-8 is prone to form large aggregates or filaments in vitro and in cells [26], obtaining a high resolution structure by X-crystallography is difficult. However, the tandem DED domain of a viral caspase-8/-10 and FLICE/caspase-8 inhibitory protein (FLIP) from poxvirus shows a dumb bell shaped structure common to all tandem DEDs (Fig. 2c) [27, 28]. Analysis by mass spectrometry, quantitative western blots, and modeling, has shown that the amount of procaspase-8/10 and c-FLIP in cells exceeds that of FADD by seven to nine folds [29, 30]. Considering that some DD superfamily domains form helical filaments, the DED chain assembly may drive caspase-8 dimerization and activation, leading to cell apoptosis [15, 31, 32].

### Structural basis of the DISC

Similar to TNFR1, the transmembrane protein Fas induces cell apoptotic death. When ligand FasL binds to the extracellular domain of Fas, the cytosolic region of Fas recruits FADD via the DD interaction, forming a large complex [21]. FADD in turn engages with caspase-8/10 via the DED interactions. Fas, FADD, and caspase-8/10 form a ternary complex, traditionally named the death inducing signaling complex (DISC), which is essential for Fas-mediated apoptosis [33].

The Fas DD structure reveals an antiparallel six helices bundle, whereas the Fas DD: FADD DD complex shows an oligomeric architecture (Fig. 2d) [21, 33]. Similar to the structures of the DD/DD complexes in the PIDDosome and Myddosome, and the PYD/PYD complex in ALR [15, 18, 23], the class projection averages of EM study reveals that the Fas DD:FADD DD complex has an asymmetric oligomeric structure. Using nanoflow electrospray ionization and tandem mass spectrometry, the complex has been shown to be mostly a mixture of 5 FADD:5 Fas, 5 FADD:6 Fas, and 5 FADD:7 Fas complexes. Mutations associated with autoimmune lymphoproliferative syndrome in humans directly affected the formation of Fas/FADD complex [21]. As the ripoptosome core RIP1/FADD forms a large complex with high similarity to the DISC, a structural model was proposed for the RIP1/FADD complex (Fig. 2e) [22].

### Cell programmed necrosis induced by TNFR1

Cell programmed necrosis, or necroptosis, is referred to the cell death induced by death receptor TNFR1 when both RIP1 dependent NF- $\kappa$ B activation and caspase-8 dependent cell apoptosis are inhibited or blocked. Cell necroptosis is

involved in many human diseases, including lymphoproliferative diseases, Crohn's disease, acute liver injury, ischemic brain injury, and myocardial ischemia–reperfusion injury [34]. Cell necroptosis is initiated by the formation of the RIP1/RIP3 necrosome when the NF- $\kappa$ B activation and apoptosis pathways are blocked [35–37]. RIP1 and RIP3 play important roles in host defense against viral and bacteria infections [38–40]. The downstream effectors of cell necroptosis include the mixed lineage kinase like protein (MLKL) and the mitochondrial protein phosphatase PGAM5 [41, 42]. Upon phosphorylation by RIP3, MLKL trimerizes and in turn makes it able to bind phosphatidylinositol phosphates and cardiolipin. Oligomerized MLKL translocates to the plasma membrane and functions directly as a pore or indirectly as an ion channel regulator [43–45]. In contrast to apoptosis, necroptosis can proceed independently of mitochondria [46].

### The RIP1/RIP3 necrosome

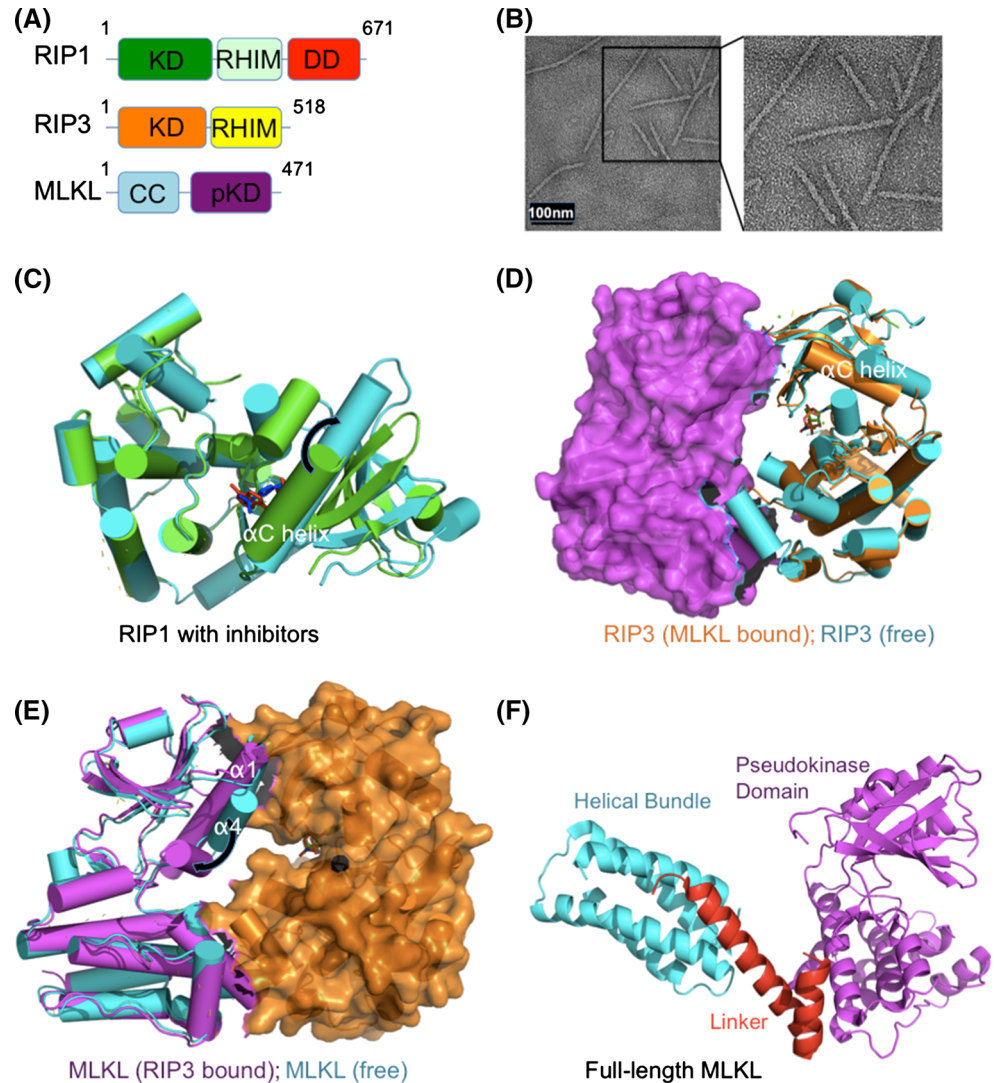
RIP1 and RIP3 have an N-terminal kinase domain (KD) and a RIP homotypic interaction motif (RHIM), whereas RIP1 has one DD at its C-terminal end (Fig. 3a). RIP1 and RIP3 form a necrotic signaling complex via the RHIM interaction [47, 48]. In vitro and in vivo studies have shown that the RIP1/RIP3 necrosome forms an amyloid structure [49]. As the RHIM interaction and kinase activity of RIP1/RIP3 are required for TNF-induced programmed necrosis [35, 49], phosphorylation and necrosome formation may be mutually reinforcing. The amyloid scaffold functions as a crucial platform for recruiting other components, such as MLKL, and triggers the downstream execution of cell necroptosis (Fig. 3b) [49].

The KDs of RIP1 and RIP3 share 33 % sequence identity and 53 % similarity, whereas they have similar 3-D structures [50–52]. The KDs have a typical kinase domain architecture common to other kinases, which is composed of an N-lobe and a C-lobe. Necrostatin is the first identified inhibitor that can specifically inhibit the kinase activity of RIP1, in turn preventing the downstream RIP3 dependent cell necrosis and caspase-8 dependent cell apoptosis. Structures of RIP1 KD bound to necrostatin and its different analogs have revealed that the RIP1 KD is in inactive conformation. The inhibitors are caged in a hydrophobic pocket between the N- and C-lobes, interacting with the highly conserved amino acids in the active loop (Fig. 3c) [52].

As necrostatins have poor pharmacokinetic properties that render them suitable for therapeutic development, Harris et al. developed a series of new kinase inhibitors targeted to the inactive DFG (Asp-Phe-Gly) conformation [53, 54]. The crystal structure of RIP1 with 1-aminoisoquinoline 8

**Fig. 3** Structures of the RIP1/RIP3/MLKL necrosome.

**a** Domain organization of human RIP1, RIP3, and MLKL. *KD* kinase domain, *RHIM* RIP homotypic interacting motif, *pKD* pseudokinase domain, *DD* death domain, *CC* coiled-coil domain. **b** Filamentous structure of RIP1/RIP3 is visualized by electron microscopy (EM); **c** Structural comparison of RIP1 KD bound with necrostatin-1 (green, PDB: 4ITH) and with 1-aminoisoquinoline (cyan, PDB: 4NEU). Major differences exist in the  $\alpha$ C helix. **d** Conformational changes in RIP3 upon binding to MLKL. Free RIP3 is shown in cyan, MLKL-bound RIP3 is shown in orange. MLKL is shown in purple and AMP-PNP is represented by sticks (PDB: 4M66 and 4M69). **e** Structural comparison of free MLKL and RIP3-bound MLKL. Free MLKL is shown in cyan and the RIP3-bound MLKL is colored in purple. RIP3 is shown in orange and AMP-PNP is represented by sticks (PDB: 4M68 and 4M69). **f** Structure of full-length MLKL reveals an N-terminal helical bundle (cyan), a two-helix brace (red), and a C-terminal pseudokinase domain (purple) (PDB: 4BTF)



revealed a different inactive conformation with those bound with necrostatins [52, 53]. The 1-aminoisoquinoline heterocycle of 8 forms hydrogen bonds with residues Glu63, Met 95, and Asp156 in the DLG motif, whereas necrostatins bind with Val76, Met92, Asp156, and Ser161 [53].

### The RIP3/MLKL kinase complex

MLKL consists of an N-terminal coiled-coil domain and a C-terminal pseudokinase domain [50]. Necrosulfonamide covalently links to Cys86 of MLKL and blocks necrosis downstream of RIP3 activation [41]. The KD of RIP3 and the KD-like domain of MLKL form a stable complex after MLKL is phosphorylated by RIP3 at Ser457 and Thr458.

The structures of RIP3 and MLKL KDs revealed that they adopt the canonical kinase fold (Fig. 3d, e) [51]. The RIP3 KD is an active conformation, whereas MLKL bound

RIP3 is in inactive conformation stabilized primarily by nucleotide AMP-PNP located in a pocket close to the P-loop and catalytic loop of RIP3 [51]. Compared to free RIP3, the MLKL-bound RIP3 undergoes a drastic conformational change including shifting of the  $\alpha$ C helix and rearrangement of the DFG motif in the activation loop [51]. The movement of the  $\alpha$ C helix leads to disruption of the salt bridge between Lys51 and Glu61 in RIP3 (Fig. 3d). On the other hand, a structural comparison of free MLKL and RIP3-bound MLKL revealed dramatic conformational changes involving in the  $\alpha$ 1 and  $\alpha$ 4 helices, corresponding to the  $\alpha$ C helix and the activation loop in RIP3 KD respectively. The rearrangement of  $\alpha$ 1/ $\alpha$ 4 helices and the following loop may facilitate the interaction between RIP3 and MLKL (Fig. 3e).

The full-length MLKL structure reveals that it is composed of a four-helical bundle tethered to the pseudokinase domain (Fig. 3f) [50]. Despite being able to binding ATP,

MLKL is catalytically inactive [50]. The N-terminal helical bundle is important for oligomerization of MLKL [43–45]. The C-terminal KD of MLKL adopts a typical kinase-like fold, comprised of a five antiparallel beta strands at the N-lobe, the brace  $\alpha$ C helix, and the C-lobe  $\alpha$ -helices. Different from canonical kinases, MLKL lacks the typical Asp in the HRD motif of the catalytic loop and the Asp in the DFG motif that is responsible for  $Mg^{2+}$  coordination, which explains the structural basis for the pseudokinase family.

## Conclusions

The signal transduction pathway triggered by TNF induces different cell fates. Structural studies on cell apoptosis and programmed necrosis have greatly helped us understand the elaborate mechanism involved in cell signal transduction in innate immunity. The recent progress in studying the DISC, ripoptosome, and necrosome reveals that the high-order signaling machines are involved in many intracellular processes [31]. In addition, the DD superfamily can form helical filaments to execute signal transduction and signal amplification [15, 55]. These higher order structures may open up new avenues for understanding cell signal transduction in innate and adaptive immunity.

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