^{FADD}DED filaments coordinate complex IIa assembly during TNF-induced apoptosis

Ying Chen[1†*], Vinh Thang Huynh[1†*], Lihua Lai[1†], Tongyang Li[1], Yaw Bia Tan[3,4], Che Shin Chew[3,4], Amhed Missael Vargas Velazquez[1], Ping Liu[3,4], Firdaus Samsudin[7], Jan K. Marzinek[7], Peter J. Bond[7,8], Wu Bin[4,5], Dahai Luo[3,4,6*], Vinay Tergaonkar[1,2,*]

Affiliations:

[1]Institute of Molecular and Cell Biology (IMCB), Agency for Science, Technology and Research (A*STAR); Singapore, 138673, Singapore.

[2]Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore (NUS); Singapore, 117596, Singapore.

[3]Lee Kong Chian School of Medicine, Nanyang Technological University, EMB 03-07, 59 Nanyang Drive, Singapore 636921, Singapore.

[4]NTU Institute of Structural Biology, Nanyang Technological University, EMB 06-01, 59 Nanyang Drive, Singapore 636921, Singapore.

[5]School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551, Singapore.

[6]National Centre for Infectious Diseases, Singapore, 308442, Singapore

[7]Bioinformatics Institute (BII), Agency for Science, Technology and Research (A*STAR), 30 Biopolis Street, #07-01 Matrix, Singapore 138671, Republic of Singapore

[8]Department of Biological Sciences, National University of Singapore, Singapore 117543, Singapore

†These authors contributed equally to this work

*Correspondence: cheny3@imcb.a-star.edu.sg; vhthang@imcb.a-star.edu.sg; LuoDahai@ntu.edu.sg; vinayt@imcb.a-star.edu.sg

Lead contact: vinayt@imcb.a-star.edu.sg

Abstract

Extrinsic apoptosis is initiated by signaling from death receptors, leading to the assembly of RIPK1, FADD, and caspase-8 complex. Subsequently, caspase-8 forms a filamentous structure through the oligomerization of its tandem death effector domain (tDED), resulting in caspase activation and cell death. Although the DED of FADD (^{FADD}DED) is homologous to the tDEDs of caspase-8 (^{casp8}tDED), the functional form of ^{FADD}DED in extrinsic apoptosis remains unclear. Here, using cryogenic-electron microscopy, we elucidate the structure of ^{FADD}DED filaments comprising three helical chains assembled through three types of iterative interactions. Mutations disrupting ^{FADD}DED filament formation impair the recruitment of RIPK1 and caspase-8, and abrogate the cell death response, suggesting that ^{FADD}DED filamentation is essential in extrinsic apoptosis. Contrary to the belief that the homotypic death domains of RIPK1 and FADD are solely responsible for their interaction, we here show this interaction requires ^{FADD}DED filamentation. Furthermore, cFLIP can disrupt ^{FADD}DED filaments, uncovering an additional anti-apoptotic mechanism of cFLIP beyond its disruption of caspase-8 filament. Atomic resolution molecular dynamics simulations reveal that ^{FADD}DED filament thermodynamically favors ^{casp8}tDED monomer over ^{FADD}DED monomer, thus explaining the hierarchy and stoichiometry of FADD/caspase-8 complex assembly. These findings highlight the hitherto unappreciated roles of ^{FADD}DED filament formation in extrinsic apoptosis.

Keywords: Extrinsic apoptosis, TNFR1, RIPK1, FADD, caspase-8, filament

Significance

The mechanism of how TNF—a potent mediator of inflammation—triggers extrinsic apoptosis is key to understanding both physiological homeostasis and inflammatory ailments. Upon TNF receptor ligation, it is believed that merely one or three molecules of FADD are sufficient to recruit RIPK1 and nucleate caspase-8 filamentation, forming a multiprotein complex that mediates apoptosis. Using cryo-EM, we determined the filament structure comprising death effector domains (DED) of FADD (^{FADD}DED) and revealed crucial interfaces mediating ^{FADD}DED assembly, including a serine-rich motif unique to FADDDED filament. Disruption of ^{FADD}DED filamentation by structure-guided mutagenesis abrogates the interactions of FADD with RIPK1 and caspase 8 and consequently impairs extrinsic apoptosis. These findings establish ^{FADD}DED filament formation as a key mechanistic step for TNF-induced apoptosis.

Introduction

Extrinsic apoptosis, a well-known form of regulated cell death (RCD), plays a critical role in embryonic development and tissue homeostasis in multicellular organisms (1). It is initiated by the engagement of members of the death receptor superfamily, such as FAS, TRAIL receptors, and Tumor necrosis factor receptor superfamily member 1A (TNFR1). Activation of these cell surface receptors by their respective ligands triggers the assembly of multiprotein cytoplasmic complexes comprising FADD, caspase-8, and cFLIP, responsible for caspase-8 processing and subsequent apoptosis. Deregulation of extrinsic apoptosis is associated with various diseases, including autoimmune diseases, neurodegenerative disorders, and cancers (2). For instance, genetic mutations that cause loss-of-function of FAS lead to autoimmune lymphoproliferative syndrome (ALPS) in humans (3, 4). In particular, TNF-induced cell death is implicated in a myriad of inflammatory diseases (5), contributing to the success of anti-TNF biologics as one of the best-selling pharmaceuticals in history. However, the molecular mechanisms involving FADD and caspase-8 downstream of TNFR signaling remain underexplored, as most research on this complex has concentrated on FAS and TRAIL signaling (6-9).

In the absence of E3 ligases such as cellular inhibitor of apoptosis proteins (cIAPs) (10, 11), TNF engagement with TNFR1 could robustly trigger the formation of complex IIa, comprising RIPK1, FADD, and caspase-8. Structurally, FADD consists of an N-terminal death domain (DD) and a C-terminal death effector domain (DED), which mediates the homotypic interaction with the DD of RIPK1 (^{RIPK1}DD) and tandem DED of caspase-8 (^{casp8}tDED), respectively. The association of ^{FADD}DED with ^{casp8}tDED induces ^{casp8}tDED filament formation, resulting in caspase-8 autoproteolysis and subsequent apoptosis (12) —a process that can be inhibited by cFLIPs which are enzymatically inactive paralogs of caspase-8 (13). The interactions between the complex components are key determinants of the cell death decisions downstream of TNFR1, including apoptosis and necroptosis. For example, the loss of FADD or caspase-8 paradoxically leads to embryonic lethality due to RIPK3-mediated necroptosis (14-16), supporting the role of FADD and caspase-8 in inhibiting necroptosis. Particularly, the enzymatic activity of caspase-8 is crucial to restrain aberrant activation of RIPK1 and RIPK3 as caspase-8 inhibition switches TNF-induced apoptosis to necroptosis (17-19). Moreover, RIPK1 also plays a key role in determining cell fate. RIPK1-null mice exhibit postnatal lethality that can be rescued by the combined loss of caspase-8 and RIPK3 (20), suggesting that apoptosis and necroptosis drive embryonic lethality in the absence of RIPK1 and that RIPK1 inhibits these two pathways during development. Recent findings suggest that RIPK1 exerts the protective function via its interaction with FADD, as mutations in the ^{RIPK1}DD that disrupt the interaction lead to the activation of both caspase-8 and RIPK3, causing embryonic lethality (21, 22). These findings highlight the complex interplay between various cell death mediators in orchestrating cell fate decision-making. In particular, uncovering the factors that govern RIPK1-FADD interactions and the subsequent effects will be key for understanding both homeostasis and disease.

Despite containing the orthologous DED similar to caspase-8, structural determination has only been achieved for monomeric ^{FADD}DED (23, 24) and for a trimeric chain of ^{FADD}DED (23). Furthermore, the prevailing model suggests that ^{FADD}DD and ^{FADD}DED independently mediate the interactions with RIPK1 and caspase-8, respectively, and one to three FADD

molecules are thought to be sufficient to seed the formation of ^{casp8}tDED filaments (23, 25). Therefore, the formation and cellular function of ^{FADD}DED filaments remain to be fully elucidated.

Here, we solved the filamentous structure of ^{FADD}DED using cryogenic-electron microscopy (cryo-EM) and found that three typical interactions underpin the filament assembly. Structure-guided mutagenesis targeting each type of interaction disrupted ^{FADD}DED filament formation, complex IIa assembly and consequentially protected cells against extrinsic apoptosis signals, indicating that all these three types of interfaces are equally important. In addition, all-atom molecular dynamics simulations (MD) demonstrated that ^{FADD}DED filaments favor the incorporation of ^{casp8}tDED monomers over ^{FADD}DED monomers through electrostatic interactions, explaining the previously documented hierarchical assembly and stoichiometry of the FADD/caspase-8 complex. Furthermore, we showed that cFLIP inhibits ^{FADD}DED filament formation, thus revealing another anti-apoptotic mechanism beyond the conventional model wherein ^{cFLIP}tDED intercalates and disrupts ^{casp8}tDED filamentation. Our study unveils the structure of ^{FADD}DED filaments and elucidates their role in mediating complex IIa assembly.

Results

^{FADD}DEDs assemble into a filamentous structure.

The recombinantly expressed full-length FADD is prone to aggregation (26), which poses significant challenges for expression, purification, and atomic-resolution structure determination. To overcome this problem, we constructed a recombinant FADD protein with N-terminal hexa-Histidine, StrepII, and SUMO tags and a C-terminal fusion with ^{casp8}tDED carrying F122G/L123G (His-StrepII-SUMO-FADD-^{casp8}tDED^{mut}) **(SI Appendix, Fig. S1A)**. After sequential His/StrepII affinity column purifications, we successfully obtained soluble His-StrepII-SUMO-FADD-^{casp8}tDED^{mut} proteins. By resolving the purified recombinant FADD fusion proteins on a Superdex 200 size-exclusion chromatography (SEC) column, we observed a broad protein peak starting from the 8 ml elution **(SI Appendix, Fig. S1B)**. This suggested that the FADD fusion protein existed in various oligomeric forms. Examination of the recombinant FADD fusion protein eluted from the void fraction **(SI Appendix, Fig. S1B, fraction D1)** by negative-staining electron microscopy (EM) revealed filamentous structures **(SI Appendix, Fig. S1C)**. Since the F122G/L123G mutant ^{casp8}tDED cannot form filaments (27), the observed filamentous structures might be composed of ^{FADD}DED.

Cryo-EM structure of ^{FADD}DED filaments

Based on the analysis of power spectra and symmetry search, we elucidated a 3.04-Å resolution cryo-EM map that presents a tube-like filamentous structure (**SI Appendix, Table S1**). The filament possesses a typical C3 helical symmetry with an azimuthal angle of 49.53°, an axial rise of 13.97 Å per subunit, and a helical pitch of 101.45 Å. The ^{FADD}DED filament measures an internal diameter of ~26 Å and an external diameter of ~75 Å **(SI Appendix, Fig. S1C)**. From the resolved filamentous cryo-EM map, Model Angelo assisted in *de novo* model building identified ^{FADD}DED as the building unit. Model building of ^{FADD}DED monomer consisting of residues 1-81 was followed by structural refinement (**SI Appendix, Fig. S2**).

^{FADD}DED filament **(Fig. 1A and SI Appendix, Fig. S3A)** is composed of three DED oligomer chains that assume a helical coil conformation **(Fig. 1B).** The individual DED unit within one chain consists of six anti-parallel alpha helices (H1 to H6), resembling a typical W shape **(Fig. 1C).** H1 and H2 helix pair interlocks with H4 and H5 helix pair while H3 and H6 position on the sides **(SI Appendix, Fig. S3B)**. Previously, the structures of monomeric ^{FADD}DED with F25 mutations (PDB: 1A1W and 1A1Z) and a chain of three ^{FADD}DED copies (PDB: 8YBX) were determined by NMR and cryo-EM, respectively (23, 24). Upon aligning the structure of a single DED within the ^{FADD}DED filaments to these existing structures of the monomeric ^{FADD}DED or the ^{FADD}DED chain, we observed no significant structural rearrangements (**SI Appendix, Fig. S3C-E)**, implying that neither F25 mutation nor DED oligomerization causes appreciable alterations in the conformation of DED monomers.

Three types of interaction underpin ^{FADD}DED filament structures **(Fig. 1D-E)**, including type I which is necessary for chain elongation, and type II and III which dictate chain-to-chain dynamics. To improve clarity, we describe these interactions by focusing on a central monomer (orange) and its interactions with three neighboring monomers **(Fig. 1E-H, green, pink, and cream-colored monomers).** Within a chain, the green monomer's H1 helix (M1, L5, L8, H9 residues), and H4 helix (L43) form the Ib interface. On the other hand, the orange monomer's H2 (F25, L26) forms a highly conserved "FL" motif, constituting the Ia interface **(Fig. 1F)**. Together these hydrophobic interactions constitute the type I interface. In the type II interaction, the orange monomer's H4 (E51, Q52, and N53) forms the IIb interface while the cream-colored monomer's H6 (R71, R72, H73, D74) makes up the IIa interface **(Fig. 1G)**. In type III interaction, the orange monomer's H3 (R34, K33) constitutes the IIIb interface while the linkage between H1 and H2 (S12, S13, S14, L15, S16) of the pink monomer makes up the IIIa interface **(Fig. 1H)**. These interaction interfaces are reiterated across the filament. Residues involved in each interface were corroborated by structural analysis with PDBePISA (28) **(Fig. 1I and SI Appendix, Fig. S4)**.

Interface residues are crucial for ^{FADD}DED filament formations *in cellulo* and *in vitro*

We next asked whether ^{FADD}DEDs could form filamentous structures in cells. Ectopic expression of mCherry-^{FADD}DED **(SI Appendix, Fig. S5A)** in 293T cells led to robust filament formation **(SI Appendix, Fig. S5B)**, reminiscent of the filaments formed by ectopic expression of ^{casp8}tDED (27, 29). Notably, the filaments were distinctly phase-separated from the cytoplasmic homogenates, observable by differential interference contrast (DIC) microscopy **(SI Appendix, Fig. S5B)**. The same ^{FADD}DED filaments were observed in caspase-8 knockout (casp8^{KO}) MDA-MB-231 cells **(SI Appendix, Fig. S5C-D)**, suggesting that ^{FADD}DEDs can form filaments independent of caspase-8. Next, we conducted structure-guided mutagenesis **(Fig. 2A)** to assess the roles of interface residues involved in ^{FADD}DED filament formation. Among the tested residues, all except for M1G at the Type Ib interface **(Fig. 2B)**, Q52G at the Type IIb interface **(Fig. 2C)**, and S12D at the Type IIIa interface **(Fig. 2D)**, effectively disrupted the formation of ^{FADD}DED filaments when mutated individually. The residues critical for ^{FADD}DED filament formation are highly conserved across various species (**SI Appendix, Fig. S5E)**, underscoring their evolutionary significance in filament assembly. While many residues mediating the three types of interactions correspond to those in ^{casp8}tDED filament formation (**SI Appendix, Fig. S5F)**, residues L8, L15, and S16 were

identified as uniquely critical for the formation of ^{FADD}DED filaments.

We next generated His-StrepII-SUMO-FADD-^{casp8}tDED^{mut} constructs with L8G, H9G, F25G, R34E mutations on FADD and purified the resulting recombinant proteins to evaluate DED oligomerization. Despite disrupting filament formation in cells **(Fig. 2B-D),** these single mutations did not abolish DED-DED interactions based on the SEC elution profile (**Fig. 2E**). Subsequent analysis of the void fractions using negative-staining revealed random aggregates **(SI Appendix, Fig. S5G-J)** rather than filaments observed with the wildtype DED construct **(SI Appendix, Fig. S1C),** confirming that these mutations disrupt ^{FADD}DED filament formation. Together these results suggested that the cryo-EM structure of ^{FADD}DED filaments was consistent with those formed in cells upon ectopic expression and concerted Type I, II, and III interactions were necessary for the formation of ^{FADD}DED filament.

^{FADD}DED filamentation is essential for robust complex IIa assembly and TNF-induced cell death

We next examined the function of ^{FADD}DED filaments in triggering apoptosis downstream of TNFR1 signaling. Complex IIa-mediated apoptosis can be robustly induced by treating cells with the combination of TNF (T) and cIAPs antagonist birinapant (B) (30, 31) **(SI Appendix, Fig. S6A-C)**. FADD knockout (FADD^{KO}) by CRISPR technology protected cells against BT treatment (**SI Appendix, Fig. S6B-C)**, abrogated caspase-3/8 and PARP processing **(SI Appendix, Fig. S6D)**, confirming that FADD is indispensable for complex IIa mediated cell death. Furthermore, when FADD was depleted, the interaction between the cell death decision maker RIPK1 and caspase-8 was severely reduced **(SI Appendix, Fig. S6E)**. Notably, unlike the reconstitution of full-length FADD (FADD^{FL}), the reconstitution of FADD with DED deletion (FADD$^{\Delta DED}$) failed to restore BT sensitivity **(SI Appendix, Fig. S7A),** processing of apoptotic markers (**SI Appendix, Fig. S7B**), and interaction between FADD and caspase-8 **(SI Appendix, Fig. S7C)**. Unexpectedly, DED deletion largely abolished the interaction with RIPK1 **(SI Appendix, Fig. S7C)**, suggesting that ^{FADD}DD and ^{RIPK1}DD were insufficient and required ^{FADD}DED for RIPK1-FADD interaction. Similar results were observed in FADD knockdown (shFADD) 293T cells **(SI Appendix, Fig. S7D)**, wherein the transfection of StrepII-tagged FADD^{FL}, but not the empty vector or FADD$^{\Delta DED}$ resulted in robust interaction with RIPK1 and caspase-8 **(SI Appendix, Fig. S7E)**. Together, these results highlight the hitherto unappreciated role of ^{FADD}DED in orchestrating RIPK1-caspase-8 complex formation via two possible mechanisms: a) stabilization of ^{FADD}DD-^{RIPK1}DD interaction and b) recruitment of caspase-8.

To evaluate the role of ^{FADD}DED filamentation in complex IIa-mediated cell death, we transduced FADD^{KO} cells with full-length FADD constructs with either WT DED or mutant DED. Under basal conditions (untreated, UT), cells were similarly viable across the various reconstitutions (**Fig. 3A**), indicating that these FADD constructs were tolerated. Unlike those reconstituted with an empty vector, FADD^{KO} cells reconstituted with WT FADD regained sensitivity to BT treatment. On the other hand, DED mutations that abrogated filament formation —including L8G, H9G, L15G, F25G, L26G, and R72E —did not restore the apoptotic response, whereas mutations that retained filament-forming capability did **(Fig. 3B)**. The processing of PARP, caspase-8, and caspase-3 was consistent with the observed

levels of cell death in both untreated **(Fig. 3A, 3C)** and BT-treated conditions **(Fig. 3B, 3D)**. Notably, the protein levels of FADD constructs varied, indicating that the ^{FADD}DED mutations differentially affected protein stability **(Fig. 3C, 3D)**. However, the key determinant of the cell death response was the capability to form functional DED filaments. For instance, the low protein levels of WT and S12D mutant constructs were sufficient to trigger an apoptotic response upon BT treatment **(Fig. 3D, lanes 2 and 7)**. Conversely, despite high protein levels, F25G and L26G mutants were unable to facilitate an apoptotic response **(Fig. 3D, lanes 9-10)**. Together, these results suggest that FADD filament formation is a prerequisite for mediating apoptosis.

To further elucidate the role of ^{FADD}DED filament formation, we pulled down ectopically expressed WT and filament formation defective mutants from 293T cells with endogenous FADD knockdown. In contrast to WT FADD, the filamentation-defective mutants failed to robustly interact with RIPK1 and caspase-8 **(Fig. 3E, lane 1 versus lanes 2-7).** We further evaluated the interactions in the reconstituted MDA-MB-231 FADD^{KO} cells. WT FADD, but not other mutant FADD, associates with RIPK1 and caspase-8 in response to BT treatment (**Fig. 3F, lane 1 versus lanes 2-5**). These findings collectively demonstrate that FADD filament formation through DED oligomerization is essential for the effective assembly of complex IIa and the subsequent induction of a productive apoptotic response.

^{FADD}DED filament energetically favors the incorporation of ^{casp8}tDED monomers over ^{FADD}DED monomers

Since ^{FADD}DED monomers can be assembled into DED filaments, they should co-mingle with ^{casp8}tDED within the filament. However, prior observations indicate that ^{FADD}DED is restricted to one end of the FADD-caspase-8 filament (23, 27), and FADD is substoichiometric to caspase-8 (9, 32, 33). These observations suggest that there is an early switch wherein the filament only incorporates ^{casp8}tDED instead of ^{FADD}DED. To better understand the orderly recruitment of caspase-8 to ^{FADD}DED filament, we performed all-atom molecular dynamics (MD) simulations of the ^{FADD}DED filament bound to either a homomeric ^{FADD}DED or a ^{casp8}tDED in explicit solvent. Each simulation was run for 1,000 ns in triplicate (Methods). Overall, the filament was very stable in both simulations. Overlaid snapshots from an example simulation replica show that both DED subunits remained stably bound to the filament interface with small movements by the monomers and no unbinding **(Fig. 4A)**. The electrostatic interaction energy between the filament and either DED was observed to be a dominant driving force for assembly in both cases (**Fig. 4B**). However, ^{casp8}tDED displayed a more negative interaction energy to the filament compared to the ^{FADD}DED monomer, indicating more favorable interactions at the interface. The differential interaction energies are primarily attributed to the Coulombic component, whereby ^{casp8}tDED displayed almost twice the value compared to ^{FADD}DED, i.e., around -1370 kJ mol^{-1} versus -705 kJ mol^{-1}, respectively (**SI Appendix, Table S2**). Consistent with this, ^{casp8}tDED exhibited a higher buried surface area and a greater number of inter-protomeric hydrogen bonds throughout (**Fig. 4C-D**, **SI Appendix, Table S2**). We performed cluster analysis to assess the most representative binding interface of each complex, which revealed that there are five salt bridges formed at the interface of a ^{casp8}tDED with the filament compared to only two in the case of homomeric ^{FADD}DED (**Fig. 4E**). Together, these results suggest that the incorporation

of ^{casp8}tDED monomer is energetically more favorable than the incorporation of ^{FADD}DED monomer into ^{FADD}DED filament. Therefore, once the ^{FADD}DED filament is formed, a switch from ^{FADD}DED to ^{casp8}tDED occurs, which explains previous observations wherein FADD is substoichiometric to caspase-8 and only localized to one terminus of FADD-caspase-8 complex.

An interface motif unique to ^{FADD}DED filament

Our findings indicate that ^{FADD}DED filament formation is essential in establishing a stable complex with RIPK1. However, whether filament formation is necessary for effective recruitment of caspase-8 required further dissection. This is because many interface residues essential for ^{FADD}DED filament formation are also critical for the recruitment of caspase-8 via its orthologous tDED domains. To tease these molecular functions, we analyzed the recently reported structure of the ternary ^{FADD}DED/^{casp8}tDED^{mut}/^{cFLIP}tDED complex (23). We identified a serine-rich motif, LSSSE, spanning amino acid positions 15 to 19, which is part of the FADD filament Type IIIa interface but is not involved in the ^{FADD}DED-^{casp8}tDED interface (**SI Appendix, Fig. S8**). When ectopically expressed in 293T cells, the double mutant exhibited diffuse expression, indicating disrupted filament formation **(Fig. 5A)**. We then reconstituted full-length FADD with S16R/S17R mutations in FADD^{KO} MDA-MB-231 cells. Unlike WT FADD, the S16R/S17R mutant failed to restore apoptosis response to BT treatment (**Fig. 5B**) due to compromised interactions with RIPK1 and caspase-8 (**Fig. 5C**), which was further confirmed in FADD knockdown 293T cells (**Fig. 5D**). Since the LSSSE motif has not been implicated in mediating caspase-8 recruitment, these findings complemented the structure-guided mutagenesis at other filament interface residues. Together, they suggest a model whereby ^{FADD}DED filament serves as a structural platform for recruiting both RIPK1 and caspase-8 for complex IIa assembly.

cFLIP disrupts FADD^{DED} filament formation

Given the strong structural resemblance of ^{FADD}DED and ^{casp8}tDED filamentous arrangements, it is possible that cFLIP will antagonize FADD in a similar fashion as it disrupts caspase-8. Therefore, we investigated the potential antagonistic interaction between ^{FADD}DED and ^{cFLIP}tDED. We transfected 293T cells with constructs expressing mCherry-^{FADD}DED and mBFP-^{cFLIP}tDED with Cas9 as control and examined filament formation. The control cells which were co-transfected with WT ^{FADD}DED and Cas9 exhibited long filamentous structures while cells expressing ^{cFLIP}tDED and Cas9 formed either short filaments or concentrated puncta in the cytoplasm **(Fig. 5E)**. When ^{FADD}DED and ^{cFLIP}tDED were co-transfected, instead of long ^{FADD}DED filaments, we observed a pattern of expression similar to ^{cFLIP}tDED but with mCherry-^{FADD}DED colocalization **(Fig. 5E)**. These results suggest an additional mechanism wherein cFLIP can act one level upstream of caspase-8 activation by interrupting FADD filament formation.

Discussion

Previous studies suggest that FADD is recruited to the death receptor FAS or RIPK1, followed by the recruitment and filament formation of caspase-8. According to this model, ^{FADD}DD binds to either ^{FAS}DD or ^{RIPK1}DD while ^{FADD}DED primarily recruits caspase-8 via

^{FADD}DED-^{casp8}tDED homotypic interactions **(Fig. 6)**. Multiple structural studies of *in vitro* reconstituted ^{FADD}DED-^{casp8}tDED-^{cFLIP}tDED complexes showed either monomeric or linear trimeric FADD (23). Thus, FADD filament formation is neither predicted to occur nor thought to be required for cellular extrinsic signaling. However, this raises a critical question: why does monomeric FADD not spontaneously seed caspase-8 filaments in cells?

Here, we elucidated the structure of the ^{FADD}DED filament, revealing three interface interactions that are characteristic of the death domain superfamily. Structure-guided mutagenesis led to disruption of filamentation *in vitro* and *in cellulo,* corroborating the interaction interfaces. Functionally, filamentation-defective FADD mutants failed to facilitate TNF-induced apoptosis due to impaired recruitment of caspase-8 and RIPK1 **(Fig. 6)**. Furthermore, we found that cFLIP can interrupt FADD filament formation, which might have significance beyond apoptosis because FADD has an additional role in stabilizing upstream interactions with FAS or RIPK1 depending on the molecular and cellular context.

The homotypic interactions between DD domains of FADD and RIPK1 are believed to sufficiently mediate the recruitment of RIPK1 to FADD (25, 34). However, we unexpectedly observed that ^{FADD}DED filamentation contributes to the RIPK1 recruitment TNFR1 signaling pathway. Mutations that disrupted ^{FADD}DED filament formation abrogate the recruitment of RIPK1. Notably, maintaining interactions between RIPK1 and FADD is important to prevent TRADD/FADD-mediated apoptosis and ZBP1/TRIF-mediated necroptosis (21, 22). Therefore, ^{FADD}DED filament formation might be crucial to restrain both apoptosis and necroptosis. Additionally, the ability of cFLIP to prevent ^{FADD}DED filamentation suggests its role in destabilizing RIPK1-FADD interactions. This is supported by a recent study wherein knockdown of cFLIP enhances the recruitment of FADD and RIPK1 to TRAIL receptors, leading to increased production of cytokines due to upregulated inflammatory signaling such as NFκB and MAPK (13). Collectively, these findings reveal an additional role of cFLIP in inhibiting apoptosis that is independent of its effect on caspase-8 activation.

To better understand the orderly assembly of ^{FADD}DED and ^{casp8}tDED, we performed all-atom molecular dynamics simulations using either ^{FADD}DED or ^{casp8}tDED monomers bound to ^{FADD}DED filaments. The simulations demonstrate that ^{FADD}DED filaments remain stable upon incorporation of either monomer. Moreover, the energy required for incorporating ^{casp8}tDED monomers is three times lower than that for ^{FADD}DED monomers, suggesting that incorporation of ^{casp8}tDED monomers is thermodynamically favored, which supports the hypothesis of a switch from ^{FADD}DED to ^{casp8}tDED filaments. This transition further substantiates the documented model wherein FADD is substoichiometric to caspase-8 (9, 32, 33) and positioned exclusively at one end of ^{casp8}tDED filaments (23, 27). However, what instructs the assembly of ^{FADD}DED filament remains the next outstanding question.

Given the strong structural resemblance between ^{FADD}DED and ^{casp8}tDED filaments (27), which is confirmed by the monomers within our structure, it is plausible that the same residues coordinate ^{FADD}DED-^{FADD}DED might also be responsible for ^{FADD}DED-^{casp8}tDED interaction. Therefore, the impaired apoptotic signaling could originate from defects in caspase-8 recruitment rather than compromised self-association. To ascertain the role of ^{FADD}DED filament formation in extrinsic cell death signaling, we ought to distinguish between these events. By analyzing and comparing the ternary complex structure to our ^{FADD}DED filament structure, we have pinpointed a serine-rich motif at the type III interface

that is uniquely involved in FADD^{DED} filament. The mutations S16R/S17R lead to impaired filament formation and interactions with both RIPK1 and caspase-8, resulting in an abrogated apoptotic response. Together, these findings support the hypothesis that ^{FADD}DED filaments serve as a platform to nucleate caspase-8 for effective apoptosis.

FADD subcellular compartmentalization remains a conundrum (35) wherein FADD was thought to be primarily cytoplasmic to mediate programmed cell death. However, accumulating evidence suggests that FADD is also distributed in the nucleus (35), wherein it can promote the progression of certain cancers via facilitating G2/M transition (36, 37). Our findings add additional insights as mutation of the residues within the serine-rich motif L15 to G, S16 to R confined DED to cytosolic localization. While both a nuclear export signal (L20-L28) and a nuclear localization signal (K33-K35) were reported (35), WT DED and mutants other than those targeting the serine-rich motif are expressed diffusely. Therefore, the serine-rich motif might function as a molecular switch regulating FADD localization, possibly via posttranslational modification, and this serendipitous finding adds an additional dimension when considering FADD levels in response to extrinsic signal or its noncanonical function in cancer.

It is noteworthy that several constructs were employed in our study. For structure determination of the DED filament, we purified recombinant ^{FADD}DED linked to mutant ^{casp8}tDED (His-StrepII-SUMO-FADD-^{casp8}tDED^{mut}). To validate the interacting interfaces observed with the Cryo-EM structure, we ectopically expressed mCherry-^{FADD}DED in cells with corresponding mutations of interface residues and assessed filament formation by fluorescence microscopy. Furthermore, introducing some mutations that disrupt filament formation *in cellulo* to the His-StrepII-SUMO-FADD-^{casp8}tDED^{mut} architecture for purification and analysis via gel filtration and negative stain electron microscopy revealed a corroborative loss of filaments. To evaluate the functional consequence of ^{FADD}DED mutant, we reconstituted FADD^{KO} cells with full-length FADD containing both DD and DED with corresponding mutations at interface residues. Consistently, DED mutations that disrupted filament formation in both mCherry-^{FADD}DED and His-StrepII-SUMO-FADD-^{casp8}tDED^{mut} constructs also failed to initiate TNF-induced apoptosis. The corroborative results across these constructs strengthen our conclusions regarding the formation and functions of ^{FADD}DED.

Limitations of the study

We present compelling evidence for the formation and function of ^{FADD}DED filaments in TNF-induced apoptosis as a critical step within a structured protein assembly cascade. However, the contextual and structural basis for how DD oligomer assembly leads to DED oligomer assembly remains to be elucidated. We postulate that DD domains might orderly organize themselves into a structure, establishing a chain of structure-mediated transmission of extrinsic death signals. These gaps represent missing pieces in the current model of extrinsic cell death.

Materials and Methods

Cloning and protein purification

Human WT or mutant FADD fused with the mutant tDED domain of caspase-8 which is incapable of filament formation (27) were cloned into pET28a vectors. Constructs were transformed into *E. coli* BL21(DE3) cells, and recombinant protein expression was induced with 0.4 mM IPTG. The bacterial cells were lysed by sonication in a lysis buffer (25 mM Tris pH 7.5, 25 mM Potassium Phosphate pH 6.8, 500 mM NaCl, 10% Glycerol) supplemented with 1 mg/ml lysozyme and protease inhibitor cocktails (Roche). The lysates were clarified by centrifugation at 20,000 rpm, then loaded onto a Ni-NTA column. After loading the clarified lysates, the column was washed with an equilibrium buffer (10 mM Tris pH 7.5, 250 mM NaCl, 10% Glycerol) containing 30 mM imidazole, and target proteins were eluted with the equilibrium buffer containing 250 mM imidazole. The eluates were then incubated with Strep-Tactin®XT resin (IBA) and eluted with an elution buffer (20 mM Tris pH 7.5, 500 mM NaCl, 100 mM biotin). Subsequently, the eluates were injected and fractioned using a Superdex 200 column.

Cryo-EM data collection and image processing

Five microlitres of fraction D1 from SEC measured at 0.175 mg/mL was incubated on glow-discharged UltrAuFoil 300 mesh R1.2/1.3 grid (modified with monolayer graphene (38)) for 2 minutes in Vitrobot (ThermoFisher) regulated at 4ºC and 100% humidity. The grid was then blotted with blot force –1 for 20 seconds before plunging into ethane for vitrification. Cryo-EM data was collected at 300 kV Krios equipped with Falcon4i detector and Selectris-X energy filter (ThermoFisher) with total dose of $50e^-/Å^2$ and preprocessed (motion-correction and CTF estimation) within CryoSPARC environment, including all downstream processes. Blob picking strategy was used for particle picking for the first 2D classification. The best particle set from the initial 2D classification was used for Topaz training and Topaz extract (particle picking equivalent module) for further 2D classifications. The best 2D classes were selected for ab-initio construction coupled to heterogenous refinement. Meanwhile, power spectra and symmetry search was conducted on the 2D class and 3D map volumes generated. Continuing from heterogenous refinement, the determined symmetries (helical rise, rotation angle, pitch) were applied to helical refinements. The final cryo-EM map was analyzed for the helical symmetries on HI3D (39) website (https://helical-indexing-hi3d.streamlit.app/). Model was first built by ModelAngelo (40) and manually refined by Coot (41) and eventually refined by Phenix (42) generating map/model validation statistics. All cryo-EM data were summarized in Table S1 and validated/deposited at PDB/EMDB. All molecular visualizations and analyses are enabled by ChimeraX (43).

Cell lines and cell culture

HEK293T, MDA-MB-231 cell lines were cultured in DMEM media supplemented with 10% fetal bovine serum (FBS). Cells were passaged every 3 to 4 days using Trypsin (HyClone™). All cells were maintained in a humidified incubator at 37°C with 5% CO_2.

Cell stimulation

MDA-MB-231 were stimulated with the following concentration of cytokines or inhibitors: 10 ng/ml of TNF (R&D Systems), 100 nM of birinapant.

Fluorescent and differential interference contrast (DIC) microscopy imaging

Cells were seeded on microscopy slides pre-coated with poly-lysine. 48 hours after transfection, the slides were collected, washed in PBS, and mildly fixed with 4% Paraformaldehyde (PFA) for 30 minutes. Subsequently, two PBS washes were performed before mounting and imaging using Nikon Ni-E Upright microscope at 60x magnification.

Annexin V/ Propidium iodide (PI) apoptosis assay

At 16 hours after BT treatment, the media containing dead cells was collected. The cells remained attached to the wells were washed once with 1XPBS and then trypsinized for 3 minutes. The trypsinized cells were then neutralized with media and collected into the same tube. The cells were harvested by spinning down at 4000 rpm, and the resulting cell pellet was washed with 1XPBS and spun down again at the same speed. The cells were then stained with 5 ul Annexin V (BD Pharmingen™) in 1X Annexin V binding buffer (0.01M HEPES (pH 7.4), 0.14 M NaCl, 2.5 mM $CaCl_2$ solution) for 15 minutes, followed by 1 ul Propidium Iodide Staining. The percentage of live cells was analyzed using flow cytometry by measuring the percentages of Annexin V^-PI^- cells.

Western blot

Total proteins were isolated using the NucleoSpin RNA/Protein kit following the manufacturer's instructions. Protein concentrations were measured using a protein quantification assay kit (MACHEREY-NAGEL) and standardized to the same concentration across all samples. Protein extracts were separated on 4%–12% NuPAGE Bis-Tris gels in NuPAGE MOPS running buffer (Thermofisher), and transferred to PVDF membranes. The membranes were blocked with 5% nonfat milk in PBST for 1 hour, and then incubated overnight at 4°C with the specified primary antibodies. After incubation with primary antibodies, the membranes were washed three times with PBST for 10 minutes each, and then incubated with secondary antibodies for 1 hour at room temperature. The membranes were washed again with PBST (3 times, 10 minutes each) and developed using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermofisher). The samples were probed with anti-cIAP1(CST, #7065, 1:1000), anti-caspase-8 (Proteintech, 13423-1-AP, 1:1000), anti-cleaved caspase-8 (CST, #9748, 1:1000); anti-caspase-3 (CST, #9662, 1:1000), anti-cleaved caspase-3 (CST, #9662, 1:1000), anti-PARP (CST, #9541, 1:1000), anti-HSP90 (BD bioscience, #610419, 1:1000), anti-RIPK1 (CST, #3493S, 1:1000), anti-FADD (Proteintech, 14906-1-Ap, 1:1000), anti-GAPDH (Santa cruz, sc-32233, 1:500).

Immunoprecipitation (IP)

Lysis buffer (20 mM Tris (pH 8.0), 137 mM NaCl, 1 mM EDTA, 1.5 mM MgCl2, 10% glycerol, 1% Triton X-100) supplemented with protease inhibitor (Roche) and phospho-stop (Roche) were used to extract total protein. After gentle shaking for 30 mins at 4°C, the protein extracts were further cleared using Bioruptor UCD-200 (Diagenode) for 5 mins. Soluble proteins were clarified by high-speed centrifugation at 15000 rpm for 30 mins and quantified using the Bradford protein assay (Bio-Rad). 1 mg of protein extracts were incubated with 1.5 µg of the indicated antibody overnight at 4°C, followed by isolation of the antigen-antibody complex using Protein G magnetic beads (Thermo Fisher) for 2 hrs. After

washing six times with lysis buffer, IP eluates were prepared by boiling beads with 2X SDS loading dye and proceeded with immunoblotting.

Generating shRNA stable cell lines using lentiviral infection

1.2 million HEK293T cells were seeded on 10 cm plates two days before transfection. Transfections were performed using 500 µl 2X HEPES Buffered Saline (HBS), 50 µl 2.5 M $CaCl_2$, 3.6 µg VSVG, 6.5 µg pMDL, 2.5 µg pREV, and 10 µg transfer plasmid of interest. Media was changed 6 hours after transfection. Every 24 hours, the media supernatant was filtered through a 0.45 µm membrane, mixed with polybrene (10 µg/ml), and added to cells to be transduced. After 48 hrs, virus-containing media was replaced with fresh media with 1.5 µg/ml puromycin for selection.

shRNA-mediated knockdown experiments

shRNA sequences were designed using GPP Web Portal (https://portals.broadinstitute.org/gpp/public/) and cloned into pLKO.1 - TRC vector following standard manufacturer's instructions. The cloned constructs were validated by Sanger sequencing. The knockdown efficiency was evaluated using qPCR or immunoblot. Following is the shRNA sequence for knocking down FADD:

shFADD: 5'-TGAACTCAAGCTGCGTTTATT-3'

RNA isolation and qRT-PCR analysis

RNA was extracted using Trizol following the standard manufacturer's instructions. The aqueous phase after high-speed centrifuge was mixed with 100% ethanol at 1:1.5 ratio. The mixture was loaded onto column (NucleoSpin) and washed following manufacturer's instructions. The RNA concentration and purity were evaluated using nanodrop, and the integrity was assessed by 1% agrose gel. 1 µg of RNA was reverse transcribed into cDNA using HiScript III 1st Strand cDNA Synthesis Kit (Vazyme). Real-time qPCR was performed using Taq Pro Universal SYBR qPCR Master (Vazyme) and analyzed by ΔΔCT method to determine the relative expression. The sequences of primers were as follows:

*GAPDH*_Forward: 5'- GTCTCCTCTGACTTCAACAGCG -3'

*GAPDH*_Reverse: 5'- ACCACCCTGTTGCTGTAGCCAA -3'

*FADD*_Forward: 5'- ATTAATGCCTCTCCCGCACC -3'

*FADD*_Reverse: 5'- TCTCTGCTTCGCTCCGATTC-3'

StrepII pulldown

Cells were quickly washed with ice-cold PBS and lysed using lysis buffer (20 mM Tris (pH 8.0), 137 mM NaCl, 1 mM EDTA, 1.5 mM MgCl2, 10% glycerol, 1% Triton X-100) supplemented with PhosSTOP™ tablets (Roche) and cOmplete™, EDTA-free Protease Inhibitor Cocktail (Roche), and biotin blocker (IBA). The cell lysates were cleared by centrifugation at 15000 rpm for 30 mins and quantified using the Bradford protein assay (Bio-Rad). 1 mg of protein extracts were incubated with Strep-Tactin beads overnight at 4°C. The next day, the beads were washed six times with lysis buffer and resuspended with 2X NuPAGE™ LDS Sample Buffer. The eluates were proceeded with immunoblotting.

All atom molecular dynamics simulations of ^{FADD}DED filaments

To elucidate the difference in interaction energies between the ^{FADD}DED filament with homomeric ^{FADD}DED as well as with caspase-8 tDED, we performed all-atom MD simulations. Firstly, the cryo-EM structure of ^{FADD}DED filament solved in this study was aligned to the cryo-EM structure of the FADD/Caspase-8/cFLIP complex (PDB: 8YBX) (23) using PyMOL (44). The top subunit of one of the three ^{FADD}DED oligomer chains in the filament was aligned to the ^{FADD}DED subunit at the bottom of the FADD/caspase-8/cFLIP complex. An adjacent ^{FADD}DED subunit from the FADD/Caspase-8/cFLIP complex was copied into the filament structure to generate a filament plus a homomeric ^{FADD}DED complex. Similarly, an adjacent caspase-8 tDED was copied into the filament structure to generate a filament plus a caspase-8 tDED complex.

The simulation systems were prepared using CHARMM-GUI webserver (45). The ^{FADD}DED filament complexes were parameterized with the CHARMM36m all-atom forcefield (46). Termini were treated in their charged state assuming neutral pH. The complexes were placed in a cubic box of dimensions 15 x 15 x15 nm^3 and were solvated with TIP3P water model along with 0.25 M NaCl salt. Any inter-atomic clashes were removed by performing steepest descents energy minimization. A 5 ns NPT equilibration was conducted with positional restraints applied to the protein backbone atoms. Subsequently, the restraints were removed and three independent 1,000 ns production simulations were performed for each filament complex. The temperature of each system was maintained at 310 K by a velocity rescale thermostat with a time constant of 1 ps (47), while isotropic coupling to a stochastic cell rescaling barostat with a time constant of 5 ps was used to maintain the pressure at 1 atm (48). Long-range electrostatic interactions were computed using the smooth particle mesh Ewald (PME) method with a real-space cut-off of 1.2 nm (49). A short-range van der Waals cut-off of 1.2 nm was applied, switched at 1.0 nm. Periodic boundary conditions were applied in all directions. Constraints were applied on all covalent bonds involving hydrogen atoms using the LINCS algorithm (50), allowing for a 2 fs integration time step.

All simulations were performed using GROMACS 2023.2 simulation package (51). Simulations were performed on the National Supercomputing Center of Singapore (https://www.nscc.sg) using 2 nodes containing 128 cores and 256 logical cores (AMD EPYC^{TM} 7713 @ 2.0 GHz) each. Simulation snapshots were generated using VMD (52). Interaction energies were calculated as a sum of short-range Coulomb and Lennard-Jones energies. Buried solvent accessible surface area (SASA) between the filament and homomeric ^{FADD}DED or ^{casp8}tDED was calculated by summing the constituent SASA of two components before subtracting the SASA of the entire complex. Hydrogen bonds were calculated based on a 0.35 nm donor-acceptor cut-off distance and 30º hydrogen-donor-acceptor cut-off angle. All averaged values over the last 500 ns are shown in Supplementary Table 2. To determine the representative structures of filament-DED interface, we performed a structural clustering analysis using the GROMOS algorithm (53) with a root mean square deviation (RMSD) cut-off of 0.25 nm. The top clusters comprised of 77% and 50% of the total simulation time for FADD DED and caspase-8 tDED, respectively.

Statistical analysis

Statistical analyses were performed using GraphPad PRISM 9 (GraphPad Software). $P > 0.05$ was considered not significant (n.s), while $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ were considered statistically significant. The Student's *t* test (two-tailed) was used to determine the P values.

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Author contributions

Conceptualization: Y.C, V.T.H; Methodology: Y.C, V.T.H, B.W. D.L; Investigation: Y.C, V.T.H, L.H.L. T.Y.L, Y.B.T, C.S.C, P.L, F.S, J.K.M and P.J.B; Writing–Original Draft: Y.C, V.T.H; Writing–Review & Editing: V.T.H, Y.C, Y.B.T, D.L, V.T; Supervision: D.L, V.T.

Declaration of interests

The authors declare no competing interests.

Figure legends

Fig. 1: ^{FADD}DEDs assemble into a filamentous structure

(A) Surface representation of ^{FADD}DED filamentous structure from side view (left) and top view (right). Each ^{FADD}DED monomer is distinguished by a different color.

(B) Surface representation of a single ^{FADD}DED chain within the filament structure from side view (left) and top view (right).

(C) A ribbon diagram depicting ^{FADD}DED monomer. H1 to H6, helix 1 to helix 6; N, N-terminus; C, C-terminus.

(D) Schematic illustration of ^{FADD}DED filament assembly, with each monomer represented by a hexagon. The directions of the three types of interactions are labeled.

(E) Surface representation of ^{FADD}DED filamentous structure from a side view. The interfaces of each type of interaction in the perspective of a single monomer (orange) are labeled.

(F-H) Ribbon diagrams depicting type I **(F)**, type II **(G)**, type III **(H)** interfaces. Key residues at these interfaces are shown as stick models.

(I) Interaction interfaces were analyzed using PDBePISA to compute buried surface area scores for each residue, which are visualized as a yellow-to-purple (low-to-high) gradient color scheme.

Fig. 2: Interface residues are crucial for ^{FADD}DED filament formations *in cellulo* and *in vitro*

(A) Schematic representation of ^{FADD}DED with six helices. Single-mutations of interaction residues at the corresponding interfaces are indicated (Type I, brown; Type II, purple; Type III, blue).
(B-D) 293T cells were transfected with indicated mCherry-^{FADD}DED fusion constructs. Fluorescence and Differential interference contrast (DIC) microscopy was performed 48 hours after transfection, with DAPI staining the nuclei. Left: Representative fluorescence microscopy images. Right: Representative images with merged DIC and mCherry channels. Scale bar =10 μm.
(E) Recombinant His-StrepII-SUMO-FADD-^{casp8}tDED^{mut} with indicated DED mutations were expressed and purified from *E.coli*. Left: SEC profile of purified proteins fractionated on a Superdex 200 column. Right: Fractions corresponding to SEC peaks were analyzed by SDS-PAGE.

Fig. 3: ^{FADD}DED filamentation is essential for effective complex IIa assembly and TNF-induced extrinsic apoptosis.
(A-B) MDA-MB-231 cells with FADD knockout (FADD^{KO} MDA-MB-231) were reconstituted with empty vector (EV), wild-type (WT) FADD, or FADD with indicated DED mutations. The percentage of live cells without treatment (UT, **A**) or with BT treatment **(B)** were analyzed by PI staining and flow cytometry.
(C-D) FADD^{KO} MDA-MB-231 cells reconstituted with indicated FADD constructs were treated with (**D**) or without (**C**) BT for 3 hrs. Total cell lysates were probed for PARP, cIAP1, caspase-8 (C8), cleaved caspase-8 (CC8), caspase-3 (C3), cleaved caspase-3 (CC3), FADD, and GAPDH.
(E) 293T cells with FADD knockdown (FADD^{KD}) were transfected with constructs expressing StrepII-tagged WT FADD or the indicated mutant FADD. Total cell lysates and StrepII-pulldown were probed for total RIPK1 (tRIPK1), caspase-8 (C8), and FADD.
(F) FADD^{KO} MDA-MB-231 cells reconstituted with WT or indicated FADD mutants were treated with BT for 3 hrs. Total cell lysates and StrepII-pulldown were probed for total RIPK1 (tRIPK1), caspase-8 (C8), and FADD.

Fig. 4: ^{FADD}DED filament energetically favors the incorporation of ^{casp8}tDED monomers over ^{FADD}DED monomers
(A) Three independent repeats of 1,000 ns all-atom MD simulations were performed for ^{FADD}DED filament (grey) in complex with homomeric ^{FADD}DED (cyan) and ^{casp8}tDED (pink). The figure shows overlaid snapshots taken every 50 ns from one replica of each simulation to demonstrate stable binding of the DEDs to the filament.
(B) Average Coulombic (top) and Lennard-Jones (LJ, bottom) interaction energies between the filament and homomeric ^{FADD}DED (blue) or ^{casp8}tDED (red). Standard deviations between three repeat simulations are shown as shaded areas.
(C) Three independent repeats of 1,000 ns all-atom MD simulations were performed for ^{FADD}DED filament in complex with homomeric ^{FADD}DED or ^{casp8}tDED. Buried surface area between the ^{FADD}DED filament and ^{FADD}DED monomer (blue) or ^{casp8}tDED monomer (red) throughout the simulations.
(D) The number of hydrogen bonds made by the DEDs with the ^{FADD}DED filament.

^{FADD}DED, blue; ^{casp8}tDED, red.

(E) Cluster analysis was performed on concatenated trajectories to assess the most representative binding interface during simulations. The figure shows the central structure of the top cluster with residues at the interface visualized in stick representation. Oppositely charged residues forming salt bridges are labelled.

Fig. 5: An interface motif unique to ^{FADD}DED filament

(A) 293T cells were transfected with indicated mCherry-^{FADD}DED fusion constructs. Fluorescence and Differential interference contrast (DIC) microscopy was performed 48 hours after transfection, with DAPI staining the nuclei. Left: a representative fluorescence microscopy image. Right: an image with merged DIC and mCherry channels. Scale bar =10 μm.

(B) FADD^{KO} MDA-MB-231 cells reconstituted with indicated FADD constructs were treated with BT for 16 hrs. The percentage of live cells was analyzed by PI staining and flow cytometry.

(C) FADD^{KO} MDA-MB-231 cells reconstituted with indicated FADD constructs were treated with BT for 3 hrs. Total cell lysates and StrepII-pulldown were probed for total RIPK1 (tRIPK1), caspase-8 (C8), and FADD.

(D) 293T cells with FADD knockdown were transfected with constructs expressing StrepII-tagged WT FADD or S16R/S17R mutant FADD. Total cell lysates and StrepII-pulldown were probed for total RIPK1 (tRIPK1), caspase-8 (C8), and FADD.

(E) Two constructs among those expressing mCherry-^{FADD}DED, mBFP-^{cFLIP}tDED, and Cas9 protein were transfected into 293T as indicated. Cas9 expressing construct was used as a placeholder control. Representative images from fluorescence and DIC microscopy with the indicated channels are displayed. Scale bar =10 μm.

Fig. 6: Graphical abstract illustrating the current understanding and new roles of ^{FADD}DED filaments in mediating interactions with RIPK1 and caspase-8 during TNF-induced extrinsic apoptosis.

The current prevailing model of TNF-induced apoptosis requires the assembly of a multiprotein complex comprising RIPK1, FADD, caspase-8, and ^{FADD}DD and ^{FADD}DED individually mediates the homotypic interaction with ^{RIPK1}DD and ^{casp8}tDED, respectively. Additionally, ^{FADD}DED monomers are believed to be sufficient for recruiting ^{casp8}tDED and inducing ^{casp8}tDED filament. However, our study suggests that ^{FADD}DED forms filaments to recruit ^{casp8}tDED and stabilize the interaction with RIPK1. Mutations at the interaction interfaces that disrupt ^{FADD}DED filament formation impairs apoptotic response by abolishing the interactions with RIPK1 and caspase-8.

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