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Peer Review File

Reverse Hierarchical DED Assembly in the cFLIP-Procaspase-8 and cFLIP-Procaspase-8-FADD Complexes

Corresponding Author: Dr Su-Chang Lin

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Peer review comments:
This paper describes a reverse hierarchical assembly process of cFLIP by presenting the structural details of cFLIP, an important protein that prevents apoptosis by interfering with the FADD-procaspase-8 complex, in complexes with procaspase-8, which can recruit FADD, which further induces caspase-8 activation and RIPK1 cleavage. Mutagenesis studies also found the key residues and interactions at the binding interface of the complex. The mechanism is closely related to cell fate, cell survival and resistance to apoptosis, and sheds lights on cell death regulation.
Overall, I think the framework of the paper is clear. The content is also informative and comprehensive. I have a few minor comments, mainly on figures.
First of all, for the structural ones, like figure 1-3, there are multiple molecules for both Caspase-8 and cFLIP. I can see that the authors are trying to make things clear. However, the current color scheme and numbering is a little bit confusing at first glanc, although I believe that is also what you used in your previous publication. Is there a better way to make it more organized?
For Fig 3.h, before showing the readers type II-III-II and III-II-III, it would be great to show a schematic diagram with the most basic idea, like showing a three neighboring subunits interpreting the Ia, Ib, IIa, IIb, IIIa and IIIb interfaces.
Figure 4 is also too crowded with tons of information and it is hard to find the key points. The residues and labels are overlapping to each other. I wonder if there is a way to make things easier to read. Like a table listing different mutations?
For the discussion part, I would like to see more on how your findings could be utilized in diseases, since the structures may serve as potential bases for drug developments in cancer and other dysregulated apoptosis diseases. The significant of the study could be highlighted.
Please also explain the novelty about the study, like the uniqueness of the findings.

Reviewer #2

(Remarks to the Author)
Review 28th July 2024

- You should be trying to help the work get published, not necessarily in this journal but ultimately.
- Don't criticize an experiment unless you can tell the authors how they could do it better:
My mentor would say, “If you just want to throw darts, go to the pub”.
- Keep in mind that no one ever built a statue to a critic.
- Try to act as a peer in the process of peer review.
Science Signaling 2009 Michael Yaffe

Title: Reverse Hierarchical DED Assembly in the cFLIP-Procaspase-8 and cFLIP-Procaspase-8- FADD Complexes

Manuscript #NCOMMS-24-34471

General Remarks

This is a structural study combining X-ray crystallography, cryo-EM and analysis of mutants in vitro to investigate caspase-8 cFLIP complexes. I am not a structural biologist therefore my remarks are confined to observations about the biological relevance of the findings. Overall, although difficult for a non-structural biologist to fully understand, I think the manuscript makes some salient observations that fit with our knowledge of how cFLIP can both inhibit and activate caspase-8. The reverse assembly model is plausible, depending on levels of cFLIP, but does beg the question how complex formation is normally prevented and regulated, and if it is physiological, since the authors are forced to examine the process with mutants of caspase-8 that don't self assemble. Nevertheless, the authors acknowledge this limitation and I believe the information generated is, notwithstanding this caveat, useful to the field.

Re communicating their message, I do feel that some sort of video for researchers who do not have access or expertise in looking at these structures would be extremely helpful.

Reviewer #3

(Remarks to the Author)
Yang et al reported the structures of cFILP-Procaspae-8 and cFLIP-Procaspase-8-FADD complexes by focusing on the DED assembly in those complexes. This paper has the advantage of providing a lot of structural information and explaining the still-debated DED assembly of DISC. However, there are a few points that need to be considered, and appropriate explanations for the following issues are necessary.

1. In the structures of various protein DED domain complexes, particularly given the low resolution characteristic of Cryo-EM structures, is it possible to distinguish the actual DEDs? An explanation of sequence comparison and sequence similarity between cFLIP-DED, Procaspase-8-DED, and FADD-DED is necessary. Additionally, it is essential to explain how these distinctions can be clearly made.
2. An explanation is needed for why various types of DED complexes are distributed in the same solution and their actual presence and functions within the cell.
3. In actual cells, the complexes are not just DED domains but whole proteins. It is necessary to compare the likelihood of complex formation when the Caspase domain is present in a DED complex and when FADD full-length, which includes the FADD DD, is present.
4. Many artificial mutants have been used for the structural study of DED complexes. It is necessary to explain whether these mutants could realistically affect the protein complexes that occur in actual cells. Additionally, the limitations of this study regarding the potential biases introduced by the mutants used for structural research need to be addressed.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed the comments raised. For clarity, how about changing the symbols "I", "II" and "III" in Fig. 3g from white to black?

Reviewer #3

(Remarks to the Author)
The authors adequately answered all my comments and concerns.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Point-by-point response to reviewer's comments:

General Response:

We sincerely appreciate the constructive feedback provided by Reviewers #1, #2, #3, and #4. After carefully considering their suggestions, we have made several revisions to enhance the clarity and quality of the manuscript. Specifically, we have updated and created new figures to improve visual presentation and ensure that the content is more accessible. Additionally, we have addressed each of the reviewers' concerns and revised the manuscript accordingly. We apologize for any confusion caused by the previous version of the manuscript.

REVIEWER COMMENTS

General comment of Reviewer #1:

This paper describes a reverse hierarchical assembly process of cFLIP by presenting the structural details of cFLIP, an important protein that prevents apoptosis by interfering with the FADD-procaspase-8 complex, in complexes with procaspase-8, which can recruit FADD, which further induces caspase-8 activation and RIPK1 cleavage. Mutagenesis studies also found the key residues and interactions at the binding interface of the complex. The mechanism is closely related to cell fate, cell survival and resistance to apoptosis, and sheds lights on cell death regulation. Overall, I think the framework of the paper is clear. The content is also informative and comprehensive. I have a few minor comments, mainly on figures.

Specific comment 1 of Reviewer #1:

First of all, for the structural ones, like figure 1-3, there are multiple molecules for both Caspase-8 and cFLIP. I can see that the authors are trying to make things clear. However, the current color scheme and numbering is a little bit confusing at first glanc, although I believe that is also what you used in your previous publication. Is there a better way to make it more organized?

Response to specific comment 1 of Reviewer #1:

To improve clarity and organization in the figures, we have implemented a few key changes. First, we applied the protein ID color scheme from Figures 5 and 7 to create new diagrams for Figures 1-3. We believe this consistent color coding will make it easier for readers to grasp the distribution of cFLIP and Caspase-8 at first glance. Additionally, these new diagrams allow for side-by-side comparisons with the original chain ID color scheme in Figures 1-3, providing a clearer understanding of the complex assembly. These updated diagrams are now included in Figures 1c, 2c, and 3a. Second, we have reorganized Figures 1c, 1d, 2c, and 3 to emphasize the conserved regions across different structures and presentations while maintaining consistency with our previous publications. The figure legends of Figures 1 to 3 were modified accordingly.

Specific comment 2 of Reviewer #1:

For Fig 3.h, before showing the readers type II-III-II and III-II-III, it would be great to show a schematic diagram with the most basic idea, like showing a three neighboring subunits interpreting the Ia, Ib, IIa, IIb, IIIa and IIIb interfaces.

Response to specific comment 2 of Reviewer #1:

We have created a schematic diagram to illustrate the basic concept behind the Ia, Ib, IIa, IIb, IIIa, and IIIb interfaces. We believe that, as suggested by Reviewer #1, this addition will enhance readers' understanding of the differences between the type II-III-II and III-II-III arrangements. However, to enhance clarity in Fig. 3, we have relocated the new diagram, along with Fig. 3h and 3i, to Supplementary Fig. 3, as referenced, for example, at Line 202.

Specific comment 3 of Reviewer #1:

Figure 4 is also too crowded with tons of information and it is hard to find the key points. The residues and labels are overlapping to each other. I wonder if there is a way to make things easier to read. Like a table listing different mutations?

Response to specific comment 3 of Reviewer #1:

We apologize for the crowded appearance of Figure 4. To address this, we have remade the diagrams, providing more zoomed-in views of the interfaces in Figures 4a and 4b. The updated diagrams feature less crowded residues and labels, improving readability. Additionally, as suggested by Reviewer #1, we have included a list that outlines the locations of all mutations on the various interfaces, making the information easier to follow.

Specific comment 4 of Reviewer #1:

For the discussion part, I would like to see more on how your findings could be utilized in diseases, since the structures may serve as potential bases for drug developments in cancer and other dysregulated apoptosis diseases. The significant of the study could be highlighted.

Response to specific comment 4 of Reviewer #1:

We have expanded the discussion to explore the potential applications of our findings in disease treatment, particularly in cancer and inflammatory diseases. Specifically, preventing complex formation could reduce the anti-apoptotic and anti-necroptotic effects in tumor cells with elevated cFLIP levels. A recent study demonstrated that using a peptide mimicking a helix of Caspase-8 DED2 to target and disrupt the "type I" DED-DED interaction can inhibit apoptosis (König et al., 10.1016/j.chembiol.2024.06.014). In this context, our structural data could inform the rational

design of peptides aimed at selectively targeting apoptotic complexes, such as the FADD-Caspase-8 intermediate complex (Yang et al. 10.1038/s41467-024-47990-2), while avoiding anti-apoptotic complexes.

Additionally, another study employed a homology-modeled cFLIP DED2 for in silico screening to identify cFLIP inhibitors, aiming to restore apoptosis in TRAIL-resistant cancer cells (Yaacoub et al., 10.3390/cimb46010046). Our structures could contribute to drug screening efforts or the design of peptides that selectively target complexes assembled in reverse order, focusing on the DED-DED interactions unique to cFLIP double-layer formation or the Caspase-8-recruiting CBS of the cFLIP double-layer complex. The goal is to selectively inhibit cFLIP self-oligomerization or its recruitment of Caspase-8 as a potential strategy for treating cancers with elevated cFLIP levels, while preserving apoptotic complexes. Similarly, targeting the FADD-recruiting CBS of the cFLIP-Caspase-8 complex could also be a viable approach for treating such cancers. Based on Reviewer #1's insightful suggestions, we have included these points at Line 690 in the last paragraph of the discussion section to emphasize the significance of this study.

Specific comment 5 of Reviewer #1:
Please also explain the novelty about the study, like the uniqueness of the findings.

Response to specific comment 5 of Reviewer #1:

The novelty of this study lies in the discovery of a reverse hierarchical DED assembly, which differs both in hierarchy and directionality from the previously known FADD-mediated unidirectional Caspase-8 polymerization (Fu et al. 10.1016/j.molcel.2016.09.009) and the FADD-Caspase-8-cFLIP DED assembly (Yang et al. 10.1038/s41467-024-47990-2). Typically, the directionality and unidirectionality of multiprotein DD-fold assembly align with the downstream signaling hierarchy in DD-fold-mediated processes, such as the MyD88-IRAK4-IRAK2 DD assembly (Lin et al., 10.1038/nature09121), NLRP3-ASC PYD assembly (Hochheiser et al., 10.1126/sciadv.abn7583), ASC-Caspase-1 or NLRC4-Caspase-1 CARD assembly (Li et al., 10.1073/pnas.1810524115), and RIG-I-MAVS CARD assembly (Peisley et al., 10.1038/nature13140). A unique aspect of our findings is that, unlike these other DD-fold complexes, the FADD-Caspase-8-cFLIP DED assembly can occur in reverse order, forming a cFLIP-Caspase-8-FADD DED assembly.

Another distinctive aspect of our findings is the significant role of CBS formation in controlling the directionality of multiprotein DD-fold assembly. When CBS appears on the oligomeric FADD-Caspase-8 intermediate complex, the subsequent DED assembly proceeds downstream by recruiting cFLIP. Conversely, when CBS appears on the oligomeric cFLIP intermediate complex, the DED assembly uniquely proceeds in reverse, binding first to Caspase-8 and then to FADD.

These findings were made possible by our discovery of the intrinsic ability of cFLIP oligomers to recruit procaspase-8, followed by FADD, as well as by our structural studies. The concept of reverse hierarchical DED assembly offers a more comprehensive understanding of the complex regulatory and assembly mechanisms involving cFLIP, beyond the conventional hierarchical DED assembly initiated by FADD. It is tempting to speculate that the co-occurrence of this reverse assembly of the cFLIP-Caspase-8-FADD complex under certain conditions may contribute to different models proposed in previous studies in this field and impact cell fate determination. Based on Reviewer #1's suggestion, we have included these points in the discussion section at Line 579 to emphasize the uniqueness of our findings.

General comment 1 of Reviewer #2:
Review 28th July 2024

- You should be trying to help the work get published not necessarily in this journal but ultimately.
- Don't criticize an experiment unless you can tell the authors how they could do it better. "If you just want to throw darts," he would say, "go to the pub."
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This is a structural study combining X-ray crystallography, cryo-EM and analysis of mutants in vitro to investigate caspase-8 cFLIP complexes. I am not a structural biologist therefore my remarks are confined to observations about the biological relevance of the findings. Overall, although difficult for a non-structural biologist to fully understand, I think the manuscript makes

some salient observations that fit with our knowledge of how cFLIP can both inhibit and activate caspase-8. The reverse assembly model is plausible, depending on levels of cFLIP, but does beg the question how complex formation is normally prevented and regulated, and if it is physiological, since the authors are forced to examine the process with mutants of caspase-8 that don't self assemble. Nevertheless, the authors acknowledge this limitation and I believe the information generated is, notwithstanding this caveat, useful to the field.

Response to general comment 1 of Reviewer #2:

We appreciate Reviewer #2's insightful comments and acknowledge the inherent limitations of our study design, particularly regarding the challenge of overcoming caspase-8's dominant aggregation during overexpression. A similar point was raised by Reviewer #3, and we have addressed it in detail in **Response to specific comment 4 of Reviewer #3**.

Regarding the regulation and prevention of cFLIP-Caspase-8 complex formation under physiological conditions, our model, as illustrated in Fig. 7a, offers some hypotheses. We speculate that under normal conditions, cFLIP levels are kept low to minimize the formation of the binary cFLIP-Caspase-8 complex, thereby maintaining cellular sensitivity to apoptotic signals, such as those triggered by CD95. Conversely, in situations where TNF induces cFLIP upregulation, the formation of the binary complex is promoted, leading to reduced cellular sensitivity to both apoptotic and necroptotic pathways. In tumor cells with consistently high levels of cFLIP, this anti-apoptotic and anti-necroptotic cFLIP-Caspase-8 complex likely persists, contributing to the cells' resistance to stress and their continued survival. These speculations, while not included in the discussion, provide a possible explanation for the regulation of cFLIP-Caspase-8 complex formation under different conditions.

General comment 2 of Reviewer #2:

Re communicating their message, I do feel that some sort of video for researchers who do not have access or expertise in looking at these structures would be extremely helpful.

Response to general comment 2 of Reviewer #2:

Following Reviewer #2's suggestion, we have created seven supplementary videos to assist researchers who may not have access to or expertise in analyzing these structures. The videos include:

1. An overview of the overall structure of the binary DED complex (at Line 151).
2. A breakdown of the three DED layers that compose the complex (at Line 196).

3. A demonstration of how two layers of cFLIP DED form another CBS to recruit cFLIP DED, assembling the third cFLIP DED layer at the bottom end (at Line 216).
4. A demonstration of how the Caspase-8 DED layer exhibits an incomplete or impaired CBS (at Line 237).
5. A visualization of how two layers of cFLIP DED form a CBS to recruit Caspase-8 DED, constructing the Caspase-8 DED layer at the top end (at Line 245).
6. A model illustrating how the 9:4 cFLIP-Caspase-8 binary complex recruits FADD, leading to structural jamming and the formation of the 4:3:3 cFLIP-Caspase-8-FADD complex (at Line 622).
7. A depiction of the structural morphing within the 4:3 cFLIP-Caspase-8 segment during the transition from the 9:4 cFLIP-Caspase-8 binary complex to the 4:3:3 cFLIP-Caspase-8-FADD complex (at Line 622).

General comment of Reviewer #3:

Yang et al reported the structures of cFILP-Procaspae-8 and cFLIP-Procaspase-8-FADD complexes by focusing on the DED assembly in those complexes. This paper has the advantage of providing a lot of structural information and explaining the still-debated DED assembly of DISC. However, there are a few points that need to be considered, and appropriate explanations for the following issues are necessary.

Specific comment 1 of Reviewer #3:

1. In the structures of various protein DED domain complexes, particularly given the low resolution characteristic of Cryo-EM structures, is it possible to distinguish the actual DEDs? An explanation of sequence comparison and sequence similarity between cFLIP-DED, Procaspase-8-DED, and FADD-DED is necessary. Additionally, it is essential to explain how these distinctions can be clearly made.

Response to specific comment 1 of Reviewer #3:

We apologize for not clarifying earlier that a structure-based sequence comparison between cFLIP-DED, procaspase-8-DED, and FADD-DED was performed and presented in our previous publication (refer to Fig. S1, Yang et al. 10.1038/s41467-024-47990-2). The sequence identity among these DEDs ranges from 16.2% to 34.1%. To ensure clarity, we have now included this

information in the manuscript at Line 154, along with a diagram in Supplementary Fig. 1c, which references the aforementioned structure-based sequence alignment.

However, to distinguish the DEDs in cryo-EM envelopes, we rely on their distinct structural features. For instance, FADD DED is composed of six helices, while both cFLIP and Caspase-8 tandem DEDs have an additional helix (helix H7) that connects DED1 and DED2. The absence of an envelope corresponding to helix H7 in the cryo-EM structure clearly indicates the presence of FADD DED. Additionally, in both cFLIP DED1 and DED2, the region corresponding to helix H3 is disordered, similar to what is observed in vFLIP MC159. In contrast, helix H3 is present in Caspase-8 DED1 and DED2. By fitting each model into the cryo-EM envelopes and confirming their identities based on these structural characteristics, we can accurately distinguish between the DEDs. These explanations were included in the Methods section under the subheading "Cryo-EM image processing and model refinement." at Line 839.

Specific comment 2 of Reviewer #3:

2. An explanation is needed for why various types of DED complexes are distributed in the same solution and their actual presence and functions within the cell.

Response to specific comment 2 of Reviewer #3:

In our cryo-EM studies of the ternary complex, which involved WT cFLIP tDED, full-length Casp-8 with FGLG and CADA mutations, and His-tagged full-length FADD with the F25G mutation, we identified five distinct cryo-EM structures within the dataset. This finding suggests the presence of multiple DED complexes in the same solution, likely due to a dynamic process, as cryo-EM can resolve multiple structures within such processes (Tsai et al. 10.1146/annurev-biophys-100121-075228). Since we used His-tagged FADD to isolate the ternary complex for cryo-EM studies, the appearance of four binary cFLIP-Casp-8 complex structures after 3D reconstruction indicates that the FADD F25G mutant dissociated early from the ternary complex, which is consistent with previous observations that the FADD F25G mutant has weak binding affinity for Casp-8 and cFLIP. This early dissociation was detailed in the results section under the subheading "Early dissociation of FADD F25G yields binary byproducts." For additional clarity, we have included the following sentence at Line 405: "Importantly, the appearance of multiple structures in a cryo-EM data set also suggests a dynamic process was involved (Tsai et al. 10.1146/annurev-biophys-100121-075228)."

To further explain this process, we propose that the 8:3, 6:3, 5:3, and 4:3 binary complexes are byproducts resulting from the initial dissociation of the FADD F25G mutant. This suggests that the ternary complex undergoes a transition following the binding of the binary 9:4 cFLIP-Casp-8 complex to FADD. Considering that the cryo-EM structure of the ternary 5:3:3 complex contains only five cFLIP molecules, it is plausible that this transition is FADD-dependent and results in a reduction of cFLIP molecules through a FADD-binding-induced structural jamming mechanism, as discussed in the Discussion section and Supplementary Fig. 8. Consequently, the resultant binary complexes might cease to transition and become stable byproducts in the solution. For additional clarity, we have inserted the following sentence at Line 408: "after the 9:4 cFLIP-Casp-8 binary complex binds to FADD, the resultant ternary complex undergoes a FADD-dependent transition."

In cells with WT FADD, we speculate that this transition would be completed, thereby preventing the byproducts observed in our study from being present. To clarify this point, we have added the following sentence to Line 636 in the Discussion section: "It should also be noted that the byproducts of the binary complexes observed here may be absent in cells with WT FADD due to the completion of the transition."

Specific comment 3 of Reviewer #3:

3. In actual cells, the complexes are not just DED domains but whole proteins. It is necessary to compare the likelihood of complex formation when the Caspase domain is present in a DED complex and when FADD full-length, which includes the FADD DD, is present.

Response to specific comment 3 of Reviewer #3:

We appreciate Reviewer #3's insightful suggestion. To address the complexity of the full-length proteins in cellular contexts, we utilized available structural data to model the locations of the caspase domains and the FADD death domain (DD).

For FADD, we referred to the NMR structure of full-length FADD (PDB: 2GF5, Carrington et al. 10.1016/j.molcel.2006.04.018) to predict the spatial arrangement of the FADD DD. We created a figure illustrating the potential interaction sites for Fas DD or RIPK1 DD with FADD DD, which is included in Supplementary Fig. 7c. We have added the following sentence to Line 381: " The potential locations of the FADD DD were modeled using the previously determined full-length structure of FADD (PDB: 2GF5) (Supplementary Fig. 7c)."

Regarding Casp-8, we used predictions of the caspase domain's location based on the FADD-Casp-8 intermediate complex (Fig. 5c in Yang et al. 10.1038/s41467-024-47990-2). We hypothesized that the caspase domain of Casp-8, which may be disordered, could form a dimer at specific "aligned dimer" sites between the N^{th} and $(N+2)^{th}$ asymmetric units (Figs. 7b and 7c, in Yang et al. 10.1038/s41467-024-47990-2). Other regions of Casp-8's caspase domain might remain disordered, as predicted in Fig. 5c of the same publication.

To clarify these details, we have added the following sentence to the figure legend of Supplementary Fig. 7c: "For the possible location of Casp-8's caspase domain and its heterodimer with cFLIP's pseudo-caspase domain, please refer to Figs. 5c, 7b, and 7c in Yang et al. 10.1038/s41467-024-47990-2."

Specific comment 4 of Reviewer #3:

4. Many artificial mutants have been used for the structural study of DED complexes. It is necessary to explain whether these mutants could realistically affect the protein complexes that occur in actual cells. Additionally, the limitations of this study regarding the potential biases introduced by the mutants used for structural research need to be addressed.

Response to specific comment 4 of Reviewer #3:

The mutants employed in our structural study include cFLIP tDED H7G, Casp-8 tDED F122G/L123G (FGLG), full-length Casp-8 FGLG_CADA, and full-length FADD F25G. Previous research (see below) supports the usefulness of these mutants to understanding protein interactions in certain cell lines. For example, the Casp-8 FGLG mutant has been shown to form a complex with cFLIP and inhibit apoptosis and necroptosis by cleaving RIPK1 in MEF and BMDM cells (Tummers et al. 10.1016/j.immuni.2020.04.010), which aligns with our observations of a ternary complex involving FADD, Casp-8 FGLG, and cFLIP (Yang et al. 10.1038/s41467-024-47990-2). Similarly, the cFLIP H7G mutant effectively interacts with FADD, is recruited to the DISC, and inhibits TRAIL-induced caspase activation with efficiency comparable to the wild-type protein in HCT116 and H460 cells (Majkut et al. 10.1038/ncomms4350), consistent with our structural data showing equivalent numbers of cFLIP WT and H7G molecules in ternary complexes (Yang et al. 10.1038/s41467-024-47990-2). Additionally, the FADD F25G mutant has reduced binding to Casp-8 and impaired apoptotic induction in T47D cells (Eberstadt et al. 10.1038/31972), which is reflected in our data showing a significant loss of FADD F25G mutant particles in the cryo-EM study. Casp-8 CADA mutations, located on the caspase domain, do not affect DED complex

formation. Therefore, our structures may offer a structural basis for the behaviors observed in certain cell lines with the corresponding mutant proteins but may not fully account for those observed in cells with WT proteins.

We acknowledge the need to address the study's limitations. We have revised our discussion to include the following clarification: "Using Casp-8 FGLG and cFLIP H7G mutations, along with overexpression of cFLIP tDED, was intended to enhance sample homogeneity for structural studies (Yang et al. 10.1038/s41467-024-47990-2). The FADD F25G mutation was employed to bypass the hierarchical binding process and study the complex assembled in reverse order. Consequently, our structures may not fully represent the diverse native complexes found in cells. Additionally, the byproducts observed with the FADD F25G mutation are unlikely to be present in cells with WT FADD." at Line 640.

General comment of Reviewer #4:

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response to general comment of Reviewer #4:

We thank Reviewer #4 for their efforts in co-reviewing the manuscript and for their contribution to the peer review process.

Point-by-point response to reviewer's comments:

General Response:

We sincerely thank all Reviewers for their valuable feedback.

REVIEWER COMMENTS

Comment of Reviewer #1:

The authors have addressed the comments raised. For clarity, how about changing the symbols "I", "II" and "III" in Fig. 3g from white to black?

Response to comment of Reviewer #1:

Thank you for the helpful suggestion. We have changed the symbols "I", "II", and "III" in Fig. 3g to black for improved clarity.

Comment of Reviewer #3:

The authors adequately answered all my comments and concerns.

Comment of Reviewer #4:

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.