nature portfolio

Peer Review File

The structure of mouse RIPK1 RHIM-containing domain as a homo-amyloid and in RIPK1/RIPK3 complex

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors describe the fibrillar structure of the RHIM-containing core of mouse RIPK1, determined by solid state NMR (ssNMR). The structural basis of necroptosis and formation of the necrosome is of significant interest, in light of the necrosome being a functional amyloid and also because necroptosis is associated with significant pathology when dysregulated.

Noteworthy results, how it compares to established literature:
The authors have determined the structure of homomeric amyloid fibrils formed by a domain encompassing residues 485 to 566 of mouse RIPK1. This segment corresponds closely to the segment of human RIPK1 (497-583) which has been studied previously in the heteromeric amyloid formed by hRIPK1:hRIPK3 (Li et al. 2012; Mompean et al 2018). The ssNMR structure of mRIPK1 shows an ordered core which adopts an N-shape with 3 major beta-strands, forming a parallel, in-register fibril. This is similar to the structures of other homomeric fibrils of human and mouse RIPK3, determined by ssNMR and cryoEM, with the only difference being that in mRIPK1, the 3rd strand does not pack against tightly against the 2nd strand. The authors speculate that this may be due to sequence differences immediately preceding the core tetrad IQIG however there have been no studies included in this work to probe this further. Additional evidence is needed.
Mass-per-length measurements and AFM are consistent with a fibrillar structure with one RIPK1 monomer per 4.7A along the fibril long axis.
Additionally, the authors have prepared a sample that contains both mRIPK1 and mRIPK3 and have collected ssNMR data, which shows some changes in the RIPK3 spectra. Changes in ThT profile may indicate heteromeric fibril assembly but are not definitive and the study lacks analysis of the supernatant and controls with mixtures of homomeric fibrils. Therefore there is no verification that the sample contained 100% heteromeric fibrils and the observed loss in RIPK3 signal raises questions about the extent of amyloid assembly and co-assembly. The change in assembly conditions for homomeric and heteromeric samples also makes interpretation/conclusions of the significance of observed minor structural changes difficult. Critical experimental details for RIPK3 are lacking.

Points to be addressed:
1. Clarity of expression could be improved throughout.
2. The orientation of the strands should be consistent throughout the main figures and supplementary, to allow for more effective comparison of multiple structures.
3. No details are provided about the expression and purification of the mRIPK3

construct. The length and corresponding residues in the mRIPK3 fragment are only provided in a figure label in Supp Fig S8.
4. The method of fibril assembly of the homomeric RIPK1 fibrils and the heteromeric mixture are different, one from GuHCl/Tris/NaCl/imidazole, pH8 and the others from acetic acid. Given the weight of evidence that assembly conditions can impact polymorphism of fibrils, similar assembly conditions should be used. Why were acidic conditions chosen for the mixture?
5. The ThT experiments were performed at pH 7.4 so may not reflect the mixture, which was prepared from 30% acetic acid by dialysis into unbuffered water.
6. The ThT experiments should include comparison of mixtures of homomeric fibrils prepared at the same concentrations and then mixed, post-assembly.
7. The section discussing region G527-I530 is not clear. What is meant by "if anti-parallel b-sheet conformation was assumed"? How could an anti-parallel arrangement be accommodated here? Each layer of the fibril consists of beta-arches, the strands in each layer are not forming a beta sheet. All of the strands appear to be involved in parallel beta-sheets. The authors should explain this and include models of the proposed alternative structures.
8. The ordered fibril core determined by ssNMR has dimensions of ~3.1 x 2.8 nm. AFM results indicate a height of ~3.12 nm. Fig S4 legend indicates a fibril diameter of about 3.1 nm. This appears to be a histogram of height measurements, not diameter. Authors should comment on the fact that this does not account for the other residues which are not observed. Fig S4b may give an indication of the additional structures, with distance from edge-to-edge about 40nm.
9. There is no validation of the formation of heteromeric amyloid. How much of each protein remained in the supernatant after dialysis? How were the fibrils pelleted after assembly, before treatment with urea and SDS-PAGE?
10. The changes in RIPK3 peaks in the RIPK1:RIPK3 mixed sample are only described in the text and quite difficult to visualise without additional figures or perhaps colour changes in Fig 6 to highlight the ones that could be indicative of changes in RIPK3 3rd b-strand. How significant are these changes, given the large decrease in intensity from RIPK3 signals?
11. It would be helpful if the authors could indicate whether the NMR data collected here for the homomeric RIPK3 is closely similar to that reported previously (https://www.nature.com/articles/s41467-021-21881-2). From the structure presented in Fig 7, it is not clear whether the authors are suggesting that in the mixed sample, the 3rd strand is disordered or in the same more open conformation seen previously.
12. All structure figures would benefit from labels being offset to improve readability, and all structures being presented in the same orientation. For Fig 7, please include residue numbers.
13. The authors raise the possibility that residues immediately before the IQIG tetrad could be the difference for the different orientations of the 3rd strand. This may be true

but is speculative, in the absence of any mutagenesis data. The evidence they cite regarding RHIM-A and RHIM-B of ZBP1 is not directly comparable, given the mutations introduced to the ZBP1 RHIMs were AAAA in the core tetrad and did not alter the preceding residues.
14. The discussion of the relative stability of RIPK1:RIPK3 hetero-amyloids is interesting but should also consider whether the stability of RIPK3 in homomeric amyloids would be similar/different, given this more extended or disordered strand, and the impact of HSPA8 on homomeric RIPK3 fibrils.
15. Experimental details for preparation of RIPK3 are lacking.

Reviewer #2:
Remarks to the Author:
The manuscript by Liu et al describes the structure determination of the core of homomeric amyloid fibrils formed by mouse RIPK1 (mRIPK1) by solid state NMR (SSNMR). In addition, spectroscopic data reflecting changes upon heteromeric fibril assembly when mRIPK1 combines with mRIPK3 are informed and discussed. The presented 3D structure of mRIPK1 as well as the SSNMR data on mRIPK1-mRIPK3 fibrils are important pieces to advance our understanding of amyloid signaling in immunity. While RIPK3 and RIPK1-RIPK3 amyloid fibrils have been previously studied, the structure of RIPK1 has remained unsolved. Therefore, before recommending publication, the following two major points (in addition to minor points) should be addressed.

(A) Collection of distance restraints to define the fold in mRIPK1 fibrils.

The 3D structure presented is based on several assumptions that require verification through experiments. Although the N-shape appears to be the most plausible fold based on previous published work, supporting data must be obtained from experimental distance restraints. More precisely:

(i) The structural model encompasses residues 516-539, whose sequence DLIKYTIFNSSG*IQIG*NHNYMDVG contains the canonical RHIM tetrad I528QIG531 (between "*") that is crucial for mRIPK1 self-assembly. However, the authors hardly found long-range cross-peaks involving these two most relevant isoleucines (I528 and I530) that would enclose a hydrophobic core with the first β-strand as depicted in their N-shaped structure.

In line with the preceding paragraph, on page 2 the authors state that a number of

resonances have been tentatively assigned: “these resonances were probably from residues A514, D515, D516, L517, 518, (…)”, and acknowledge that “the assignment for these residues was sometimes ambiguous since not all the correlation peaks could be found”. Given the essential role of this hydrophobic core in defining the proposed N-shape structure, the authors should prioritize assigning these residues and obtaining unambiguous distance restraints (currently is only supported by one unambiguous contact, vide infra). The reviewer questions why the authors did not evolve the third dimension in their 13C-detected 2D NCACX and NCOCX experiments, as done with their CONCA experiment. With these three 3D experiments for sequential backbone assignments, the ambiguities mentioned above could potentially be resolved in a short timeframe. According to Table 4: solid-state NMR experimental conditions, these experiments took 8 (NCACX) and 12 hours (NCOCX), making it feasible (time-wise) to record the corresponding 3D versions within 1.5 days (the CONCA is more demanding and took less than 2 days).

(ii) Building upon the previous point (i), the authors have identified only one unambiguous distance restraint between the first β-strand and the canonical RHIM core, specifically involving Y520CZ and I530CG2&CD1. The reviewer expects at least contacts involving Y530 CE & CD, according to the authors’ assignment table, but they were not listed. Therefore, it is important that the authors show the aromatic region of their spectra at short and long DARR mixing, as this region displays little overlap and according to the assignment table (Table 1) and the structural model depicted in Fig. 5, essential restraints are expected.

(iii) The classification of cross peaks as intra- and intermolecular is not consistently justified. Given that amyloids consist of stack of monomers, intramolecular contacts can only be reliably assigned when experiments are conducted on samples containing mixtures of [12C,14N]-mRIPK1 and [13C,15N]-mRIPK1 at a diluted ratio (e.g., 1:4, see PMID 33046908).

To clarify this point, the authors would agree in that a contact between two nuclei (e.g., CA-CB) within a given residue (e.g., I530) can be both intra- (I530CA-I530CB from same monomer) and inter-residue (I530CA in one monomer with I530CB from the monomer in the strand immediately above or below). This is also true for distinct residues, either sequential (e.g., I530 and G531) or more distant (e.g., G527 and M536). Thus, when the authors assert that certain cross peaks, such as those between G527CA and M536 “were all considered intramolecular interactions defining the folding of the subunit”,

they need to provide appropriate experimental evidence. In fact, it is noteworthy that, according to the structure, the G527CA -M536CG distance is 5.8Å if belonging to the same monomer (intra), but 6.3Å if belonging to distinct molecules (inter), with both values within the quite broad 5.5 +/- 2.5Å distance range listed in Table 2.

To address these ambiguities, the authors are encouraged to prepare an isotopically diluted sample of [13C,15N]-mRIPK1 : [12C,14N]-mRIPK1 to accurately distinguish intra- from intermolecular contacts. This step is particularly crucial given the multiple uncertainties present in the current dataset.

(iv) The authors stated, “The inter-molecular arrangement of the hydrogen-bonded subunits was investigated with 13C-13C correlation experiments on sparsely 13C-labeled mRIPK1 fibrils using [2-13C]-labeled glycerol as a carbon source”. However, this statement might cause confusion, as the elucidation of the H-bonded arrangement was not possible with the glycerol-derived samples, but rather from their study of a 1:1 mixed sample composed of [12C,15N]-mRIPK1 and [13C,14N]-mRIPK1.

The comparison of an NCA (on a U-13C15N sample) with an hNHHC spectrum ([12C,15N] : [13C,14N] mixed sample), as shown in Fig. S7, cleverly demonstrates the parallel arrangement, should cross peaks be coincident upon superposition of the two spectra. However, according to Table 4, the authors conducted both experiments using the same 3.2 mm HCN probe, but at significantly distinct temperatures (ca. 40 degrees, 309K for NCA vs. 271K for hNHHC). It is essential to clarify whether the authors adjusted peak positions in Fig. S7 to show the overlay, and if so, why this adjustment is not explicitly mentioned in the text. To strengthen the evidence of parallel stacking, the authors should repeat these experiments at the same temperature and present the matching of peaks. Given the importance of this experiment, the revised results should be incorporated into the main text.

(v) The sample generated using [2-13C]-labeled glycerol as a carbon source should be combined with one prepared using [1,3-13C]-labeled glycerol to measure exclusively intermolecular contacts for precise collection of distance restraints. This approach represents the most direct way to incorporate the necessary information into the workflow for structure calculation (e.g., PMID 27165577 or 29627359).

(B) Restraints sorting and structure calculation.

(i) With the introduction of two new samples (diluted with 1:4 ratio of [13C15N] : [12C14N] to establish the monomer fold, and a 1:1 ratio from [13C-1,3] : [13C-2] glycerol for intermolecular contacts), the nature of the assignments reported in Table 2 could be ascertained (prior to 13C-detected confirmation of the assignments).

Regarding the structure calculation process, the authors should provide clarification on how many rounds of calculations were performed before arriving at the final model. The current methods section may give the impression that a single calculation led to the displayed structural model. However, upon examining the deposited restraint file (8ib0.mr), it appears that, at an initial stage of their protocol, HN–O and N–O distances for residues 7 to 8, 15 to 16, and 19 to 20 were restrained to 2.30 and 3.30Å, respectively. This implies explicit definition of hydrogen bonds from I522 (HN) to F523 (O), I530 (HN) to G531(O), and N534 (HN) to Y535 (O). It's noteworthy that these residues, based on geometric quality criteria in the PDB validation report, are considered outliers. While the explicit inclusion of hydrogen-bonding restraints is a common procedure, it should be explicitly described in the text for the sake of reproducibility. The authors should detail how they implemented hydrogen-bonding restraints and update Table 3 accordingly.

(ii) In relation to Table 3, the authors should reconsider the table using curated data from both diluted and mixed samples. Currently, they differentiate between unambiguous and ambiguous intramolecular contacts. However, given the current experimental design, it is impossible to distinguish whether these contacts are intra- or intermolecular. This introduces a new layer of ambiguity to their dataset.

(iii) The authors observed that certain distances in their structural models are longer than expected, surpassing typical experimental cutoffs (e.g., > 9Å for cross peaks in 400 ms DARR). Based on this observation, they proposed the possibility of monomers stacking antiparallel to each other along the fibril axis. For instance, the authors suggested that “The inter-molecular distance between G527 to I530 could be closer (< 9Å) if an antiparallel β-sheet conformation was assumed for the mRIPK1 I528-I530 segment”.

To address the question of the antiparallel arrangement, the authors conducted a series of CHHC experiments, assuming that the absence of N-ter to C-ter (with respect to the RHIM region) contacts is indicative of a parallel arrangement. However, this interpretation is not straightforward, especially given the number of residues within the N-terminus that could not be assigned unambiguously. The authors should consider

curating their restraints with the new samples to refine their analysis. Additionally, it is advisable for the authors to employ CHHC in conjunction with DARR/PDSD for the characterization of the new samples, as CHHC provides a spectrum with much less spin diffusion.

(iv) In line with the previous point, the authors may consider using an antiparallel model in their calculations, and see whether their experimental restraints (the one that they actually see) could be accommodated within such an alternative model. This approach would be more systematic than disregarding conformations through visual inspection.

(v) MPL measurements suggest one monomer per layer, but what if antiparallel dimers stack along the fibril axis with a parallel arrangement? The lack of sequential connectivities within the first β-strand, contacts to the IQIG tetrad, the presence of longer distances for these few residues, and the overall parallel arrangement may be spectroscopically compatible. In their prior work, the authors employed MD simulations to evaluate the stability of such a fibril of dimers. Could the I528-I530 segment (third β-strand) act as a dimeric interface, aligning with the absence of cross-peaks (only with a single nucleus of Y530, as mentioned earlier)? In scenarios where distinct species coexist, it's plausible that only the smaller species are attached to copper grids. To mitigate potential biases in the resulting conformations, the authors are encouraged to explore different models that could potentially accommodate the experimental restraints. The set of ambiguous restraints may align with alternative models, suggesting the presence of distinct yet spectroscopically similar species that can be only explored through calculations (e.g., PMID 25395337 for an examination of fibrils with 2- and 3-fold symmetry). Currently, many unresolved ambiguities and unexpectedly large distances may encode a secondary minor species (e.g., N532 to I518, G531 to K519, or G531 to Y520).

(v) Back calculations of the expected cross peaks from the determined structures (for each of the alternative models) are needed to verify the correlation between structure and spectroscopic data.

Minor points:

(i) The authors should display traces of selected 2D dataset for a SNR assessment.

(ii) The Methods section should comprehensively describe how the samples were packed into the rotors. Currently, it is just mentioned that lyophilized samples were packed and rehydrated inside the rotor. How many mg the packed? Did they rehydrate in a single step? During packing? How many µL? In both rotors?

(iii) Dissolving the hetero-amyloid in 8M urea for SDS-PAGE analyses revealed a 1.7:1 ratio of mRIPK1 to mRIPK3. However, it is noted that 8M urea might not be sufficient to fully dissolve these fibrils, leaving many small seeds. The authors should highlight this point, unless they plan to revisit these numbers using formic/acetic acid or NaOH, which have superior depolymerization abilities.

(iv) The mRIPK1 construct used in this study spans 82 residues, about which ~25% are visible in CP-based experiments. The authors could discuss the residue types identified through TOBSY/INEPT and potential matching with positions outside the RHIM core (e.g., the observation of CB patterns for His, Ala, etc)

(v) Related to the previous point, what happened to the His-tag? According to the Methods section, the protein contained a Hexa-His tag that was not cleaved. This should be indicated in Fig. 1A.

(vi) The results concerning mixing of mRIPK1 with mRIPK3 are not entirely clear. Does a molecular ratio of mRIPK1 to mRIPK3 of 1.7:1 suggest there would be 5 molecules of mRIPK1 every 3 molecules of mRIPK3? If so, how is this related to “a reduction of mRIPK1 of 60% in the hetero-amyloid”? These observations are intriguing, and the text could be rephrased for clarity.

REVIEWER COMMENTS

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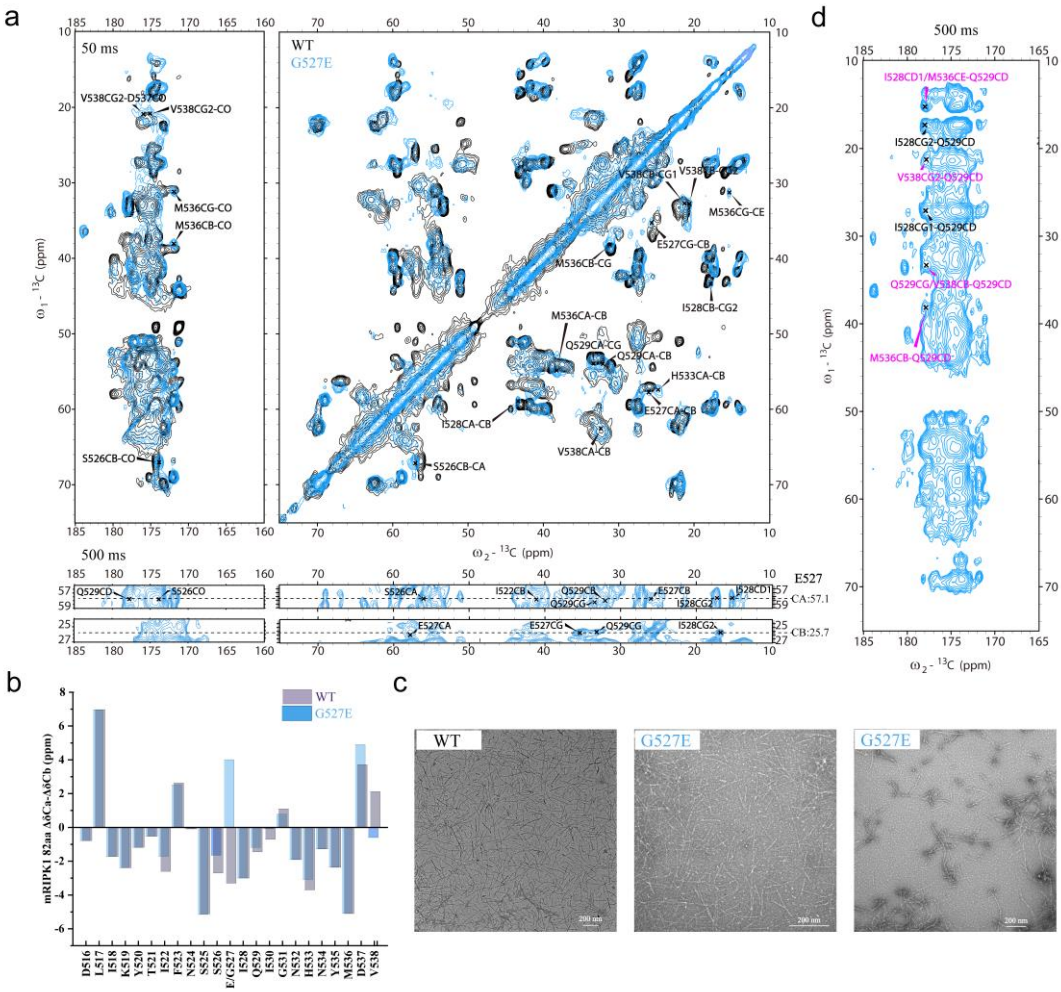
From our previous results on human and mouse RIPK3, and current results on mouse RIPK1, we found great similarity in the formed fibril structures. There is a slight difference in mouse RIPK3 (not mouse RIPK1) with its third β-strand slightly away from the center β-strand (There is still NMR correlation peaks between these two strands, indicating the third β-strand still has restricted motion). By comparing the sequence, we speculated the E before tetrad IQIG in mouse RIPK3 caused the last strand away from the central strand. Since this manuscript is on mouse RIPK1 fibril and the difference is on mouse RIPK3, we only discussed this issue. In order to provide more evidence on this issue, we prepared a single-site mutation of mouse RIPK1 (G527E) to mimic the mouse RIPK3 sequence and obtained ssNMR spectra on the fibril. We found the 13C-13C 2D spectra exhibited peak position changes for the mutation site, at the turn, and other nearby sites (I522, S526, G527E, G531, N532, H533, D537, V538, figure below). Although the chemical shifts still indicated 3 major β-strands, the correlation between M536 and E527 was not found. The correlations between M536 and G527 can be easily found for wild type mouse RIPK1 fibril. On the other hand, we found correlations between Q529 and M536/V538, suggesting a close association between the center tetrad and the last β-strand. Therefore, it needs further investigation on what determines the orientations between these two β-

strands. We did not include this mutation studies in the paper since it is not the focus of the paper and requires further studies. We modified the corresponding discussion and pointed out other sequence differences that could affect the structure collectively. We thank the reviewer for suggesting this study.



The mRIPK1 mutant G527E fibril study supports the interaction of the center β-strand with the last β-strand still exists. (a) The 2D ^{13}C-^{13}C 50 ms DARR spectrum of mRIPK1 G527E fibrils. (below) No correlation of E527 with M536, V538 was found in 2D ^{13}C-^{13}C 500 ms DARR spectrum, and the spectra were collected at 273 K. (b) The comparison in the secondary chemical shift (ΔδCA-ΔδCB) between WT and the mutant. (c) Electron microscopy images showed that the mutant G527E was still able to form fibrils. (d) The 2D ^{13}C-^{13}C spectra of uniformly ^{15}N, ^{13}C-labeled mRIPK1 G527E using 500 ms DARR mixing showing correlation peaks between Q529 and M536/V538.

Mass-per-length measurements and AFM are consistent with a fibrillar structure with one RIPK1 monomer per 4.7A along the fibril long axis.

Additionally, the authors have prepared a sample that contains both mRIPK1 and mRIPK3 and have collected ssNMR data, which shows some changes in the RIPK3 spectra. Changes in ThT profile may indicate heteromeric fibril assembly but are not definitive and the study lacks analysis of the supernatant and controls with

mixtures of homomeric fibrils. Therefore there is no verification that the sample contained 100% heteromeric fibrils and the observed loss in RIPK3 signal raises questions about the extent of amyloid assembly and co-assembly. The change in assembly conditions for homomeric and heteromeric samples also makes interpretation/conclusions of the significance of observed minor structural changes difficult. Critical experimental details for RIPK3 are lacking.

We added control experiments of post-assembly fibril sample using two homo-amyloid mixture at 1:1 ratio and analyzed the supernatant of RIPK1/RIPK3 hetero-amyloid sample. The change in assembly conditions won't change the fibril structures and NMR spectra. A comparison on the NMR spectra was added in figures shown below. The detail explanation was addressed below for each point. The experimental conditions for RIPK3 were added in the method.

Points to be addressed:

1. Clarity of expression could be improved throughout.

We carefully edited the manuscript.

2. The orientation of the strands should be consistent throughout the main figures and supplementary, to allow for more effective comparison of multiple structures.

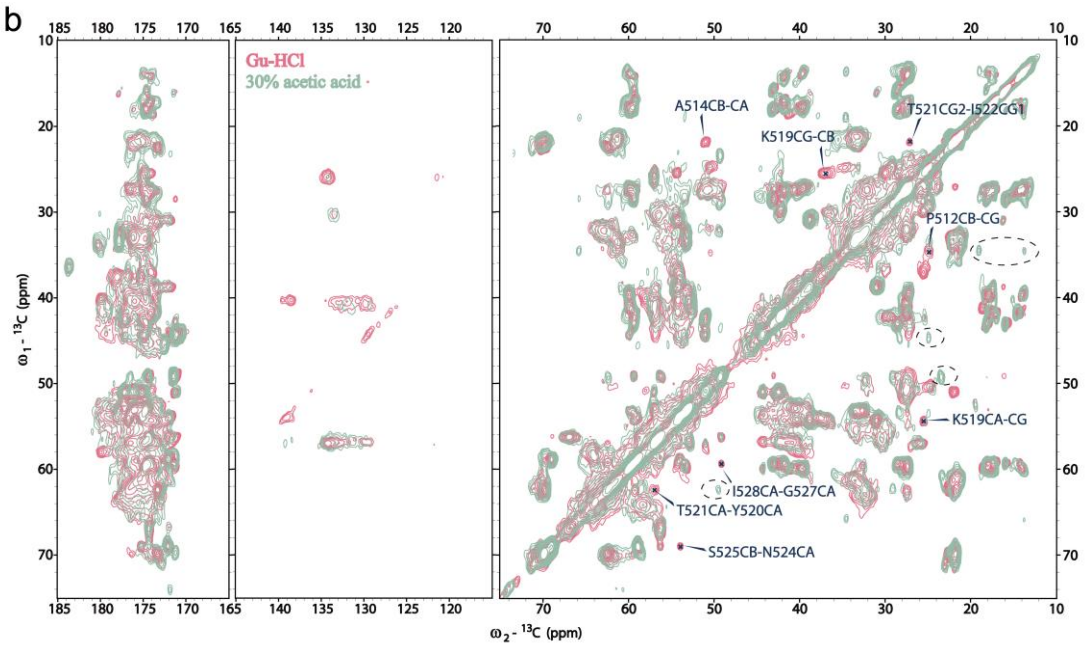
The figures are updated accordingly.

3. No details are provided about the expression and purification of the mRIPK3 construct. The length and corresponding residues in the mRIPK3 fragment are only provided in a figure label in Supp Fig S8.

The details on the expression and purification of the mRIPK3 construct were added in the experiments and referenced.

4. The method of fibril assembly of the homomeric RIPK1 fibrils and the heteromeric mixture are different, one from Gu-HCl/Tris/NaCl/imidazole, pH8 and the others from acetic acid. Given the weight of evidence that assembly conditions can impact polymorphism of fibrils, similar assembly conditions should be used. Why were acidic conditions chosen for the mixture?

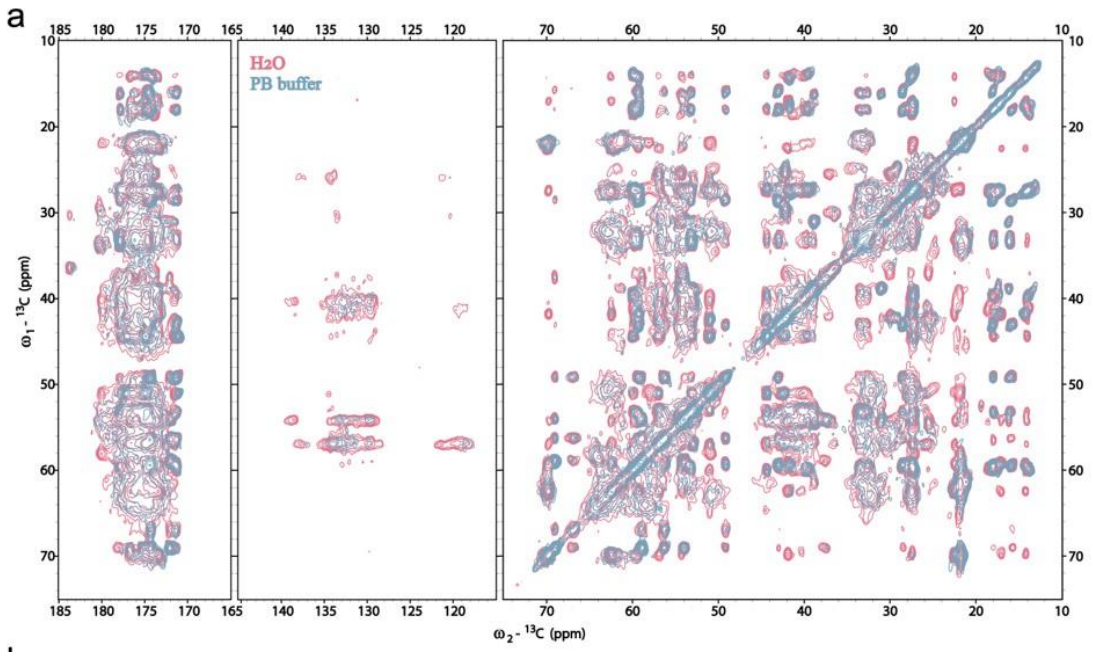
Gu-HCl/Tris/NaCl/imidazole, pH8 is our Ni column buffer condition. Using the protein directly from this step, homomeric RIPK1 fibrils and homomeric RIPK3 fibrils were prepared. HPLC could be used after Ni column which provided us dry protein powder after lyophilization. Then the dry protein was then solubilized using acetic acid for fibril preparation. Both methods generated the same fibril structures with the same NMR spectra (figure below and figure S12 in the paper). We preferred using the first method since it saved some time and increased the yield. But Gu-HCl/Tris/NaCl/imidazole, pH 8.0 is not a good condition for a long-term storage of proteins. When the heteromeric fibrils or other fibrils requiring to mix two types of proteins was prepared, we prepared the two proteins separately and stored them in the freezer. So acetic acid was used to dissolve the protein.



2D 13C-13C spectra of mRIPK1 homo-amyloid with 50 ms DARR mixing under two different assembly conditions. 30% acetic acid dissolving the protein (light green) and Gu-HCl/Tris/NaCl/imidazole, pH8 condition for protein solution (pink). Both were followed by water dialysis. Both NMR spectra were collected at 293 K and ωr = 15 kHz. There are intensity differences at several peaks, labeled in the figure with assignments for the pink spectrum and dashed circles for the green spectrum.

5. The ThT experiments were performed at pH 7.4 so may not reflect the mixture, which was prepared from 30% acetic acid by dialysis into unbuffered water.

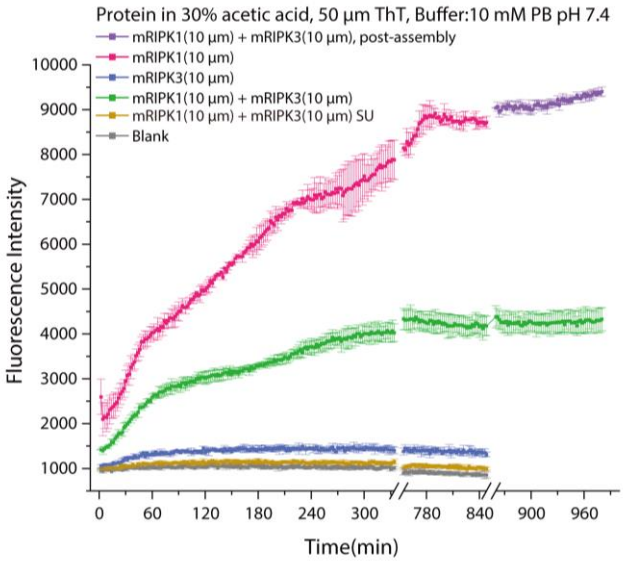
First, THT experiment will not work at very acidic conditions. Second, the buffered condition for THT mimics the dialysis into unbuffered water better. Although the hetero-amyloid for NMR was prepared from 30% acetic acid by dialysis into unbuffered water, the water was in a big volume (1: 1000) and changed many times. Finally, the water pH after dialysis was close to pH 6.8. Since THT experiment was carried out in a 96-well plate with very small volume, buffer was used to maintain its pH. The NMR spectra using the two conditions display the same peak positions, shown below.



2D ^{13}C–^{13}C spectra of mRIPK1 homo-amyloid with 500 ms DARR mixing under two different assembly conditions. mRIPK1 solution dialyzed against H_2O (pink) or PB at pH 7.4 (light blue) conditions. Light blue represented mRIPK1 fibrils prepared using ^{13}C-labeled protein mixed with ^{15}N-labeled protein in 30% acetic acid, prepared by dialyzed against 10 mM PB buffer at pH 7.4. Pink represented a fully ^{13}C-labeled mRIPK1 sample in Gu-HCl/Tris/NaCl/imidazole, pH 8.0 prepared by dialysis against Milli-Q water. The ^{13}C-labeled sample in pink was twice as much as in light blue, thus showing a higher number of relevant peaks and higher intensities than light blue spectrum.

6. The ThT experiments should include comparison of mixtures of homomeric fibrils prepared at the same concentrations and then mixed, post-assembly.

The post-assembly sample (purple in the figure below) using two homomeric fibrils in 1:1 ratio was prepared and the result was added to the figure. The comparison showed the THT value of the post-assembly sample was very different from heteromeric fibrils (green) with the same protein concentration.



7. The section discussing region G527-I530 is not clear. What is meant by "if anti-parallel b-sheet conformation was assumed"? How could an anti-parallel arrangement be accommodated here? Each layer of the fibril consists of beta-arches, the strands in each layer are not forming a beta sheet. All of the strands appear to be involved in parallel beta-sheets. The authors should explain this and include models of the proposed alternative structures.

This part was indeed very confusing. We deleted this part. After carefully analysis of the data, there is no evidence of anti-parallel conformation. It is a parallel conformation. We have shown the evidence in the manuscript.

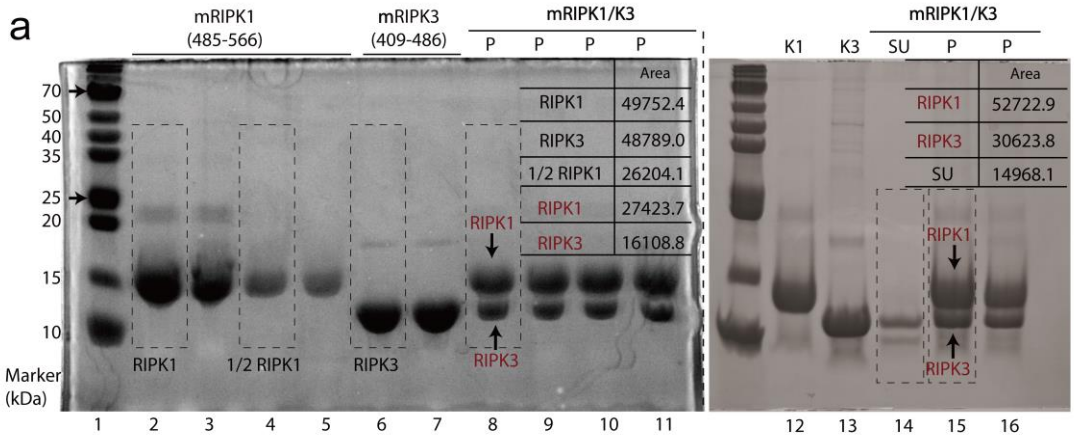
8. The ordered fibril core determined by ssNMR has dimensions of ~3.1 x 2.8 nm. AFM results indicate a height of ~3.12 nm. Fig S4 legend indicates a fibril diameter of about 3.1 nm. This appears to be a histogram of height measurements, not diameter. Authors should comment on the fact that this does not account for the other residues which are not observed. Fig S4b may give an indication of the additional structures, with distance from edge-to-edge about 40nm.

AFM reading on the fibril height is more accurate than its edge-to-edge reading and usually is used to describe the dimension of the fibril in the literature. Because tap-mode was used in AFM, it cannot tell accurately where edge rises. We did not want to distinguish "height" and "diameter" in this case, so we change the word to "height" to be consistent. Other residues that were not in the fibril core may distribute randomly around the fibril. But these residues do not have a folding structure, they probably only contribute little to the height. We added the comments in the manuscript.

9. There is no validation of the formation of heteromeric amyloid. How much of each protein remained in the supernatant after dialysis? How were the fibrils pelleted after assembly, before treatment with urea and SDS-PAGE?

From the THT reading, the heteromeric amyloid displayed a fluorescence value

different from any one of the homomeric fibril or post-assembly of the two homomeric fibrils, indicating a different structure from the homomeric fibrils. We measured the remained protein in the supernatant after dialysis (figure S11A, lane 14). Using SDS-PAGE, we found RIPK3, but no RIPK1 in the supernatant, consistent with a lower ratio of RIPK3 in the fibril pellet. The fibril was pelleted using 225,000× g (Beckman Optima XPN-100, USA) for 1h at 4℃ before treatment with urea.



10. The changes in RIPK3 peaks in the RIPK1:RIPK3 mixed sample are only described in the text and quite difficult to visualise without additional figures or perhaps colour changes in Fig 6 to highlight the ones that could be indicative of changes in RIPK3 3rd b-strand. How significant are these changes, given the large decrease in intensity from RIPK3 signals?

We added zoomed in views and 1D slices on several regions and adjusted the color in figure 6. The expanded region clearly showed RIPK3 correlation peaks disappearing or decreases significantly in the hetero-amyloids. We agreed with the reviewer that the large decrease in intensity from RIPK3 signals itself could also cause the difficulty to observe these inter-residue cross-peaks. Therefore, we adjusted our conclusion in this part as "Although the signal intensity decrease in general for mRIPK3 may cause the disappearance of inter-residue cross-peaks, the results all together suggest that mRIPK3 in the hetero-amyloid adopted a less rigid structure compared to homo-amyloid".

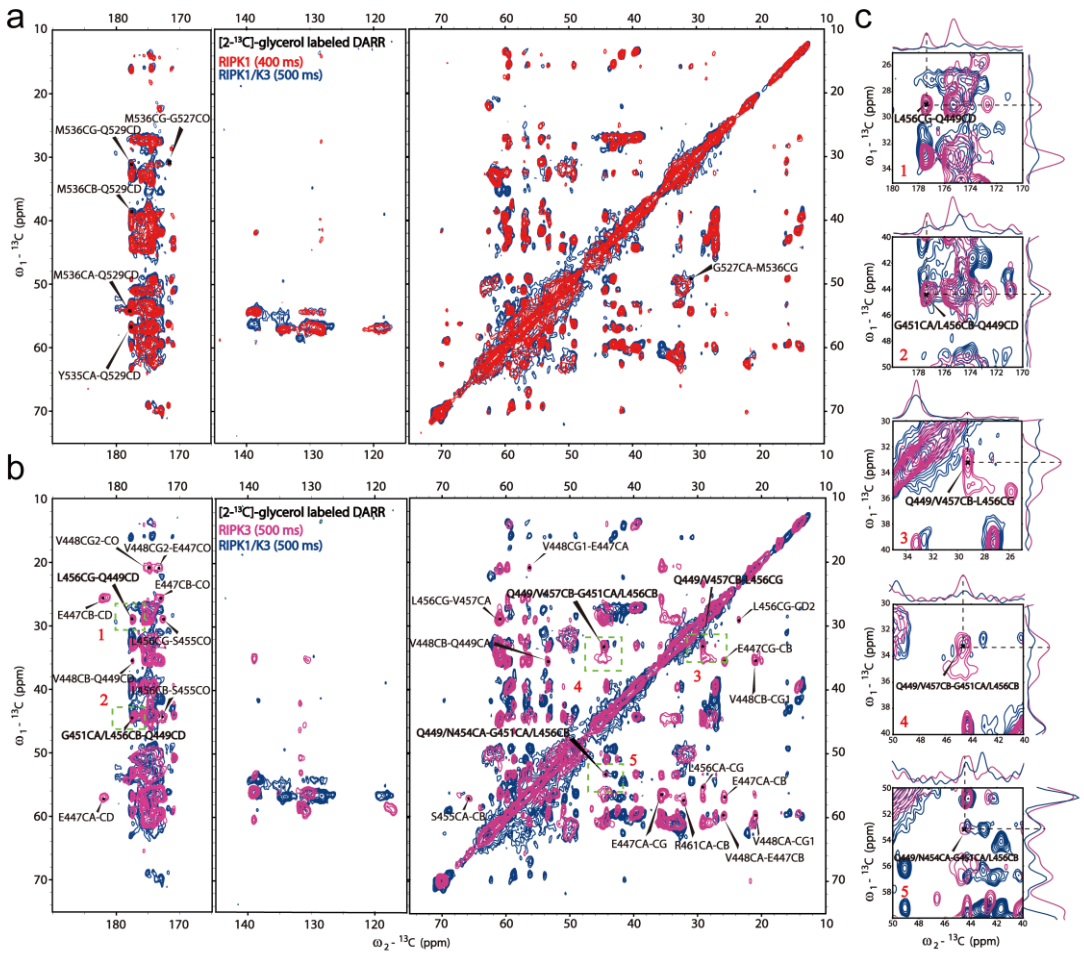
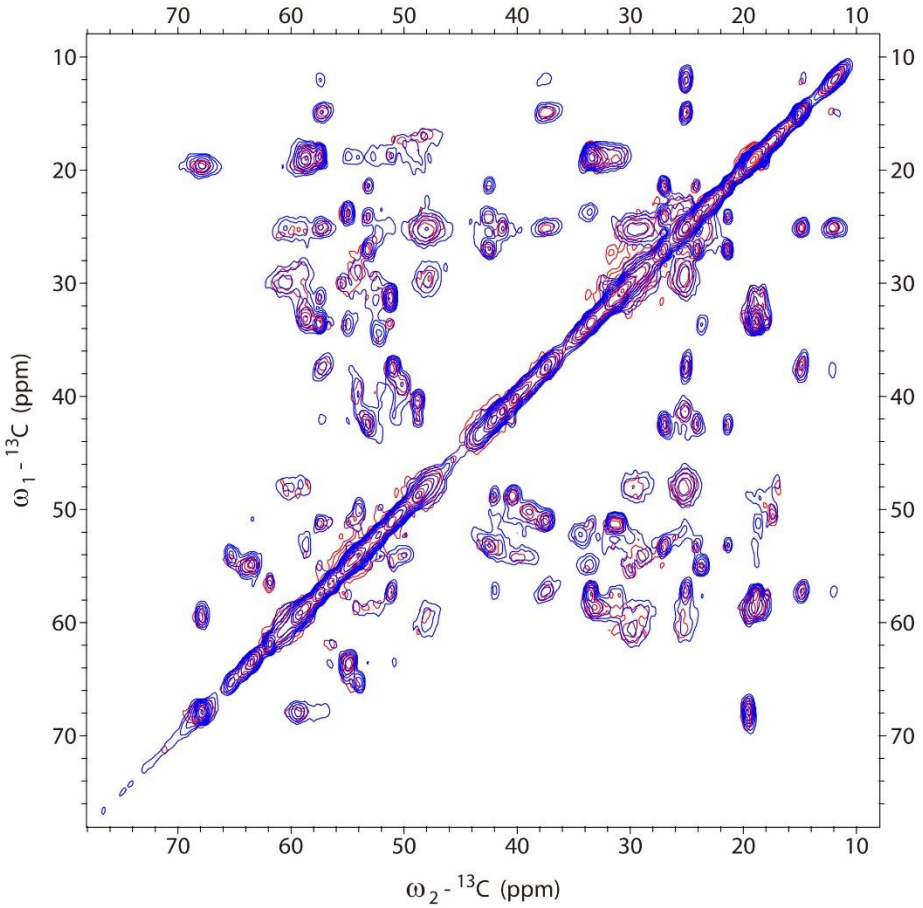


Figure. 6 2D ^{13}C–^{13}C correlation spectra of [2-^{13}C]-glycerol labeled protein fibrils with 400 or 500 ms DARR mixing, indicating different changes for mRIPK1 and mRIPK3 in the hetero-amyloid. Recorded on the mRIPK1 fibrils (red), the mRIPK3 fibrils (magenta), and the mRIPK1/mRIPK3 fibrils (dark blue). (a) The correlation peaks of mRIPK1 basically matched with those in the hetero-amyloid. (b) Many correlation peaks of mRIPK3 disappeared or attenuated in the mRIPK1/mRIPK3 spectrum, indicating by the bold labeling for the mRIPK3 fibrils. The peaks for L456Cγ-Q449Cδ, G451Cα/L456Cβ-Q449Cδ, Q449/V457Cβ-L456Cγ, Q449/V457Cβ-G451Cα/L456Cβ, and Q449/N454Cα-G451Cα/L456Cβ were highlighted in (c).

11. It would be helpful if the authors could indicate whether the NMR data collected here for the homomeric RIPK3 is closely similar to that reported previously (https://www.nature.com/articles/s41467-021-21881-2). From the structure presented in Fig 7, it is not clear whether the authors are suggesting that in the mixed sample, the 3rd strand is disordered or in the same more open conformation seen previously.

The spectra of homomeric mouse RIPK3 is quite repeatable. The comparison is shown below for the uniformly ^{13}C-labeled mouse RIPK3 fibril (blue color is from the reference and red is from this paper). RIPK3 in the heteromeric fibril still keeps the same secondary structure as RIPK3 homomeric fibril since no big changes in the chemical shifts upon mixing with RIPK1. The chemical shifts comparison is shown in figure S15. We think upon mixing with RIPK1, the last β-strand of

RIPK3 has a more open conformation, but it is still a β-strand conformation.



12. All structure figures would benefit from labels being offset to improve readability, and all structures being presented in the same orientation. For Fig 7, please include residue numbers.

Changed

13. The authors raise the possibility that residues immediately before the IQIG tetrad could be the difference for the different orientations of the 3rd strand. This may be true but is speculative, in the absence of any mutagenesis data. The evidence they cite regarding RHIM-A and RHIM-B of ZBP1 is not directly comparable, given the mutations introduced to the ZBP1 RHIMs were AAAA in the core tetrad and did not alter the preceding residues.

Replied above on the mRIPK1 mutant G527E study. We deleted the discussion referenced to ZBP1. We thank the reviewer for the suggestion and will carry out more work to investigate this issue.

14. The discussion of the relative stability of RIPK1:RIPK3 hetero-amyloids is interesting but should also consider whether the stability of RIPK3 in homomeric amyloids would be similar/different, given this more extended or disordered strand, and the impact of HSPA8 on homomeric RIPK3 fibrils.

The discussion of the relative stability of RIPK1:RIPK3 hetero-amyloids was

compared to RIPK3 homomeric amyloids and the RIPK3 in hetero-amyloids was less rigid than it in homo-amyloid. The secondary structure of peptides in the hetero-amyloids was not changed since the chemical shifts were still the same compared to the homo-amyloid. In our hand, the last two strands of RIPK3 in the hetero-amyloids showed attenuated cross peak signals, indicating less interactions with each other. At the same time, homomeric RIPK3 is also less compact than homomeric RIPK1. Both cases would probably facilitate their interactions with HSPA8.

15. Experimental details for preparation of RIPK3 are lacking.

Added

Reviewer #2 (Remarks to the Author):

The manuscript by Liu et al describes the structure determination of the core of homomeric amyloid fibrils formed by mouse RIPK1 (mRIPK1) by solid state NMR (SSNMR). In addition, spectroscopic data reflecting changes upon heteromeric fibril assembly when mRIPK1 combines with mRIPK3 are informed and discussed. The presented 3D structure of mRIPK1 as well as the SSNMR data on mRIPK1-mRIPK3 fibrils are important pieces to advance our understanding of amyloid signaling in immunity. While RIPK3 and RIPK1-RIPK3 amyloid fibrils have been previously studied, the structure of RIPK1 has remained unsolved. Therefore, before recommending publication, the following two major points (in addition to minor points) should be addressed.

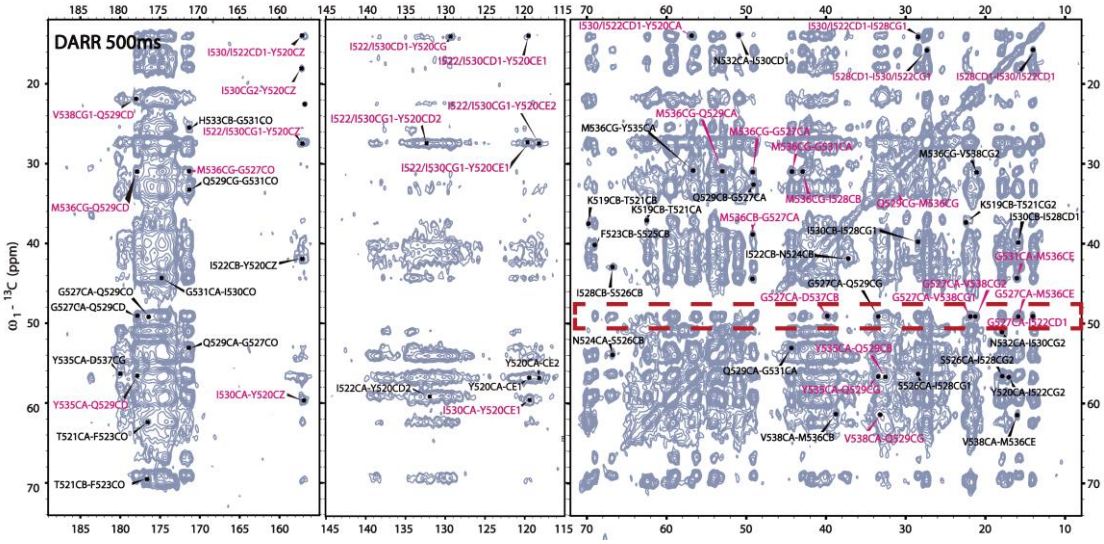
(A) Collection of distance restraints to define the fold in mRIPK1 fibrils.

The 3D structure presented is based on several assumptions that require verification through experiments. Although the N-shape appears to be the most plausible fold based on previous published work, supporting data must be obtained from experimental distance restraints. More precisely:

(i) The structural model encompasses residues 516-539, whose sequence DLIKYTIFNSSG*IQIG*NHNYMDVG contains the canonical RHIM tetrad I528QIG531 (between "*") that is crucial for mRIPK1 self-assembly. However, the authors hardly found long-range cross-peaks involving these two most relevant isoleucines (I528 and I530) that would enclose a hydrophobic core with the first β-strand as depicted in their N-shaped structure.

There are isoleucine correlation peaks. Actually, the fibril core has four isoleucine residues, with correlation peaks to each other in a small crowded region. I518 is the only isoleucine displays relatively weaker intensity, probably caused by its location near the edge of the fibril core. We added some

assignments at isoleucine region in figure S8. For example, there are I522/I530 to I528 correlation peaks. Those correlation peaks corroborated with G527 to I522/I530 correlation peaks and I530 to Y520 peaks, supporting our structural model for RIPK1.



In line with the preceding paragraph, on page 2 the authors state that a number of resonances have been tentatively assigned: “these resonances were probably from residues A514, D515, D516, L517, 518, (…)”, and acknowledge that “the assignment for these residues was sometimes ambiguous since not all the correlation peaks could be found”. Given the essential role of this hydrophobic core in defining the proposed N-shape structure, the authors should prioritize assigning these residues and obtaining unambiguous distance restraints (currently is only supported by one unambiguous contact, vide infra). The reviewer questions why the authors did not evolve the third dimension in their 13C-detected 2D NCACX and NCOCX experiments, as done with their CONCA experiment. With these three 3D experiments for sequential backbone assignments, the ambiguities mentioned above could potentially be resolved in a short timeframe. According to Table 4: solid-state NMR experimental conditions, these experiments took 8 (NCACX) and 12 hours (NCOCX), making it feasible (time-wise) to record the corresponding 3D versions within 1.5 days (the CONCA is more demanding and took less than 2 days).

3D NCANX, NCONX were carried out and the assignments on A514, D515, D516, L517, I518 were confirmed. The sequential assignments from A514 to K519 was shown in figure S3. The number of scans for each 3D is 64.

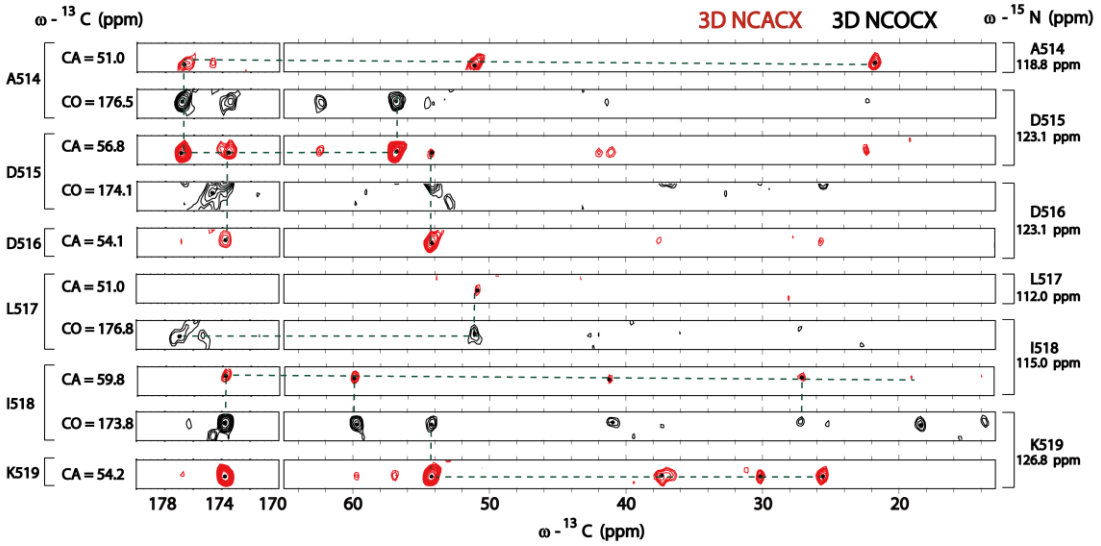


Fig. S3 3D NCACX (red) and NCOCX (black) spectra of uniformly ^{15}N, ^{13}C-labeled mouse RIPK1 fibrils, collected at 700 MHz field strength, 15 kHz MAS and 273 K.

(ii) Building upon the previous point (i), the authors have identified only one unambiguous distance restraint between the first β-strand and the canonical RHIM core, specifically involving Y520CZ and I530CG2&CD1. The reviewer expects at least contacts involving Y530 CE & CD, according to the authors' assignment table, but they were not listed. Therefore, it is important that the authors show the aromatic region of their spectra at short and long DARR mixing, as this region displays little overlap and according to the assignment table (Table 1) and the structural model depicted in Fig. 5, essential restraints are expected. **There are correlation peaks between Y520 CE & CD and I522/I530CD1. We modified the Y520CZ and I530CD1 peak assignment to Y520CZ and I522/I530CD1. We added the aromatic area in figure S8 for uniformly labeled sample with 500 ms mixing time. We also added the aromatic region in figure S1 with 50 ms mixing time.**

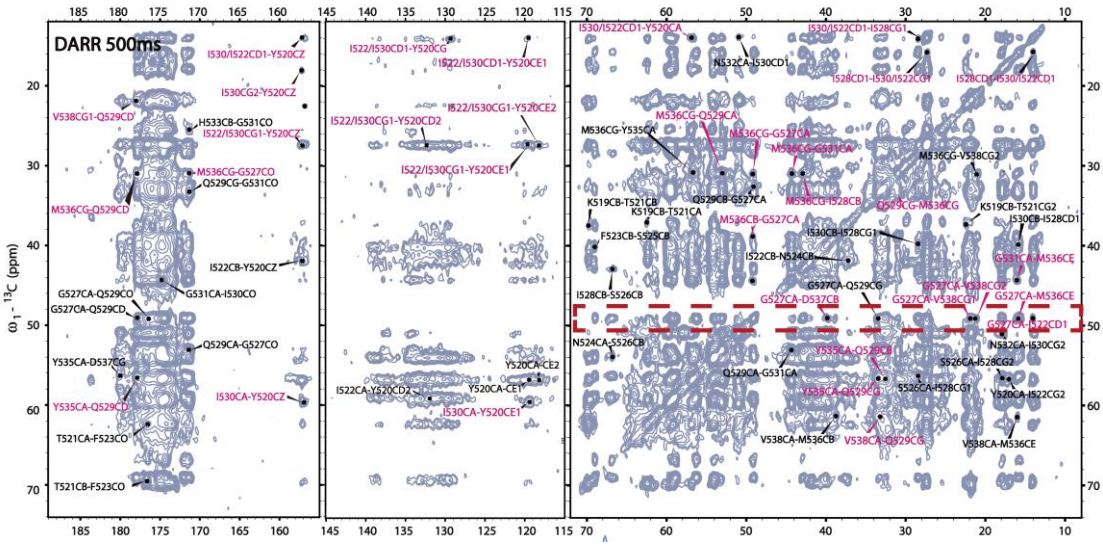


Fig. S8 2D ^{13}C-^{13}C DARR spectrum of uniformly ^{15}N, ^{13}C-labelled mRIPK1 fibrils with long mixing time, showing long-range correlation peaks for mRIPK1 fibril.

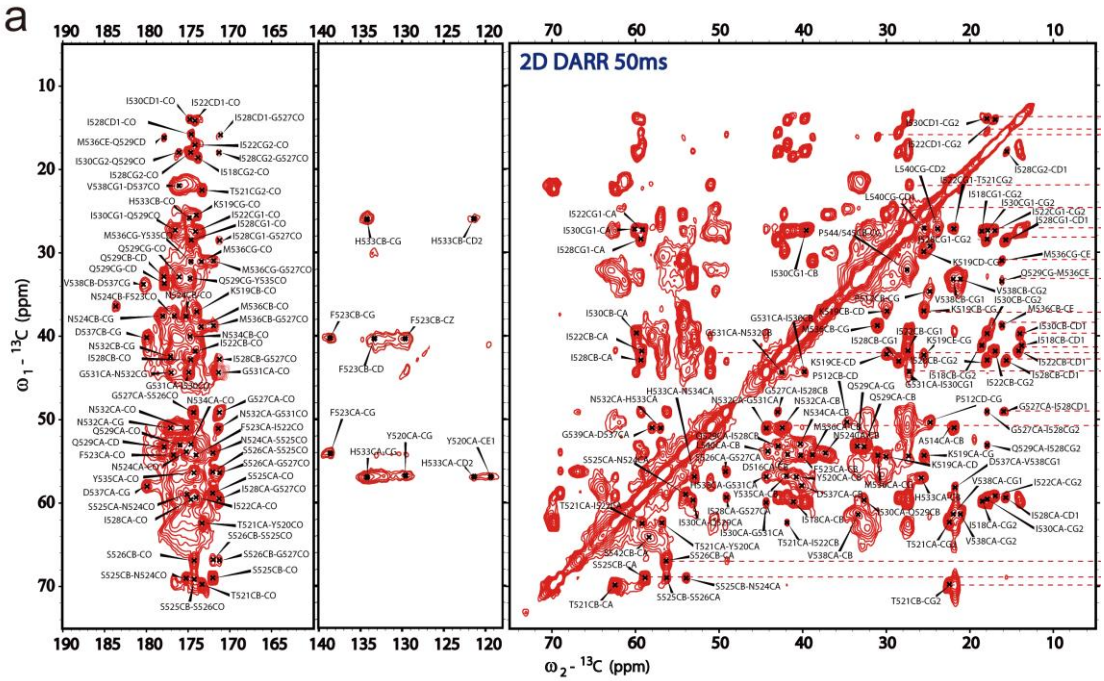


Fig. S1 ^{13}C ssNMR spectra of uniformly ^{15}N, ^{13}C-labeled mouse RIPK1 fibrils. (a) 2D ^{13}C-^{13}C DARR spectra with 50 ms mixing time.

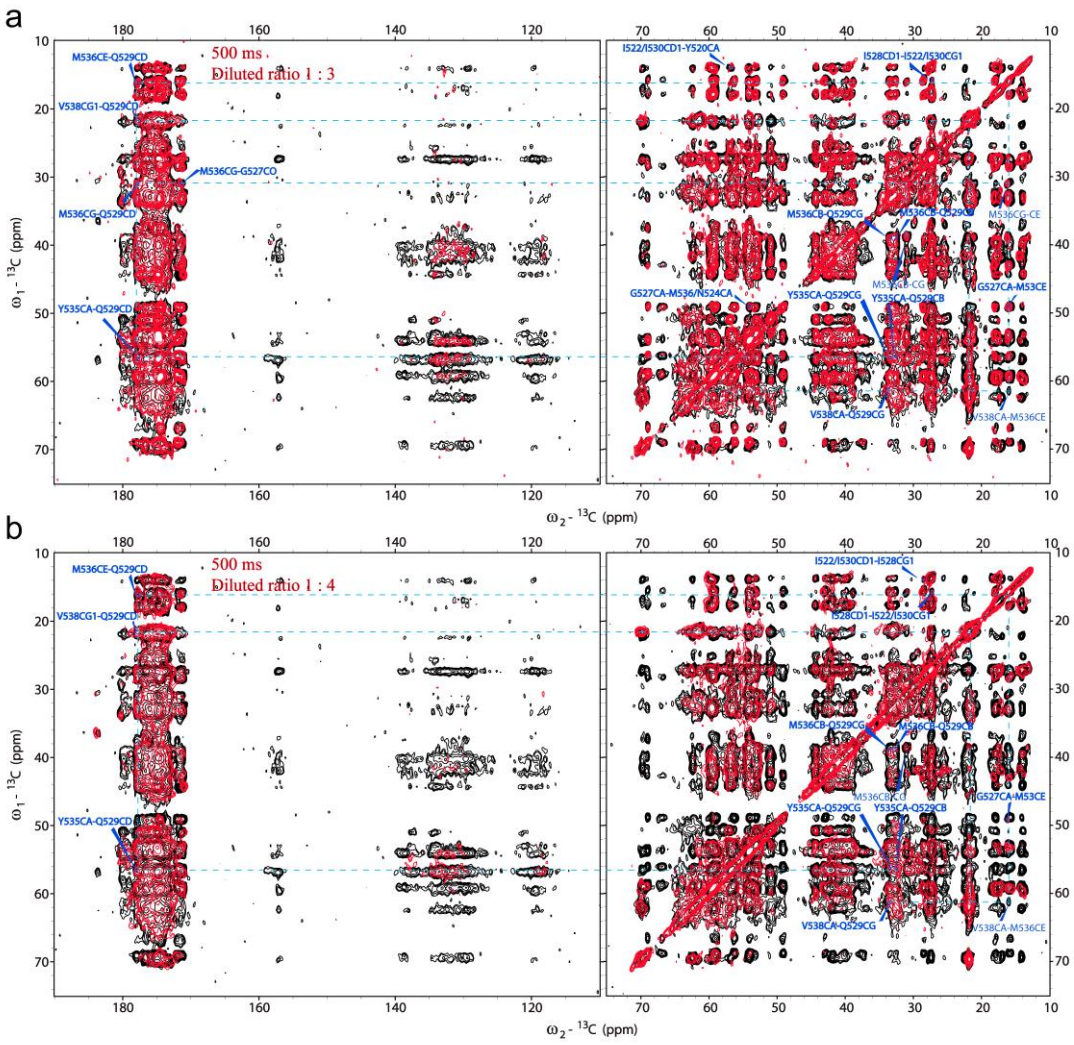
(iii) The classification of cross peaks as intra- and intermolecular is not consistently justified. Given that amyloids consist of stack of monomers, intramolecular contacts can only be reliably assigned when experiments are conducted on samples containing mixtures of [12C,14N]-mRIPK1 and [13C,15N]-mRIPK1 at a diluted ratio (e.g., 1:4, see PMID 33046908).

To clarify this point, the authors would agree in that a contact between two nuclei (e.g., CA-CB) within a given residue (e.g., I530) can be both intra- (I530CA-I530CB from same monomer) and inter-residue (I530CA in one monomer with I530CB from the monomer in the strand immediately above or below). This is also true for distinct residues, either sequential (e.g., I530 and G531) or more distant (e.g., G527 and M536). Thus, when the authors assert that certain cross peaks, such as those between G527CA and M536 “were all considered intramolecular interactions defining the folding of the subunit”, they need to provide appropriate experimental evidence. In fact, it is noteworthy that, according to the structure, the G527CA -M536CG distance is 5.8Å if belonging to the same monomer (intra), but 6.3Å if belonging to distinct molecules (inter), with both values within the quite broad 5.5 +/- 2.5Å distance range listed in Table 2.

To address these ambiguities, the authors are encouraged to prepare an

isotopically diluted sample of [13C,15N]-mRIPK1 : [12C,14N]-mRIPK1 to accurately distinguish intra- from intermolecular contacts. This step is particularly crucial given the multiple uncertainties present in the current dataset.

The isotopically diluted samples were prepared in 1:3 ratio and 1:4 ratio. 1:4 ratio resulted in very weak signals and the spectra comparison was shown here. The 1:3 ratio sample displayed a better spectrum. Both were added in the supplement figure S9 in the paper. We found important correlation peaks such as G527Ca-M536Ce, Q529Cb/Cg-M536Cb in the sample with 1:3 or 1:4 dilution, proving these are intra-molecular interactions. The correlations between I528CD1-I522/I530CG1, I522/530CD1-I528CG1 can still been observed upon dilution. However, the peak intensity at aromatic region was significantly attenuated, preventing a confirmative conclusion. In all, the results support intra-molecular interactions between β-strands. Therefore, our original assumption was kept. We modified this part in the paper.



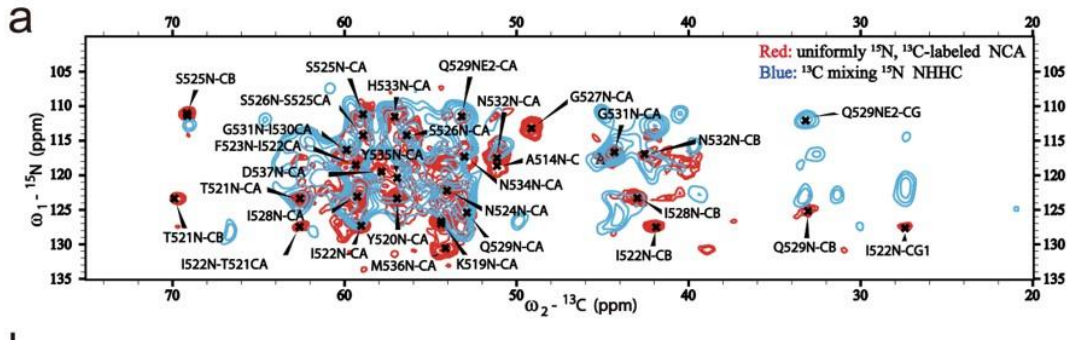
(iv) The authors stated, “The inter-molecular arrangement of the hydrogen-bonded subunits was investigated with 13C-13C correlation experiments on sparsely 13C-labeled mRIPK1 fibrils using [2-13C]-labeled glycerol as a carbon source”.

However, this statement might cause confusion, as the elucidation of the H-bonded arrangement was not possible with the glycerol-derived samples, but rather from their study of a 1:1 mixed sample composed of [12C,15N]-mRIPK1 and [13C,14N]-mRIPK1.

The statement was modified as "The inter-molecular residue alignment of the neighboring subunits was...". The description on the experiment on a 1:1 mixed sample composed of [^{12}C, ^{15}N]-mRIPK1 and [^{13}C, ^{14}N]-mRIPK1 was also moved here to support the conclusion.

The comparison of an NCA (on a U-13C15N sample) with an hNHHC spectrum ([12C,15N] : [13C,14N] mixed sample), as shown in Fig. S7, cleverly demonstrates the parallel arrangement, should cross peaks be coincident upon superposition of the two spectra. However, according to Table 4, the authors conducted both experiments using the same 3.2 mm HCN probe, but at significantly distinct temperatures (ca. 40 degrees, 309K for NCA vs. 271K for hNHHC). It is essential to clarify whether the authors adjusted peak positions in Fig. S7 to show the overlay, and if so, why this adjustment is not explicitly mentioned in the text. To strengthen the evidence of parallel stacking, the authors should repeat these experiments at the same temperature and present the matching of peaks. Given the importance of this experiment, the revised results should be incorporated into the main text.

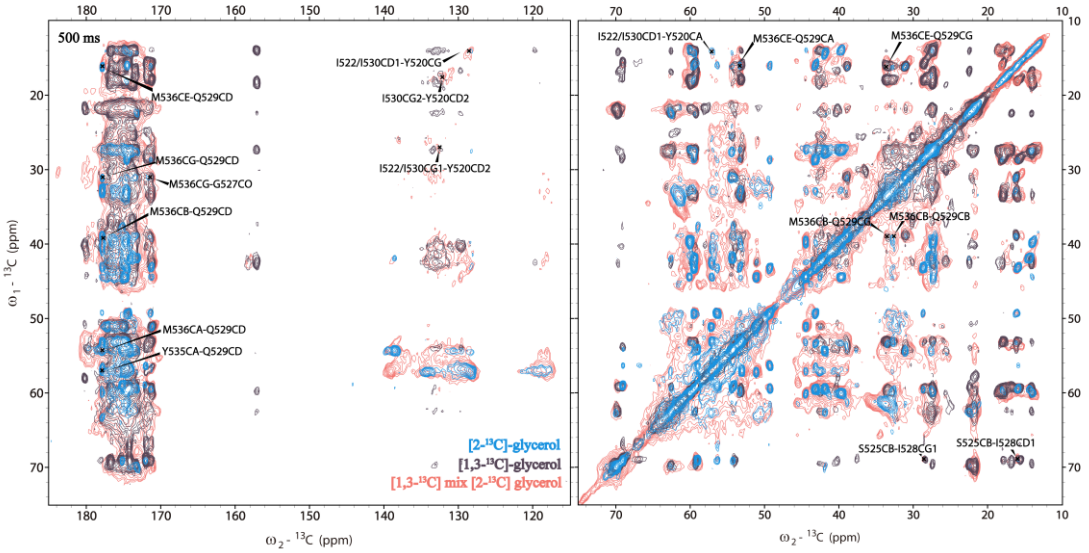
NCA on uniformly labeled sample was repeated at 255 K and the figure was updated to compare with hNHHC. The comparison showed most of N-Ca correlation peaks were overlapped. No chemical shift adjustment was made.



(v) The sample generated using [2-13C]-labeled glycerol as a carbon source should be combined with one prepared using [1,3-13C]-labeled glycerol to measure exclusively intermolecular contacts for precise collection of distance restraints. This approach represents the most direct way to incorporate the necessary information into the workflow for structure calculation (e.g., PMID 27165577 or 29627359).

We prepared one sample using [1,3-^{13}C]-labeled glycerol, one sample using [2-^{13}C]-labeled glycerol and one using 1:1 ratio of peptides using two types of labeling schemes. In particular, we found many inter-residue peaks observed using [1,3-^{13}C]-labeled glycerol or [2-^{13}C]-labeled glycerol remained even after

mixing of two different peptides. Therefore, the results support those inter-residue correlations are intra-molecular. The aromatic peaks were attenuated. But some weak peaks corresponding to correlation between Y520 CD &CG and I522/530 can be observed.



(B) Restraints sorting and structure calculation.

(i) With the introduction of two new samples (diluted with 1:4 ratio of [13C15N] : [12C14N] to establish the monomer fold, and a 1:1 ratio from [13C-1,3] : [13C-2] glycerol for intermolecular contacts), the nature of the assignments reported in Table 2 could be ascertained (prior to 13C-detected confirmation of the assignments).

Addressed above.

Regarding the structure calculation process, the authors should provide clarification on how many rounds of calculations were performed before arriving at the final model. The current methods section may give the impression that a single calculation led to the displayed structural model. However, upon examining the deposited restraint file (8ib0.mr), it appears that, at an initial stage of their protocol, HN－O and N－O distances for residues 7 to 8, 15 to 16, and 19 to 20 were restrained to 2.30 and 3.30Å, respectively. This implies explicit definition of hydrogen bonds from I522 (HN) to F523 (O), I530 (HN) to G531(O), and N534 (HN) to Y535 (O). It's noteworthy that these residues, based on geometric quality criteria in the PDB validation report, are considered outliers. While the explicit inclusion of hydrogen-bonding restraints is a common procedure, it should be explicitly described in the text for the sake of reproducibility. The authors should detail how they implemented hydrogen-bonding restraints and update Table 3 accordingly.

Two rounds of calculations were carried out. We did not use explicit definition of hydrogen bonds from I522 (HN) to F523 (O), I530 (HN) to G531(O), and N534 (HN) to Y535 (O) although it was in the list. There were “#” sign before each line, therefore, it was not used in xplor-nih. We are sorry for this. We should have deleted these lines if we did not use them.

(ii) In relation to Table 3, the authors should reconsider the table using curated data from both diluted and mixed samples. Currently, they differentiate between unambiguous and ambiguous intramolecular contacts. However, given the current experimental design, it is impossible to distinguish whether these contacts are intra- or intermolecular. This introduces a new layer of ambiguity to their dataset.
Addressed above. There is more evidence for intra-molecular interactions although some are from ambiguous assignments. The possibility for two layers of parallel sheets arranged in anti-parallel (like hRIPK1/hRIPK3) is not tested since we still see the cross-peaks between the first and the center β-strands and between the last and the center β-strands. These interactions would prevent the center β-strand interacting with other partners.

(iii) The authors observed that certain distances in their structural models are longer than expected, surpassing typical experimental cutoffs (e.g., > 9Å for cross peaks in 400 ms DARR). Based on this observation, they proposed the possibility of monomers stacking antiparallel to each other along the fibril axis. For instance, the authors suggested that “The inter-molecular distance between G527 to I530 could be closer (< 9Å) if an antiparallel β-sheet conformation was assumed for the mRIPK1 I528-I530 segment”.

To address the question of the antiparallel arrangement, the authors conducted a series of CHHC experiments, assuming that the absence of N-ter to C-ter (with respect to the RHIM region) contacts is indicative of a parallel arrangement. However, this interpretation is not straightforward, especially given the number of residues within the N-terminus that could not be assigned unambiguously. The authors should consider curating their restraints with the new samples to refine their analysis. Additionally, it is advisable for the authors to employ CHHC in conjunction with DARR/PDSD for the characterization of the new samples, as CHHC provides a spectrum with much less spin diffusion.

By carefully study our results, we do not see any evidence on anti-parallel conformation. We modified the peak (G527CA to I530CD1) to an ambiguous assignment (G527CA to I530/I522CD1). We carried out 13C-detected 3D experiments to confirmed some assignments. CHHC results also support a parallel conformation.

(iv) In line with the previous point, the authors may consider using an antiparallel model in their calculations, and see whether their experimental restraints (the one that they actually see) could be accommodated within such an alternative model. This approach would be more systematic than disregarding conformations through visual inspection.

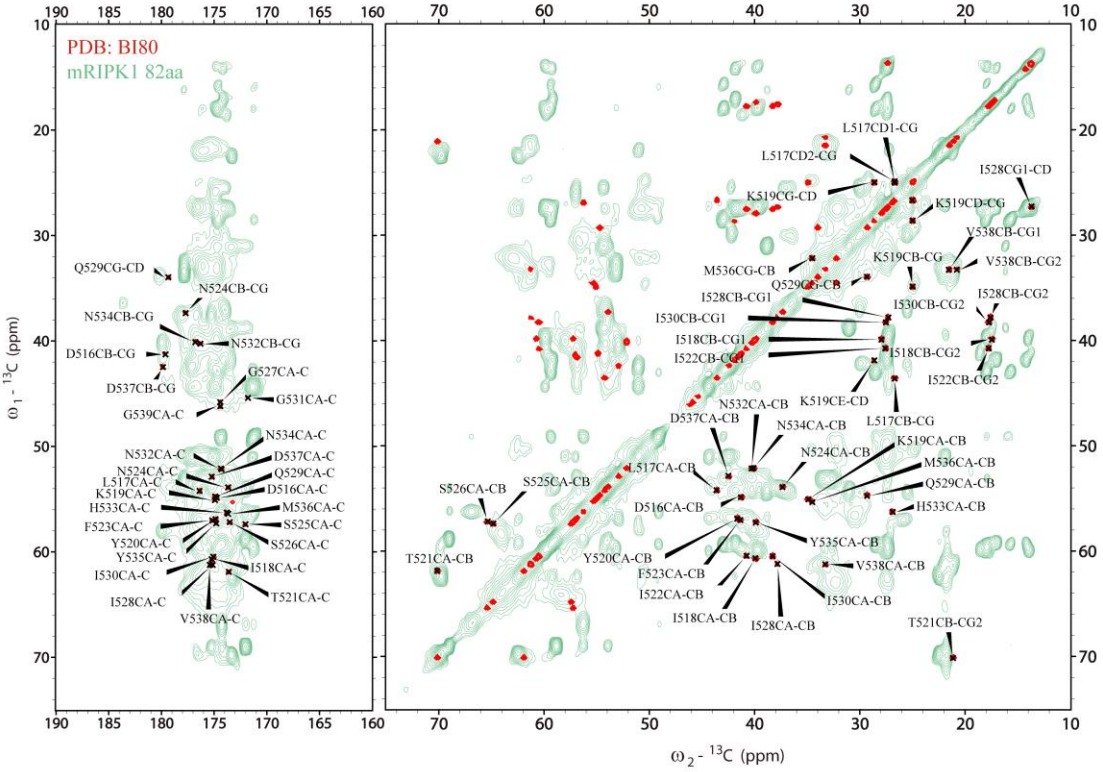
Addressed above. We tried previously, and did not find a possible model for the antiparallel conformation.

(v) MPL measurements suggest one monomer per layer, but what if antiparallel dimers stack along the fibril axis with a parallel arrangement? The lack of sequential connectivities within the first β-strand, contacts to the IQIG tetrad, the presence of longer distances for these few residues, and the overall parallel arrangement may be spectroscopically compatible. In their prior work, the authors employed MD simulations to evaluate the stability of such a fibril of dimers. Could the I528-I530 segment (third β-strand) act as a dimeric interface, aligning with the absence of cross-peaks (only with a single nucleus of Y530, as mentioned earlier)? In scenarios where distinct species coexist, it's plausible that only the smaller species are attached to copper grids. To mitigate potential biases in the resulting conformations, the authors are encouraged to explore different models that could potentially accommodate the experimental restraints. The set of ambiguous restraints may align with alternative models, suggesting the presence of distinct yet spectroscopically similar species that can be only explored through calculations (e.g., PMID 25395337 for an examination of fibrils with 2- and 3-fold symmetry). Currently, many unresolved ambiguities and unexpectedly large distances may encode a secondary minor species (e.g., N532 to I518, G531 to K519, or G531 to Y520).

Based on our current data, especially the added data in this reply, we think all indicate a parallel conformation.

(v) Back calculations of the expected cross peaks from the determined structures (for each of the alternative models) are needed to verify the correlation between structure and spectroscopic data.

The ^{13}C chemical shifts were back calculated from the deposited structure (PDB: 8IB0) by the SHIFTX2 (http://www.shiftx2.ca/index.html), and these predicted chemical shifts were mapped on the experimentally measured ^{13}C-^{13}C correlation spectra for comparative analysis.



Minor points:

(i) The authors should display traces of selected 2D dataset for a SNR assessment.

1D slices were shown in figure S8, S14 and figure 6

(ii) The Methods section should comprehensively describe how the samples were packed into the rotors. Currently, it is just mentioned that lyophilized samples were packed and rehydrated inside the rotor. How many mg the packed? Did they rehydrate in a single step? During packing? How many µL? In both rotors?

The wet fibril was packed into the rotor directly without lyophilization and rehydration. The method was modified in "Assembly of mRIPK1 fibrils in vitro for solid-state NMR studies". Extra water was removed during packing. Usually 20 mg wet sample can be packed into a 3.2 mm standard-wall rotor, 30 mg wet sample can be packed into a 3.2 mm thin-wall rotor and 1.2-1.6mg wet sample can be packed into a 0.7mm rotor.

(iii) Dissolving the hetero-amyloid in 8M urea for SDS-PAGE analyses revealed a 1.7:1 ratio of mRIPK1 to mRIPK3. However, it is noted that 8M urea might not be sufficient to fully dissolve these fibrils, leaving many small seeds. The authors should highlight this point, unless they plan to revisit these numbers using formic/acetic acid or NaOH, which have superior depolymerization abilities.

Added in the method "Urea was chosen here since it worked better in SDS-PAGE, however, fibril dissolved more thoroughly in 30% acetic acid.". We also added this to the discussion "Our SDS-PAGE study contains several error sources too, such as the integration method in the gel analysis, how thoroughly the amyloid could be dissolved by urea, etc., However, the errors were not expected to be significant."

(iv) The mRIPK1 construct used in this study spans 82 residues, about which ~25% are visible in CP-based experiments. The authors could discuss the residue types identified through TOBSY/INEPT and potential matching with positions outside the RHIM core (e.g., the observation of CB patterns for His, Ala, etc)

Added assignments on TOBSY and it cannot suggest a special region for the flexible residues.

(v) Related to the previous point, what happened to the His-tag? According to the Methods section, the protein contained a Hexa-His tag that was not cleaved. This should be indicated in Fig. 1A.

Added His tag in Fig 1A

(vi) The results concerning mixing of mRIPK1 with mRIPK3 are not entirely clear. Does a molecular ratio of mRIPK1 to mRIPK3 of 1.7:1 suggest there would be 5 molecules of mRIPK1 every 3 molecules of mRIPK3? If so, how is this related to "a reduction of mRIPK1 of 60% in the hetero-amyloid"? These observations are intriguing, and the text could be rephrased for clarity.

The description was modified as "Figure S14 showed that the cross peaks of mRIPK1 in hetero-amyloid displayed slightly less than 60% intensities compared to mRIPK1 homo-amyloids. Since the amount of mRIPK1 was also reduced to about 60% in the hetero-amyloid (1.7/(1.7+1)=0.63), it suggested the signal of mRIPK1 generally remained in the hetero-amyloid".

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors have made significant changes in their response to reviewers' comments and suggestions. The additional data and clarifications will make it easier for the community to understand the results and implications of the work.

Points to be addressed:
The language and style of writing throughout could be improved with editing, for example checking the tense used throughout (in many places the past tense is used to refer to figures) and replacing "etc." with a full explanation.
There is reference to X-ray diffraction data but no indication of where this is shown (line 103).
The dimensions of the ordered core structure should be included, for comparison with the height as measured by AFM.

Reviewer #2:
Remarks to the Author:
The authors have carefully considered and responded to all the comments. The manuscript is now much clearer, and the conclusions are well-supported with the new data incorporated. These efforts were crucial to establish that mRIPK1 also adopts an N-shape fold, as seen in RIPK3 in their previous work.

However, one point of concern remains, namely that the two Gly residues within the critical G527-I-Q-I-G531 RHIM core may be incorrectly assigned. While Gly does not have side chains, and thus this should not affect the N-shape observed (since no key distance restraints defining the fold involve these residues), it is essential that the assignments are accurately presented. Upon re-examining Fig. 2 (prompted by the new G527E variant presented in these revisions), it seems that the connectivities between residues 528 and 532 are not correct at the critical site G531.

Now that the authors have performed NCACX, NCOCX, and CANCOCX experiments during the revisions, which have proven more valuable for assigning residues that were ambiguous and could not be detected in their original strategy using CANH, coCAcoNH, CONH, and COcaNH, they need to revise Fig. 2 and add a complementary Figure with new strip plots for the same stretch of residues (T521 to H533) with emphasis on the G527-I-Q-I-G531 RHIM core.

As mentioned, it is acknowledged that in case of a misassignment for these glycines, this will likely not alter the N-shape fold obtained from the authors' distance restraints list. However, a revision of Fig. 2 to show the proper backbone walk in the strip plot, and an analogous representation using the new set of 13C-detected experiments, will provide a robust assessment of their NMR dataset.

I appreciate the efforts the authors have made so far and believe that addressing this point will significantly strengthen their manuscript.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have made significant changes in their response to reviewers' comments and suggestions. The additional data and clarifications will make it easier for the community to understand the results and implications of the work.

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There is reference to X-ray diffraction data but no indication of where this is shown (line 103).
The dimensions of the ordered core structure should be included, for comparison with the height as measured by AFM.

Reply to reviewer #1
We have edited the language carefully using chatgpt 3.5 and showed in the manuscript using track changes. We changed to use the present tense to refer to figures. We removed some etc. and added the full explanation if necessary.

We explained what the distances from X-ray diffraction data indicated in the structure using cyan color. "The 4.7 Å distance was used as a constraint in the structural calculation to define the distance between neighboring hydrogen-bonded β-strands. The 9.6 Å distance represents the typical distance between two β-sheets."

The dimension of the ordered core structure is 3.1×2.8 nm, matching the AFM result which shows a fibril height of 3.1±0.4 nm (figure 5b). This is added in the manuscript.

Reviewer #2 (Remarks to the Author):

The authors have carefully considered and responded to all the comments. The manuscript is now much clearer, and the conclusions are well-supported with the new data incorporated. These efforts were crucial to establish that mRIPK1 also adopts an N-shape fold, as seen in RIPK3 in their previous work.

However, one point of concern remains, namely that the two Gly residues within the critical G527-I-Q-I-G531 RHIM core may be incorrectly assigned. While Gly does not have side

chains, and thus this should not affect the N-shape observed (since no key distance restraints defining the fold involve these residues), it is essential that the assignments are accurately presented. Upon re-examining Fig. 2 (prompted by the new G527E variant presented in these revisions), it seems that the connectivities between residues 528 and 532 are not correct at the critical site G531.

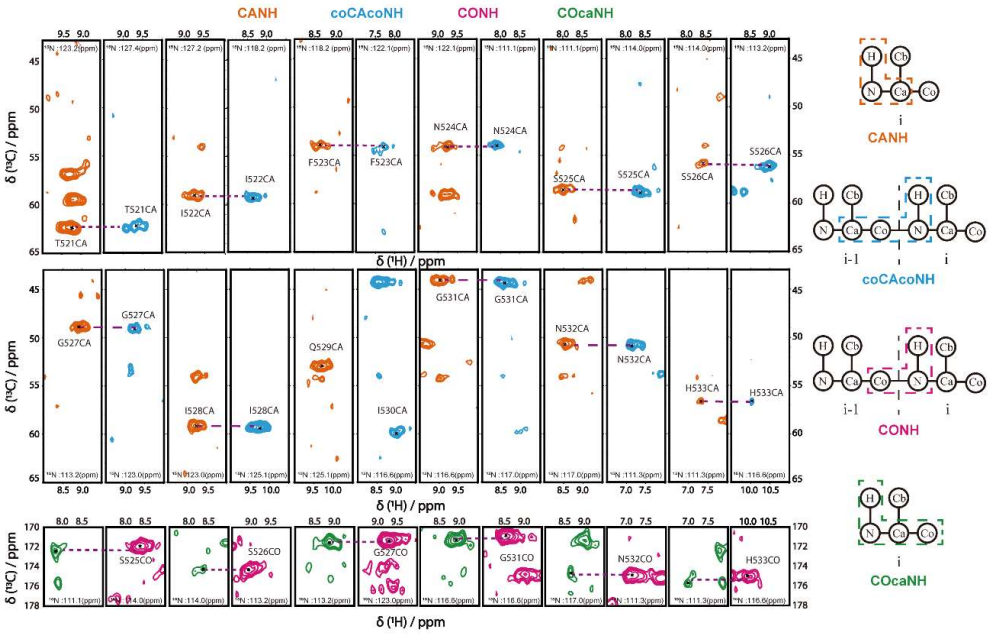
Now that the authors have performed NCACX, NCOCX, and CANCOCX experiments during the revisions, which have proven more valuable for assigning residues that were ambiguous and could not be detected in their original strategy using CANH, coCAcoNH, CONH, and COcaNH, they need to revise Fig. 2 and add a complementary Figure with new strip plots for the same stretch of residues (T521 to H533) with emphasis on the G527-I-Q-I-G531 RHIM core.

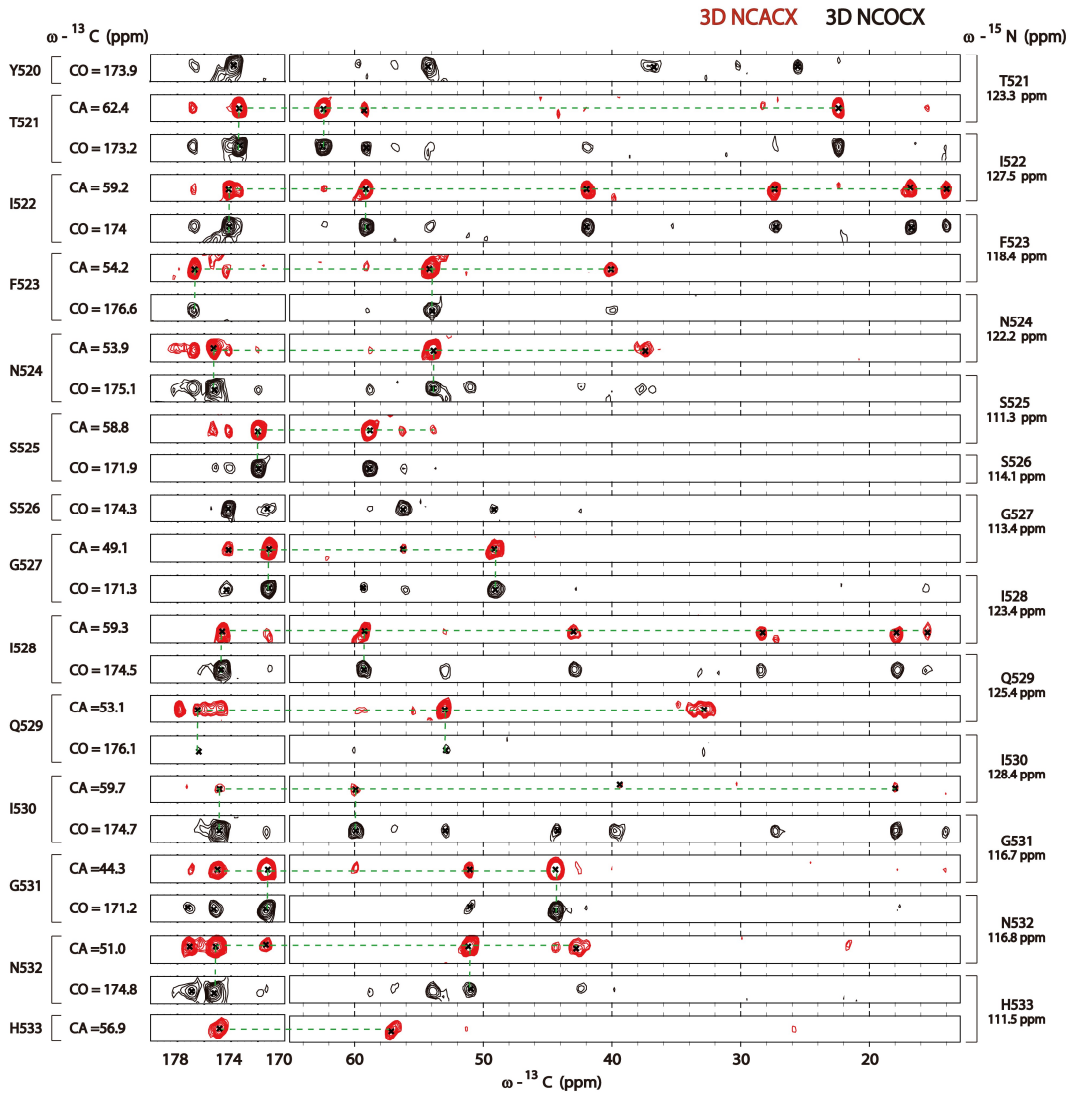
As mentioned, it is acknowledged that in case of a misassignment for these glycines, this will likely not alter the N-shape fold obtained from the authors' distance restraints list. However, a revision of Fig. 2 to show the proper backbone walk in the strip plot, and an analogous representation using the new set of 13C-detected experiments, will provide a robust assessment of their NMR dataset.

I appreciate the efforts the authors have made so far and believe that addressing this point will significantly strengthen their manuscript.

Reply to Reviewer #2

We added the strip plots (residues T521 to H533) from the sequential assignment using NCACX and NCOCX, corresponding to the region shown in figure 2 which used 1H-detected 3D experiments. The 13C-detected experiments supported our original assignments, providing the missing links. This is the new supplement figure 3. From our observation, it is the link between Q529 to I530, which showed a broken connectivity in the 3D sequential assignment in figure 2. 13C-detected experiments showed a better signal than 1H-detected experiments in this site, although it is still relatively weak. We thank the reviewer for the advice which makes the manuscript stronger in the conclusion. Here I showed both figure 2 and new supplement figure 4 for comparison.





Supplementary Figure 4. 3D NCACX (red) and NCOCX (black) spectra of uniformly ^{15}N, ^{13}C-labeled mouse RIPK1 fibrils (residues T521-H533), collected at 700 MHz field strength, 15 kHz MAS and 273 K.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors have made the suggested changes to the manuscript.

Minor point to be addressed: Line 245 Should this be upper case for However as the start of a new sentence?

Reviewer #2:
Remarks to the Author:
The authors have incorporated new strip plots to strengthen their sequential assignments based on 13C-detected experiments -although the CANCO was not included-. The manuscript reads much consistent.

From the new dataset, and upon inspection of their previous 1H-based analyses, the authors notice that the link between Q529 and I530 was missing. This is interesting since these two residues correspond precisely to the central RHIM tetrad, I538-Q529-I530-G531, which is essential for amyloid assembly during necroptosis signaling.

It is important that the authors briefly discuss this observation in their final version for two reasons: (i) the connectivities within this tetrad are expected to be the strongest based on prior literature on fungal and mammalian proteins involved in programmed cell death, and (ii) the presence of additional IG/VG pairs within the protein sequence that may differ from I530-G531 could confuse readers who notice the broken link within the RHIM tetrad I528-G531. This confusion arises since strip plot data are shown only for the segment that is N-terminal to the RHIM tetrad (T521-I528) but not for the segment C-terminal to the RHIM (N532-L540).

These additional comments will make it easier for the community to understand the consistency of their dataset.

We addressed the reviewers' comments here.

Reviewer #1 (Remarks to the Author):

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Minor point to be addressed: Line 245 Should this be upper case for However as the start of a new sentence?

It is changed in the manuscript.

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These additional comments will make it easier for the community to understand the consistency of their dataset.

Although the connection between Q529 and I530 is weak, our assignment is still with high confidence since there is no other IQ or IG residue pair in the sequence and the sequential assignments before Q529 and after I530 were done well as shown in figure 2 and figure S4. In the later secondary structure prediction, we pointed out that the 13C chemical shift prediction of I530, indicating it is at the end of a β-strand, which could lead to a decreased rigidity of I530. A less rigid structure of I530 may cause a weak connection signal between Q529 and I530.

In the 1st section of the result, the following two parts were added: "It is interesting to notice that the connection between Q529 and I530, which is at the center of RHIM domain, is weak. Considering the spectra regions showing the sequential connectivity before Q529 and after I530 are in relatively good quality, and there are no other IQ or IG residue pair in the entire

sequence, the assignment is with high confidence**.”** **“**According to the secondary chemical shift prediction, I530 is right at the edge of a β-strand with the value of $\Delta\delta^{13}C\alpha$-$\Delta\delta^{13}C\beta$ close to 0 ppm. This could lead to a decreased rigidity of I530, which might explain that only a weak connection between Q529 and I530 was observed in the 3D sequential experiments.”