

1 **The RHIM within the M45 protein from murine cytomegalovirus forms**  
2 **heteromeric amyloid fibrils with RIPK1 and RIPK3**

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15

16 **Abstract**

17

18 The M45 protein from murine cytomegalovirus protects infected murine cells from death by  
19 necroptosis and can protect human cells from necroptosis induced by TNFR activation, when  
20 heterologously expressed. We show that the N-terminal 90 residues of the M45 protein,  
21 which contain a RIP Homotypic Interaction Motif (RHIM), are sufficient to confer protection  
22 against TNFR-induced necroptosis. This N-terminal region of M45 drives rapid self-  
23 assembly into homo-oligomeric amyloid fibrils and interacts with the RHIMs of human  
24 RIPK1 and RIPK3 kinases to form heteromeric amyloid fibrils *in vitro*. An intact RHIM core  
25 tetrad is required for the inhibition of cell death by M45 and we show that mutation of those  
26 key tetrad residues abolishes homo- and hetero-amyloid assembly by M45 *in vitro*,  
27 suggesting that the amyloidogenic nature of the M45 RHIM underlies its biological activity.  
28 Our results indicate that M45 mimics the interactions made by RIPK1 with RIPK3 in forming  
29 heteromeric amyloid structures.

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31

## 32 **Introduction**

33

34 The RIP homotypic interaction motif (RHIM) was first identified in receptor-interacting  
35 protein kinase 1 (RIPK1) and receptor-interacting kinase 3 (RIPK3)<sup>1</sup>. A RHIM contains 18–  
36 19 amino acids, with a tetrad (V/I)-Q-(V/I/L/C)-G core sequence (Fig. 1a). An intact RHIM  
37 is required for the interaction between RIPK1 and RIPK3 that occurs downstream of tumour  
38 necrosis factor receptor 1 (TNFR1) activation, during the programmed cell death response  
39 known as necroptosis<sup>2,3</sup>. In addition to the TNFR1 pathway that leads to RIPK1:RIPK3  
40 association, two other pathways involving RIPK3 and specific RHIM-containing adapter  
41 proteins can result in necroptosis<sup>4</sup>. Binding of microbial pathogen-associated molecular  
42 patterns to Toll-like receptors 3 and 4 can lead to activation of RIPK3 through formation of a  
43 complex with the RHIM-containing TIR-domain-containing adapter-inducing interferon- $\beta$   
44 protein (TRIF). Additionally, the Z-DNA binding protein/DNA-dependent activator of  
45 interferon regulatory factors (ZBP-1/DAI) is a cytosolic nucleic acid sensor that binds foreign  
46 nucleic acid and acts as the RHIM adapter protein for RIPK3 in viral-induced necroptosis<sup>5-8</sup>.  
47 In all three pathways, oligomerisation and autophosphorylation of RIPK3 occurs after  
48 binding of RIPK3 to the adapter protein. The activation of RIPK3 leads to phosphorylation  
49 and oligomerisation of mixed lineage kinase domain-like protein (MLKL) and this commits  
50 the cell to membrane disruption, ion dyshomeostasis and lytic necrotic cell death<sup>9-12</sup>.

51

52 Necroptosis has essential functions in adult tissue homeostasis and innate immune defence  
53 against intracellular pathogens, however undesirable necroptosis can be triggered by  
54 ischaemic tissue damage<sup>13,14</sup> and recent evidence suggests that necroptosis contributes to  
55 neurodegenerative conditions<sup>15</sup>. Small molecules that block necroptosis are under  
56 investigation as potential modulators of ischaemia-reperfusion injury associated with

57 myocardial infarction, stroke and solid organ transplantation<sup>16</sup>. Solid cancers present with  
58 necrosis but additionally necroptosis can promote tumour cell growth through suppression of  
59 the immune response against cancer<sup>17</sup>. A number of herpesviruses express RHIM-containing  
60 proteins that are primarily implicated in inhibiting host cell necroptosis to ensure virus  
61 survival<sup>18,19</sup>. Understanding the molecular basis for this viral RHIM-driven inhibition could  
62 identify therapeutic targets and provide guidance for the development of anti-necroptosis  
63 strategies with many clinical applications.

64

65 We have characterised the properties of the RHIM within the protein M45 (Fig. 1a), also  
66 known as the viral Inhibitor of RIP Activation (vIRA)<sup>20</sup> from murine cytomegalovirus  
67 (MCMV) and the structural basis for its interactions with RIPK1 and RIPK3. MCMV inhibits  
68 host apoptosis and necroptosis pathways to sustain infection<sup>21,22</sup> and specifically thwarts  
69 necroptosis in infected endothelial cells and macrophages<sup>23,24</sup> through the actions of M45.  
70 Thus far, M45 is the only viral RHIM-containing protein known to suppress necroptosis in  
71 mouse and human cells<sup>23,25,26</sup>. The M45 protein is delivered within the infecting virions<sup>27</sup>.  
72 Inhibition of necroptosis by M45 has been shown to involve interactions between M45 and  
73 RIPK1<sup>23</sup>, RIPK3<sup>26</sup> and ZBP-1/DAI<sup>7,28</sup> and to require an intact RHIM; substitution of the  
74 IQIG tetrad within the RHIM of M45 with AAAA abolishes the ability of the virus to confer  
75 protection against necroptosis<sup>7,28</sup>.

76

77 In 2012 Wu and colleagues demonstrated that the RHIM-driven association between RIPK1  
78 and RIPK3 results in the formation of an amyloid, fibrillar structure where the RHIM  
79 residues form the  $\beta$ -sheet core of RIPK1:RIPK3 fibrils<sup>29</sup>. This necrosome complex was the  
80 first functional amyloid structure described with an active signalling role<sup>30</sup>. The report of a  
81 similar amyloid-based signalling platform in *Drosophila*, where the PGRP-LC and PGRP-LE

82 receptors and downstream Imd adapter protein interact through motifs bearing some  
83 similarity to RHIMs<sup>31</sup>, suggests that RHIM-based signalling may be widespread. The  
84 structure of the necrosome RIPK1:RIPK3 core has been determined by solid-state nuclear  
85 magnetic resonance spectroscopy (ssNMR), confirming the hetero-amyloid nature of the  
86 complex and revealing a compact hydrophobic interface involving the RHIM tetrad<sup>32</sup>.

87

88 Given the demonstrated ability of the M45 protein to protect against TNFR-initiated  
89 necroptosis in human cells<sup>25</sup> and the interest in inhibition of necroptosis in clinical settings,  
90 we have investigated the interactions between the RHIMs of M45 and human RIPK1 and  
91 RIPK3, which are highly homologous to the murine RIPKs (Fig. 1a). We find that the RHIM  
92 of M45 is itself amyloidogenic and the ability of M45 to self-assemble into fibrils depends on  
93 the presence of the core tetrad sequence IQIG. A 90-residue fragment of M45 encompassing  
94 the RHIM is sufficient to confer protection against TNF-driven necroptosis in human cells.  
95 The RHIM of M45 is readily incorporated into heteromeric amyloid fibrils with the minimum  
96 portions of human RIPK1 or RIPK3 that are necessary for necrosome formation<sup>29</sup>. The viral  
97 M45 RHIM preferentially assembles with RIPK3 over RIPK1 and based on this, we have  
98 generated a model of a hetero-amyloid structure with RIPK3. We observe that incorporation  
99 of the viral M45 RHIM alters the properties of the human RHIM-based complexes. These  
100 results suggest that the M45 protein sequesters host RHIM-containing proteins in alternative  
101 amyloid structures that are incompatible with auto-phosphorylation of RIPK3 and/or  
102 activation of the downstream necroptosis mediator MLKL. The ability of the M45 protein  
103 revealed here, to form amyloid-based structures with host RHIM-containing proteins,  
104 suggests a general mechanism for modulation of host RHIM-based signalling by other viral  
105 RHIM-containing proteins.

107 **Results**

108

109 **The M45 RHIM drives spontaneous amyloid fibril formation**

110 Given the discovery of the amyloid nature of the RIPK1;RIPK3 necrosome<sup>29</sup>, we initially sought to  
111 determine whether the RHIM in M45 is amyloidogenic. A peptide of 19 residues, corresponding to  
112 the M45 RHIM sequence, readily assembled into long fibrils with amyloid morphology (Fig. 1a, b).  
113 In order to investigate the nature of the interactions between M45 and host proteins, we prepared  
114 fusion proteins containing portions of M45, RIPK1 and RIPK3 (Fig. 1a) with different partner  
115 proteins (His<sub>6</sub>-ubiquitin (His-Ub), His<sub>6</sub>-YPet, -ECFP or -mCHERRY fluorescent proteins (FPs) or  
116 maltose binding protein (MBP)). The human RIPK protein constructs contained the minimum  
117 RHIM-containing regions shown to support interactions between these two proteins, i.e.  
118 RIPK1-(497-583) and RIPK3-(387-518)<sup>29</sup>. Two RHIM-encompassing fragments of M45 were  
119 tested, comprising 90 or 277 N-terminal residues of the viral protein. The 1–90 region of M45 was  
120 identified as the likely smallest autonomous fragment of M45 because analysis of the predicted  
121 secondary structure of the protein highlighted a clear boundary between the  $\beta$ -sheet elements  
122 flanking the RHIM and the strongly predicted helical elements from residue 93 onwards. The 277  
123 N-terminal residues of M45 have previously been shown to provide protection against some forms  
124 of cell death<sup>23</sup> and to inhibit ZBP-1 signalling<sup>7</sup>.

125

126 **RHIM-encompassing fragments of M45 inhibit necroptosis in human HT-29 cells**

127 We compared the ability of the 1–90 and 1–277 regions of M45 to protect against TNFR-induced  
128 necroptosis in human HT-29 cells. The 90-residue and 277-residue portions were expressed as  
129 fusion proteins with the B subunit of *E. coli* DNA gyrase<sup>9</sup>. Addition of the antibiotic coumermycin  
130 A1 can induce dimerization of the gyrase subunit, to substitute for the potential dimerisation of the  
131 inactive ribonucleotide reductase-like (RNR1-like) domain of M45, which may form a heterodimer  
132 with RNR large and small subunits in the intact viral protein. Fig. 1c shows that the 90-residue and

133 277-residue portions of M45 protect human cells from TNFR-induced necroptosis through the  
134 RIPK1:RIPK3 pathway to a similar extent, in the range 70–90%. This is independent of the addition  
135 of coumermycin A1, indicating that dimerisation of M45 is not necessary for its role in inhibition of  
136 host cell death. For the majority of biophysical experiments we have therefore probed RHIM-driven  
137 interactions using the minimum necrosome-forming regions of RIPK1 and RIPK3<sup>29</sup> and the 90-  
138 residue N-terminal portion of M45, which displays the ability to inhibit TNFR-induced necroptosis.

139

### 140 **The wild type core tetrad is critical for amyloid formation by M45 and RIPK1 but not RIPK3**

141 The RHIM-containing regions were expressed as fusion proteins with His-Ub or His-FP to provide  
142 a means to study the kinetics of hetero-amyloid assembly. These fusion proteins were purified and  
143 maintained in the presence of denaturant, to prevent unwanted self-assembly. Prior experience with  
144 other functional amyloid-forming proteins has demonstrated that upon dilution of the denaturant,  
145 the ubiquitin domain refolds independently to its native state, allowing the characteristics of the  
146 partner sequence to be studied<sup>33</sup>. In YPet, ECFP and mCHERRY-containing RHIM fusion proteins,  
147 the fluorescent partner refolded correctly, as judged by recovery of characteristic fluorescence  
148 profiles. Purified His-Ub-M45<sub>1-90</sub> spontaneously formed fibrils upon removal of denaturant by  
149 dialysis and the fibrils displayed a cross- $\beta$  X-ray fibre diffraction pattern, with a strong, sharp  
150 meridional reflection at 4.7 Å and a weaker, diffuse equatorial reflection at ~10.5 Å, which are  
151 characteristic of amyloid fibrils<sup>34</sup> (Fig. 2a).

152

153 The kinetics of self-assembly, or homo-oligomeric fibril formation, of the RHIM-containing fusion  
154 proteins were studied by monitoring the fluorescence of thioflavin T (ThT), a dye commonly used  
155 as an indicator of amyloid structure. ThT exhibits increased fluorescence emission at 485 nm when  
156 it binds to amyloid fibrils<sup>35</sup>. All wild type RHIM-containing His-Ub and fluorescent fusion proteins  
157 formed homomeric amyloid (Fig. 2b). Tetra-alanine substitution of the RHIM core is a commonly  
158 used strategy to probe the role of RHIM:RHIM interactions in cells and during viral infection<sup>1,23</sup>

159 (Fig. 1a). RHIM mutant constructs in which the four core amino acids were substituted by AAAA  
160 were produced and the ability of these proteins to assemble into homomeric amyloid fibrils was  
161 tested. The substitution of the IQIG tetrad by AAAA within the M45<sub>1-90</sub> and RIPK1<sub>497-583</sub> RHIM  
162 constructs abolishes amyloid assembly (Fig. 2b). In contrast, the AAAA version of RIPK3<sub>387-518</sub>  
163 retains the ability to assemble into amyloid fibrils and has a similar kinetic profile to the WT form  
164 but exhibits a reduced ThT fluorescence intensity, reflecting an alteration in the fibril structure. This  
165 result indicates that in RIPK3, interactions outside of the core four residues are also important in the  
166 formation of homomeric amyloid structures.

167

### 168 **The M45 RHIM forms the core structure within the amyloid fibrils**

169 Amyloid assembly by the RHIM-containing fragments was also characterised under native  
170 conditions, when amyloid assembly occurred spontaneously during recombinant expression. When  
171 M45<sub>1-90</sub>, RIPK1<sub>497-583</sub> and RIPK3<sub>387-518</sub> were expressed as fusions with MBP, the fusion proteins  
172 remained soluble and could be purified under native conditions. Size exclusion chromatography  
173 after affinity purification separated the material into two main fractions (Fig. 2c). The first of these  
174 eluted in the void volume, indicating the formation of structures with MW >100 000 Da. SDS  
175 PAGE showed that the peak eluting in the void volume contained MBP-RHIM fusion protein while  
176 the second peak contained MBP only. The high molecular weight material was examined by  
177 negative stain transmission electron microscopy (TEM) and for MBP-M45<sub>1-90</sub>, MBP-RIPK1<sub>497-583</sub>  
178 and MBP-RIPK3<sub>387-518</sub>, fibrillar structures of up to ~200 nm in length were observed (Fig. 2d).  
179 Congo red solution binding assays performed with this material showed increased absorbance at  
180 540 nm for all three samples and a shift in the absorbance maxima, relative to a non-amyloid  
181 control (soluble insulin), indicating that the fibrils had an amyloidogenic nature (Fig. 2e).

182

183 To probe the arrangement of the RHIM within the fibrils, we treated the fibrillar material with  
184 thrombin to cleave specifically between MBP and the RHIM-containing regions. During incubation



185 with the protease, insoluble material became visible in all three samples. Separation of this material  
186 from the soluble fraction by centrifugation, followed by SDS PAGE and SEC analysis of the  
187 soluble material, revealed that M45<sub>1-90</sub>, RIPK1<sub>497-583</sub> and RIPK3<sub>387-518</sub> had formed insoluble  
188 precipitates while the MBP remained soluble. The insoluble material was resuspended and  
189 examined by TEM and fibrils were observed that were longer and thinner than those formed by the  
190 MBP fusion proteins (Fig. 2f). These results indicate that the RHIM-containing region of M45 is  
191 amyloidogenic and forms the  $\beta$ -sheet core structure of the fibrils, in a similar fashion to that  
192 demonstrated for RIPK1 and RIPK3 by Wu and coworkers<sup>29</sup>. The MBP fusion partner is arrayed on  
193 the outside of the  $\beta$ -sheet core and limits the growth of the fibrils to ~200 nm; in its absence, the  
194 RHIM-containing segments assemble into longer fibrils.

195

#### 196 **M45 interacts with RIPK1 and RIPK3 to form heteromeric assemblies**

197 To address the possibility of hetero-amyloid formation involving the RHIM-containing portions of  
198 M45, RIPK1 and RIPK3, we applied fluorescence polarisation and co-localisation experiments.  
199 When His-Ub-RIPK1<sub>497-583</sub> was covalently labelled with Alexa Fluor488 and mixed with unlabelled  
200 His-Ub-RIPK1<sub>497-583</sub>, His-Ub-RIPK3<sub>387-518</sub> or His-Ub-M45<sub>1-90</sub> in a ratio of 1 labeled:1999  
201 unlabelled, the Alexa488-labelled RIPK1<sub>497-583</sub> was incorporated into growing His-Ub-RIPK1<sub>497-583</sub>,  
202 His-Ub-RIPK3<sub>387-518</sub> or His-Ub-M45<sub>1-90</sub> fibrils (Fig. 3a). The rates of incorporation of the  
203 RIPK1-RHIM construct into His-Ub-RIPK1<sub>497-583</sub> and His-Ub-RIPK3<sub>387-518</sub> fibrils were similar and  
204 RIPK1-RHIM was incorporated into His-Ub-M45<sub>1-90</sub> fibrils more slowly. The tetrad sequence in the  
205 RHIM of M45 is IQIG and identical to that in RIPK1, whereas the RIPK3 tetrad sequence is  
206 VQVG. The observed difference in incorporation rates indicates that residues outside the tetrad also  
207 influence the interactions between RHIM-containing proteins (Fig. 1a). Taken together with the  
208 observed different effects of the AAAA mutations on M45, RIPK1 and RIPK3 homomeric amyloid  
209 formation, we conclude that the contributions of the tetrad sequences to homomeric and heteromeric  
210 structures are distinct and differ between RHIM-containing proteins.

211

212 Fluorescence co-localisation experiments were applied to detect RHIM-based hetero-oligomers  
213 against a background of monomeric proteins and the potential competing formation of homo-  
214 oligomers<sup>36,37</sup>. In these experiments, multiple different RHIM-containing fusion proteins were  
215 mixed together and maintained in monomeric form in 8 M urea-containing buffer. Assembly or co-  
216 assembly were initiated by reduction of the urea concentration through dilution. Mixtures of YPet-  
217 and mCHERRY-tagged proteins were examined in solution, using a confocal microscope where the  
218 lasers create a very small confocal volume (~250x250x800 nm). Freely diffusing fluorophore-  
219 tagged proteins are only excited and detected when they are within the confocal volume (Fig. 3b).  
220 The number of molecules in the focal volume/millisecond over the range of concentrations used  
221 was ~10-500.

222

223 In initial fluorescence spectroscopy experiments, we analysed mixtures of YPet- and mCHERRY-  
224 tagged RIPK1<sub>497-583</sub> or YPET- and mCHERRY-tagged RIPK3<sub>387-518</sub>. We observed coincidence of  
225 the signals from both fluorophores when oligomers formed, demonstrating that homomeric fibril  
226 assembly can be detected in this way (Fig. 3c,d). As expected, mixtures of mCHERRY-RIPK1<sub>497-583</sub>  
227 and YPet-RIPK3<sub>387-518</sub> exhibited formation of heteromeric fibrils, indicated by coincidence of the  
228 signals from the two fluorophores attached to different RHIM-containing constructs (Fig. 3e). In  
229 addition, FRET could be observed between YPet and mCHERRY within the fibrils (Fig. 3f). These  
230 results provide the first direct single-molecule-level evidence for the formation of heteromeric  
231 fibrils containing RIPK1 and RIPK3 RHIM sequences, supporting the results from bulk solution  
232 previously reported<sup>29</sup>. The RIPK3<sub>387-518</sub> construct demonstrated a greater propensity to self-assemble  
233 than the RIPK1<sub>497-583</sub> construct, as evidenced by the formation of larger assemblies (Fig. 3c,d). In  
234 addition, all oligomeric RIPK1<sub>497-583</sub> appeared coincident with RIPK3<sub>387-518</sub> material, while some  
235 RIPK3<sub>387-518</sub>-only fibrils were observed (Fig. 3e).

236

237 The self-assembly of M45<sub>1-90WT</sub> into fibrils could be detected by fluorescence co-localisation and,  
238 as expected given the ThT results, substitution of the IQIG tetrad with AAAA abolished the ability  
239 of this M45 fragment to self-assemble (Fig. 4a,b). This was confirmed by electron microscopy,  
240 which detected the presence of long fibrils formed by M45<sub>1-90WT</sub>-mCHERRY and only amorphous  
241 clusters in the M45<sub>1-90AAAA</sub>-mCHERRY sample (Supplementary Fig. 1).

242

243 When M45<sub>1-90WT</sub>-mCHERRY and YPet-RIPK1<sub>497-583</sub> or YPet-RIPK3<sub>387-518</sub> were mixed under fibril  
244 assembly conditions, we observed coincidence of M45<sub>1-90WT</sub> with both RIPK fragments,  
245 demonstrating heteromeric complex formation between viral and host RHIM-containing fragments  
246 (Fig. 4c,d). The complexes containing M45<sub>1-90</sub> and RIPK3<sub>387-518</sub> appeared larger than those  
247 containing M45<sub>1-90</sub> and RIPK1<sub>387-518</sub>, as judged by the length of time taken by the heteromeric  
248 complexes to diffuse through the focal volume. The AAAA mutation of the core tetrad of M45  
249 abolished co-assembly with RIPK1 and RIPK3 (Fig. 4e,f). This result suggests that the ability of the  
250 M45 RHIM to inhibit host necroptosis is associated with its ability to form heteromeric amyloid  
251 structures with RIPK1 and RIPK3.

252

253 We added non-fluorescent His-Ub-M45<sub>1-90</sub> into mixtures of YPet- and mCHERRY-tagged RIPK1  
254 and RIPK3 fragments, to probe the effect of the amyloidogenic viral RHIM on heteromeric  
255 complex formation by the two human RHIMs. The number of times a large complex generating  
256 >5000 or >10000 photons above baseline intensity was detected in the focal volume for 1  
257 millisecond during the last 200 seconds of a 10-minute incubation was quantified for a  
258 RIPK1:RIPK3 mixture and a series of RIPK1:RIPK3:M45 mixtures. The addition of His-Ub-  
259 M45<sub>1-90</sub> resulted in a decrease in the number of the large complexes detected, correlated with the  
260 relative concentration of His-Ub-M45<sub>1-90</sub> (Fig. 4g). EM of samples formed with increasing amounts  
261 of M45<sub>1-90</sub> relative to RIPK3<sub>387-518</sub> (1:1; 2:1 and 4:1) confirmed an increase in the size of the  
262 fibrillar assemblies (Supplementary Fig. 2). The reduction in the number of large complexes

263 detected is therefore likely due to a reduction in the rate at which larger complexes diffuse in the  
264 well, which results in fewer passes through the focal volume and hence detection. Collectively,  
265 these results show that M45<sub>1-90</sub> is able to interact with RIPK1<sub>497-583</sub> and RIPK3<sub>387-518</sub> in a RHIM-  
266 dependent manner but displays a preference for co-assembly with the RHIM of RIPK3.

267

### 268 **The M45-RHIM alters the formation of fibrils by the RHIMs of RIPK1 and RIPK3**

269 Self-assembly of single types of RHIM-containing proteins resulted in predominantly unbranched  
270 single fibrils, as visualised by TEM (Fig. 5a). In contrast, when the M45<sub>1-90</sub> fragment was mixed  
271 with either of the RIPK1<sub>497-583</sub> or RIPK3<sub>387-518</sub> fragments in a 1:1 molar ratio under conditions  
272 allowing fibril assembly, extensive fibrillar networks were observed (Fig. 5a). These consisted of  
273 large bundles of multiple fibrils that branched and reformed in different combinations, resulting in  
274 an interweaved and dense assembly covering large areas of the TEM grids. By contrast, when  
275 RIPK1<sub>497-583</sub> and His-Ub-RIPK3<sub>387-518</sub> were mixed together under conditions allowing fibril  
276 assembly, isolated mostly single fibrils of <200 nm length were observed, similar to those reported  
277 by Li et al<sup>29</sup>. When M45<sub>1-90</sub> or RIPK1<sub>497-583</sub> were allowed to assemble in the presence of an  
278 unrelated protein with the same fusion partner, His<sub>6</sub>-Ub-RodA, no thick multi-fibril bundles were  
279 observed, suggesting that this is a feature associated with hetero-fibrils formed by two RHIM-  
280 containing proteins (Supplementary Fig. 3).

281

282 The different RHIM-containing components cannot be distinguished in these TEM images so we  
283 used confocal microscopy to visualise fibrils formed from mixtures of RHIM-containing fluorescent  
284 fusion proteins (Fig. 5b–e). We observed the formation of long fibrils that contained M45<sub>1-90</sub> and  
285 either one or both of human RIPK1<sub>497-583</sub> or RIPK3<sub>387-518</sub>. These assemblies are thioflavin T  
286 positive, confirming that these large heteromeric assemblies do have an amyloid cross-β  
287 substructure (Supplementary Fig. 4). Fibrils prepared from a mixture containing mCHERRY-  
288 M45<sub>1-90</sub> and YPet-RIPK3<sub>387-518</sub> were additionally imaged by super-resolution microscopy and

289 transmission electron microscopy to provide higher resolution images. We found that the viral and  
290 host RHIMs were distributed throughout the fibrillar protein network formed by these proteins (Fig.  
291 5f,g).

292

### 293 **The incorporation of M45 RHIM alters fibril stability**

294 We reasoned that the incorporation of the viral M45 RHIM into host heteromeric fibrils might alter  
295 the properties of the fibrils, in addition to changing the morphology. Other studies of functional  
296 amyloid fibrils have noted that they are less resistant to treatment with the detergent sodium  
297 dodecyl sulphate (SDS) than some disease-associated amyloid fibrils<sup>38,39</sup>. We prepared homomeric  
298 and heteromeric assemblies from M45<sub>1-90WT</sub> and M45<sub>1-90AAAA</sub> with RIPK3<sub>387-518</sub>. These assemblies  
299 were incubated with and without SDS, and then the preparations were analysed by agarose gel  
300 electrophoresis (AGE) (Fig. 6). This separated the material into (1) very large SDS-resistant fibrillar  
301 material that remained in the wells, (2) ThT-positive soluble amyloid oligomers and (3) monomeric  
302 protein. The M45<sub>1-90WT</sub> and RIPK3<sub>387-518</sub> mixtures contained soluble amyloid oligomers. The  
303 incorporation of the viral RHIM destabilised the complexes such that treatment with 2% SDS  
304 resulted in release of both constructs in monomeric form and, in the 1:1 mixtures, the generation of  
305 RIPK3-only fibrils. RIPK3-only fibrils were predominantly SDS-resistant and remained as large  
306 fibrils trapped in the wells (Fig. 6a). When the viral M45 RHIM was present in four-fold molar  
307 excess, little RIPK3-only fibrillar material remained in the wells. These results were supported by  
308 analysis of the soluble and insoluble material generated by dialysing mixtures of RHIM-containing  
309 constructs together, which showed that the presence of the M45<sub>1-90WT</sub> fragment reduced the amount  
310 of RIPK3<sub>387-518</sub> that was detected in the insoluble fraction (Supplementary Fig. 5). This activity of  
311 the M45 RHIM was dependent on the presence of the WT tetrad core sequence, as in the presence  
312 of M45<sub>1-90AAAA</sub>, a large amount of SDS-resistant RIPK3-only material remained in the wells and  
313 very low levels of monomeric RIPK3<sub>387-518</sub> were detected, even with four times as much M45<sub>1-</sub>  
314 <sub>90AAAA</sub> (Fig. 6a). The M45<sub>1-277</sub> construct displayed a similar activity (Fig. 6b). It self-assembled into

315 soluble homo-oligomers. It co-migrated with RIPK1<sub>497-583</sub> but interacted strongly with RIPK3<sub>387-518</sub>  
316 as judged by the narrow, intense orange-coloured co-localised band corresponding to soluble  
317 oligomers. In the absence of the intact RHIM core, M45<sub>1-277AAAA</sub> formed large soluble oligomers  
318 with a wide mobility range but was unable to interact with RIPK1<sub>497-583</sub> or RIPK3<sub>387-518</sub>, as judged  
319 by the presence of independently migrating oligomers of RIPK1<sub>497-583</sub> and large RIPK3-only homo-  
320 oligomeric fibrils (Fig. 6b). The M45 RHIM is therefore able to compete with, and prevent,  
321 heteromeric RIPK1:RIPK3 RHIM-based amyloid formation and homo-oligomerisation of RIPK3  
322 such as occurs after initial formation of the necrosome<sup>40</sup>.

323

324

325

326 **Discussion**

327

328 These results demonstrate that the N-terminal 90 residues of M45 are sufficient to support  
329 interactions with the RHIMs of human RIPK1 and RIPK3 and to protect against TNFR-mediated  
330 necroptosis in human cells *in vitro*. The RHIM within the MCMV M45 protein is amyloidogenic.  
331 The ability of M45 to self-assemble into homomeric fibrils or to form heteromeric amyloid-  
332 structured complexes with RIPK1 and RIPK3 depends on the presence of the core tetrad sequence  
333 IQIG within the RHIM. This core sequence is also required for the ability of M45 to inhibit  
334 necroptosis<sup>28</sup>, strongly suggesting that the amyloid-based interactions made by M45 with RIPK1  
335 and RIPK3 are associated with its anti-necroptosis activity. When probed with necroptosis-relevant  
336 RHIM-containing protein fragments, the viral RHIM preferentially interacts with the RIPK3 RHIM  
337 over the RIPK1 RHIM. In the presence of the M45 RHIM, a RHIM-encompassing region of RIPK3  
338 is incorporated into metastable heteromeric fibrils rather than into SDS-resistant homo-oligomers.

339

340 The RIPK1:RIPK3 interaction and formation of the necrosome is central to TNFR-mediated  
341 responses if caspase-8 is inhibited by viral or chemical inhibitors<sup>3</sup>. We propose that the presence of  
342 M45 in cells results in the trapping of RIPK1 and RIPK3 within alternative heteromeric “decoy”  
343 amyloid structures and prevents the native RIPK1:RIPK3 RHIM-based amyloid interactions that are  
344 required to trigger cell death.

345

346 An important difference between RHIM-based amyloids and other disease-associated or most  
347 functional amyloids is the formation of heteromeric assemblies<sup>30</sup>. Heteromeric RHIM-based  
348 amyloid is central to necroptosis induction<sup>11</sup>. RNA-binding proteins containing low complexity  
349 sequences have the capacity to co-assemble into phase-separated droplets and hetero-fibrils, driven  
350 by intermolecular interactions between the prion-like domains, however the *in vivo* significance of  
351 heteromeric fibril assembly by these proteins is currently less clear than is the case for RHIM-based

352 amyloid<sup>38,39</sup>. The multi-protein RHIM amyloid structures are similar to other higher-order  
353 assemblies such as signalosomes that form by polymerization and recruit multiple proteins with  
354 different activities<sup>41</sup>.

355

356 The key features of the heteromeric RIPK1:RIPK3 necrosome core have been revealed by  
357 ssNMR<sup>32</sup>. The RHIMs of RIPK1 and RIPK3 adopt a serpentine fold and form parallel  $\beta$ -sheets  
358 through intermolecular hydrogen binding. Two such sheets assemble to form a fibril through inter-  
359 tetrad and other interactions, which generate an oblong-shaped hydrophobic core (Fig. 6c). The  
360 structure is also stabilised by solvent-exposed side-chain-side-chain hydrogen bonds between  
361 stacked Asn and Gln residues, and Tyr and Ser/Thr side chains that form stacked ladder-like  
362 interactions on the periphery of the core.

363

364 We have used this structure to model the incorporation of the RHIM of M45. Since M45 and  
365 RIPK1 have the same core IQIG tetrad sequence, and we have determined that M45 preferentially  
366 forms heteromeric fibrils with RIPK3 over RIPK1, we have mutated the RIPK1 sequence to that of  
367 M45. These substitutions could be introduced into the complex structure without significant steric  
368 clashes (Fig. 6d). In the M45:RIPK3 RHIM model, the core tetrad interactions are unchanged from  
369 those in the RIPK1:RIPK3 structure but with the substitution with the viral protein, two Tyr and one  
370 Asn ladder are not possible. Instead, a new Asp-Asn side-chain interaction is likely and in the  
371 M45:RIPK3 structure, a Val-Cys ladder takes the place of the Ser-Cys ladder observed with  
372 RIPK1:RIPK3. Our model suggests that the M45:RIPK3 RHIM core will be similar to that formed  
373 by RIPK1:RIPK3 and hence it is likely that M45 mimics the host inter-RHIM interactions. The key  
374 role played by the tetrad residues in the hetero-fibrils is in line with our experimental observation  
375 that the AAAA mutation to M45 abolishes the ability of this protein to form a hetero-amyloid with  
376 the RHIM of RIPK3. Our experimental data demonstrate that the interactions driving M45:RIPK3  
377 heteromeric assembly *in vitro* are stronger than those leading to M45:RIPK1 or RIPK1:RIPK3



378 assembly. Further mutagenesis studies could identify the M45-derived interactions responsible. The  
379 ssNMR-derived structure does not reveal heteromeric interactions between polypeptides that  
380 occupy the corresponding “rung” of opposing sheets. It is possible that the viral protein could be  
381 involved in such interactions with host partners. Interference by the viral protein with inter-sheet  
382 interactions would explain the branching and network morphology we observe in our heteromeric  
383 assemblies (Fig. 5a,f; Supplementary Fig. 2). The concentration of viral protein in the cell may also  
384 influence the make-up of the RHIM-based structures within cells, and hence control the final  
385 cellular outcome. The structure determined by Mompean *et al.* is consistent with confocal images  
386 we have collected of isolated M45<sub>1-90WT</sub> homo-oligomeric fibrils which show fluorescent partner  
387 proteins along the two flanks of the fibril and a dark central core; an arrangement consistent with  
388 two opposed parallel  $\beta$ -sheets (Fig. 6e,f).

389

390 It is noteworthy that in our experiments the AAAA mutation of the core tetrad in RIPK3<sub>387-518</sub> does  
391 not abolish self-assembly into an amyloid structure while this mutation in RIPK1<sub>497-583</sub> and M45<sub>1-90</sub>  
392 prevents self-assembly. This indicates that residues outside the tetrad of RIPK3 can support  
393 oligomerisation. These remain to be identified by further mutagenesis and structural studies of the  
394 entire RIPK3 RHIM. Although calculations based on the RIPK1:RIPK3 core structure suggest that  
395 RIPK3:RIPK3 homo-amyloid formation is less favourable than RIPK1:RIPK3 or RIPK1:RIPK1<sup>32</sup>,  
396 the formation of the RIPK1:RIPK3 necrosome is known to nucleate further polymerisation of  
397 RIPK3 that leads to activation of RIPK3 and interaction with MLKL<sup>40</sup>. The results of other studies  
398 show that the RHIM-based interactions of M45 with cellular partners prevent ZBP1-induced RIPK3  
399 phosphorylation<sup>7</sup> and autophosphorylation of RIPK3 when co-expressed in 293T cells<sup>42</sup>. HSV-1  
400 and -2 encode RHIM-containing proteins, ICP-6 and ICP10, which have been shown to protect  
401 against necroptosis in human cells<sup>25,43</sup> and ICP10 expression is known to prevent phosphorylation  
402 of RIPK1 and RIPK3 compared to control<sup>25</sup>. The sequestration of host RHIM-containing proteins in

403 heteromeric amyloids with viral proteins may therefore be general mechanism for modulation of  
404 host RHIM-based signalling by other RHIM-expressing viruses.

405

406 A different RHIM-based anti-necroptosis strategy is operated by some bacterial pathogens.

407 Enteropathogenic *E. coli* (EPEC) is a gut pathogen that has been shown to encode a protease, EspL,  
408 that is delivered through the type III secretion effector system and which rapidly cleaves within the  
409 RHIM of RIPK1, RIPK3, ZBP-1/DAI and TRIF when these proteins are in a non -fibrillar form<sup>44</sup>.

410 Amyloid fibril formation by the endogenous RHIM-containing proteins protects them from  
411 cleavage by EspL, allowing them to signal for necroptosis. Thus EspL appears to act by disabling  
412 the amyloid-based RHIM host defense pathways that would otherwise curtail infection<sup>44</sup>.

413

414 Several host RHIM:RHIM interactions occur in the absence of infection. An intact RHIM in RIPK1  
415 suppresses undesirable ZBP-1:RIPK3 interactions during development that would lead to  
416 necroptosis<sup>45,46</sup>. The methods we have developed to probe and compare competing RHIM  
417 interactions can be applied to test the relative strengths of different RHIM:RHIM interactions and  
418 could shed light on the basis for cellular control of these potentially competing RHIM:RHIM  
419 interactions. Additionally, necroptosis is increasingly recognized in pathophysiological processes  
420 including ischaemia-reperfusion injury, and inflammation of the skin and intestinal epithelium<sup>13,47</sup>.

421 Any development of therapies that target the necroptosis pathway will benefit from an  
422 understanding of the structural basis for viral inhibition of RHIM-based signaling.

423

424

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426

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438 fluorescent proteins. We thank Pascal Krotee and David Eisenberg for helpful discussions about the  
439 use of fluorescent proteins for studying RHIM interactions and models of heteromeric and  
440 homomeric amyloids, respectively.

441

442

## 443 **Figure Legends**

### 444 **Figure 1. The M45 RHIM is amyloidogenic and 1-90 and 1-277 regions of M45 confer**

445 **protection against TNF-induced necroptosis in human cells.** (a) Schematic representation of  
446 M45, RIPK1 and RIPK3 proteins. Grey regions indicate RHIM-encompassing fragments used in  
447 this study. Black bar represents 19-residue RHIM. RNR-like represents inactive ribonucleotide  
448 reductase domain, DD represents the RIPK1 death domain. Amino acid sequences of the RHIMs of  
449 M45 and human and mouse RIPK1 and RIPK3, boxed region indicates the core tetrad that is  
450 substituted with AAAA in mutant protein constructs. (b) Negatively-stained transmission electron  
451 micrograph of fibrils formed by a synthetic peptide encompassing the M45 RHIM. Three samples  
452 were examined by microscopy. (c) HT29 cell survival 18h after treatment with TNF+BV-6+z-  
453 VAD-fmk to induce necroptosis. Cells expressed lentivirus constructs encoding 1-90 or 1-277  
454 fragments of M45 and were cultured +/- the addition of coumermycin A1 to induce dimerisation.  
455 N=2 for M45-90 gyrase and 3 for M45-277 gyrase. Error bars represent SEM, \*\*\*\* =  $p < 0.0001$ .

### 456 **Figure 2. The RHIM-containing fragment forms the amyloid fibril core.** (a) X-ray fibre

457 diffraction pattern collected from fibrils formed by His-Ub-M45<sub>1-90</sub>. Two different samples were  
458 examined by XRFD. (b) Thioflavin T fluorescence as a function of time, following dilution of WT  
459 or AAAA His-Ub-M45<sub>1-90</sub>, mCHERRY-RIPK1<sub>497-583</sub>, and YPet-RIPK3<sub>387-518</sub> from 8 M-containing  
460 buffer to 100–300 mM urea. Buffer sample contains ThT but no protein. Samples were tested in  
461 triplicate and solid line is the average of the triplicates and the error bars (in grey) represent +/-  
462 SEM. (c) Size exclusion chromatography (SEC) of MBP-M45<sub>1-90</sub>, MBP-RIPK1<sub>497-583</sub>, and MBP-  
463 RIPK3<sub>387-518</sub> following amylose affinity purification. Grey oval indicates high MW material eluting  
464 in void volume. (d) Negatively-stained transmission electron micrograph of material eluted in the  
465 void volume. These are representative micrographs of the material observed. All fusion proteins  
466 expressed and purified at least twice. (e) Absorbance spectrum from solutions containing Congo red  
467 with soluble monomeric insulin, insulin amyloid fibrils or material eluted in the void volume  
468 containing MBP-M45<sub>1-90</sub> (green), MBP-RIPK1<sub>497-583</sub> (cyan), or MBP-RIPK3<sub>387-518</sub> (blue).  
469 Experiment conducted once. (f) Representative images of insoluble material generated after  
470 treatment with thrombin removed the MBP. Experiment repeated three times.

### 471 **Figure 3. Assembly of RHIMs gives rise to homomeric and heteromeric structures.** (a)

472 Fluorescence polarization profiles indicating the rate of incorporation of Alexa488-tagged  
473 RIPK1<sub>497-583</sub> into unlabeled M45<sub>1-90</sub> (green), RIPK1<sub>497-583</sub> (cyan), or RIPK3<sub>387-518</sub> (blue), with  
474 labeled:unlabeled protein at 1:1999. The average polarization profile from triplicate readings is  
475 shown, with s.d. indicated. (b) Schematic representation of fluorescence spectroscopy experiments  
476 used to detect the formation of homo- and heteromeric amyloid fibrils. Fusion proteins containing  
477 RHIMs and different fluorescent partners are mixed together under assembly-permissive conditions  
478 and examined in the confocal volume. Co-assembly of two different fluorophores within one  
479 complex results in detection of coincident signals in the two channels. (c) Representative  
480 fluorescence intensity trace showing homomeric assembly over 40 s in a mixture of mCHERRY-  
481 RIPK1<sub>497-583</sub> and YPet-RIPK1<sub>497-583</sub>. (d) Representative fluorescence intensity trace showing  
482 homomeric assembly over 40 s in a mixture of mCHERRY-RIPK3<sub>387-518</sub> and YPet-RIPK3<sub>387-518</sub>.  
483 (e) Representative fluorescence intensity trace showing heteromeric assembly over 40 s in a mixture  
484 of mCHERRY-RIPK1<sub>497-583</sub> and YPet-RIPK3<sub>387-518</sub>. Inset is expansion of region indicated by \* on  
485 main trace. (f) Förster resonance energy transfer (FRET) signal between YPet- and mCHERRY  
486 fluorophores, detected with excitation by 488 nm laser only. All mixtures examined in at least three  
487 different experiments on three separate occasions. In panels c–f the signal from the mCHERRY-  
488 tagged protein is coloured magenta and the signal from the YPet-tagged protein is coloured green.

489 **Figure 4. Heteromeric amyloid formation including M45 RHIM requires intact core tetrad.**  
490 Representative fluorescence intensity traces collected over 50 s for mixtures of (a) mCHERRY-  
491 M45<sub>1-90</sub>WT and YPet-only; (b) mCHERRY-M45<sub>1-90</sub>AAAA and YPet-only; (c) mCHERRY-M45<sub>1-90</sub>WT  
492 and YPet-RIPK1<sub>497-583</sub>; (d) mCHERRY-M45<sub>1-90</sub>WT and YPet-RIPK3<sub>387-518</sub>; (e) mCHERRY-M45<sub>1-</sub>  
493 90AAAA and YPet-RIPK1<sub>497-583</sub> and (f) mCHERRY-M45<sub>1-90</sub>AAAA and YPet-RIPK3<sub>387-518</sub>. (g) Number  
494 of bins where intensity (photons/ms) exceeded 5000 or 10000 in the last 200 s of a 10-min  
495 incubation, where samples contained no His-Ub-M45<sub>1-90</sub> or up to a 4-fold molar excess of His-Ub-  
496 M45<sub>1-90</sub>. Coincidence experiments conducted three times or more on different days. Quantification  
497 performed once.

498  
499 **Figure 5. Co-assembly of M45<sub>1-90</sub>WT with RIPK1<sub>497-583</sub> and His-Ub-RIPK3<sub>387-518</sub> results in the**  
500 **formation of heteromeric fibrils and dense fibrillar networks.** (a) Representative electron  
501 micrographs of fibrils formed by M45<sub>1-90</sub>, RIPK1<sub>497-583</sub> or His-Ub-RIPK3<sub>387-518</sub> when dialysed  
502 individually or in mixtures, into phosphate buffer to remove urea. Confocal fluorescence images of  
503 heteromeric fibrils formed by co-assembly of (b) mCHERRY-M45<sub>1-90</sub> and YPet-RIPK1<sub>497-583</sub>; (c)  
504 mCHERRY-M45<sub>1-90</sub> and YPet-RIPK3<sub>387-518</sub>; (d) mCHERRY-RIPK1<sub>497-583</sub> and YPet-RIPK3<sub>387-518</sub>;  
505 (e) ECFP-M45<sub>1-90</sub> with mCHERRY-RIPK1<sub>497-583</sub> and YPet-RIPK3<sub>387-518</sub>. (f) STED super-resolution  
506 and (g) electron microscope images of heteromeric fibrils formed by co-assembly of mCHERRY-  
507 M45<sub>1-90</sub> and YPet-RIPK3<sub>387-518</sub>.

508  
509 **Figure 6. SDS AGE analysis of RHIM-based assemblies and model of the heteromeric**  
510 **M45:RIPK3 core.** (a) SDS AGE analysis of YPet-RIPK3<sub>387-518</sub> and mCHERRY-M45<sub>1-90</sub>WT or  
511 mCHERRY-M45<sub>1-90</sub>AAAA homomeric and heteromeric assemblies, formed by initial mixing in 8 M  
512 urea, followed by removal of urea by overnight dialysis. Dialysed samples were incubated with 0 or  
513 2% SDS at RT for 10 min before electrophoresis. Monomeric forms of the protein constructs were  
514 maintained in 8 M urea prior to electrophoresis, as indicated. Protein components as indicated  
515 above each lane. (b) SDS AGE analysis of homomeric and heteromeric assemblies of YPet-  
516 RIPK1<sub>497-583</sub>, YPet-RIPK3<sub>387-518</sub> and mCHERRY-M45<sub>1-277</sub>WT or mCHERRY-M45<sub>1-277</sub>AAAA. BSA  
517 was included in M45-only samples to maintain the overall protein concentration constant. (c)  
518 Structure of the RIPK1:RIPK3 RHIM core. Figure prepared using PyMol Graphics System from  
519 PDB 5V7Z. The RIPK3 sequence is coloured in blue and RIPK1 is coloured in green. Tetrad  
520 residue side chains are shown as sticks; Tyr, Asn/Gln, Ser and Cys side chains are shown in wheat,  
521 red, grey and yellow, respectively. Core tetrad interactions across the opposing sheets are indicated  
522 by the dotted box. (d) Model of M45:RIPK3 RHIM core, prepared by appropriate mutation of  
523 RIPK1. The M45 sequence is coloured in cyan and RIPK3 in blue. Tetrad residue side chains are  
524 shown as sticks; Tyr, Asn/Gln, Val and Cys side chains are shown in wheat, red, grey and yellow,  
525 respectively. Core tetrad interactions across the opposing sheets are indicated by the dotted box. (e)  
526 Confocal microscopy image of a single fibril composed of YPet-M45. (f) Schematic representation  
527 showing associated globular domains (e.g. kinase or RNR, coloured green) flanking either side of  
528 the amyloid core formed by RHIMs (coloured grey).

529

## 530 **Supplementary Figures**

531 **Supplementary Fig 1. The AAAA substitution of the core tetrad within the RHIM of M45<sub>1-90</sub>**  
532 **abolishes its ability to self-assemble into fibrils.** Negatively stained EM of fibrils formed by (a)  
533 M45<sub>1-90</sub>WT-mCHERRY and (b) M45<sub>1-90</sub>AAAA-mCHERRY fibrils upon removal of 8 M urea by  
534 dialysis.

535 **Supplementary Fig 2. An increase in the amount of M45<sub>1-90</sub> relative to RIPK3<sub>387-518</sub> results in**  
536 **the formation of increasingly large fibrillar assemblies.** Negatively stained EMs of fibrillar  
537 assemblies formed from mixtures of M45<sub>1-90</sub>WT-mCHERRY and YPetRIPK3<sub>387-518</sub> at (a) 1:1, (b) 2:1  
538 and (c) 4:1 relative molar concentration. Monomeric forms of the proteins were mixed together and  
539 allowed to co-assemble upon removal of 8 M urea by dialysis.

540 **Supplementary Fig 3. The presence of an unrelated His<sub>6</sub>-ubiquitin fusion protein does not**  
541 **result in generation of fibrillar networks.** Samples of either (a) His-Ub-M45<sub>1-90</sub> or (b) His-Ub-  
542 RIPK1<sub>497-583</sub> RHIM fusion proteins were mixed with the non-RHIM-containing protein His-Ub-  
543 RodA in urea-containing buffer and allowed to assemble upon removal of urea by dialysis.

544  
545 **Supplementary Fig 4. Heteromeric assemblies formed by RHIM-containing fragments are**  
546 **thioflavin T-positive, indicative of an amyloid structure.** mCHERRY-M45<sub>1-90</sub> and YPet-  
547 RIPK3<sub>387-518</sub> were allowed to coassemble during dialysis, following which thioflavin T was added  
548 and the dialysate imaged using a Cytation 3 Imager (BioTek). CFP, YFP and Texas Red filter sets  
549 were used to detect Thioflavin T fluorescence, YPet and mCHERRY respectively.

550  
551 **Supplementary Fig 5. SDS-PAGE analysis of RHIM-containing assemblies resulting from**  
552 **overnight dialysis of individual constructs (a–c) or protein pairs (d–f).** RHIM-containing fusion  
553 proteins dialysed overnight to allow fibril assembly, either individually or in pairs after initial  
554 mixing in urea in the monomeric form. All RHIM constructs carried mCHERRY as fusion protein  
555 partner. RHIM-containing individual construct or pairs as indicated in each panel. The stability of  
556 the resulting material towards SDS treatment was tested. Samples are total dialysate (total),  
557 supernatant from centrifugation at 16000g for 10 min (soluble), pellet from centrifugation  
558 resuspended in 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, 0.5 mM DTT, pH 7.4 (insoluble), pellet from  
559 centrifugation treated with 2% SDS for 10 min at 37 °C and then separated again into supernatant  
560 (SDS-soluble) and pellet (SDS-insoluble). SDS-insoluble material was incubated in 8 M urea to  
561 solubilise material for detection by PAGE. This SDS analysis was performed once.

## 562 **Methods**

### 563 **Expression of RHIM fusion proteins**

564 RHIM-containing portions of mCMV M45 (Q06A28; residues 1-90), human RIPK1 (Q13546;  
565 residues 497-583), human RIPK3 (Q9Y572; residues 387-518), were produced as maltose binding  
566 protein (MBP) fusion proteins using the pMAL vector, as His<sub>6</sub>-Ubiquitin fusion proteins using the  
567 pHUE vector system (Catanzariti et al) or as His-tagged N- or C-terminal ECFP, YPet or  
568 mCHERRY fusion proteins using a vector prepared in-house. Successful cloning was confirmed by  
569 sequencing at the Australian Genome Research Facility at the Westmead Millennium Institute.  
570 Proteins were expressed in BL21(DE3) *E. coli* grown in LB media containing ampicillin at 37 °C  
571 for 2–3 hours, with induction by 0.5–1.0 mM IPTG when the OD<sub>600 nm</sub> of the culture reached  
572 0.6–0.8. All pMAL plasmids were grown in LB media containing 0.2% glucose to prevent amylase  
573 production. SDS PAGE analysis of cell lysates following induction of protein over-expression  
574 indicated that the MBP-RHIM fusion proteins were present in the soluble cell fraction while the  
575 His<sub>6</sub>-Ub-RHIM fusion proteins and His<sub>6</sub>-ECFP, His<sub>6</sub>-YPet or His<sub>6</sub>-mCHERRY fusion proteins were  
576 located in the insoluble fraction.

### 577 **Purification of MBP fusion proteins**

578 Cells were lysed by incubation in 20 mM Tris.HCl, 200 mM NaCl, 1 mM EDTA and 0.5 mM DTT,  
579 pH 8.0 with lysozyme at 100 µg/mL, followed by a freeze thaw cycle. DNaseI and MgSO<sub>4</sub> were  
580 added to final concentrations of 100 µg/mL and 100 mM respectively, and samples were incubated  
581 with stirring for 15 min at room temperature. Following the addition of AEBSF to 0.2 mM, samples  
582 were centrifuged at 4 °C at 10,000g for 40 min, to separate soluble material from insoluble cell  
583 debris. MBP fusion proteins were purified from the soluble fraction using amylose beads from New  
584 England Biolabs (MA, USA), as per the manufacturer's instructions.

### 585 **Purification of His<sub>6</sub>-Ubiquitin fusion proteins**

586 Cell pellets were lysed by suspension and incubation in 6 M GuHCl, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 20 mM  
587 Tris.HCl, 5 mM β-mercaptoethanol, pH 8.0, and soluble material further purified on Ni-NTA

588 agarose (Life Technologies) under denaturing conditions, with exchange into 8 M urea, 100 mM  
589  $\text{NaH}_2\text{PO}_4$ , 20 mM Tris.HCl, 5 mM  $\beta$ -mercaptoethanol at pH 6.0 for washing and pH 4.0 for elution  
590 from the Ni-NTA agarose.

### 591 **Purification of His<sub>6</sub>-ECFP, His<sub>6</sub>-YPet and His<sub>6</sub>-mCHERRY fusion proteins**

592 Two methods for purification of polyhistidine-tagged fluorescent protein-RHIM fragment fusions  
593 were used during the course of this study. The first method was batch purification where cell  
594 pellets were lysed by suspension and incubation in 8 M urea, 100 mM  $\text{NaH}_2\text{PO}_4$ , 20 mM Tris.HCl,  
595 5 mM  $\beta$ -mercaptoethanol, pH 8.0. Soluble material was purified using Ni-NTA agarose beads (Life  
596 Technologies) under denaturing conditions, with 8 M urea, 100 mM  $\text{NaH}_2\text{PO}_4$ , 20 mM Tris.HCl,  
597 5 mM  $\beta$ -mercaptoethanol at pH 8.0 and in the presence of 20mM imidazole for washing steps and  
598 300mM imidazole for elution from the Ni-NTA agarose. The second method involved using a His-  
599 trap (GE Healthcare) column. For this, cell pellets were lysed as described for the purification of  
600 MBP fusion proteins. The insoluble pellet was resuspended in 8 M urea, 100 mM  $\text{NaH}_2\text{PO}_4$ ,  
601 20 mM Tris, pH 8.0 and allow to dissolve by stirring for 2 h. The sample was centrifuged at 4 °C at  
602 31,000g for 45 min, filtered and then purified on a 5mL His-Trap column (GE Healthcare) under  
603 denaturing conditions (8 M urea, 100 mM  $\text{NaH}_2\text{PO}_4$ , 20 mM Tris, pH 8.0 + 0.5mM DTT) using  
604 AKTA FPLC. The elution of protein was achieved with a gradient of imidazole. Protein elution was  
605 monitored by absorbance at 280 nm.

606 For both purification procedures, the eluted fractions were analysed by SDS-PAGE. Fractions  
607 containing the fusion proteins were pooled and concentrated using Amicon Ultra-15 Centrifugal  
608 Filter Units with MWCO 30kDa (Millipore). The concentration of the final concentrated protein  
609 sample was determined by BCA assay (Pierce).

### 610 **Removal of fusion tag (MBP and His<sub>6</sub>-Ubiquitin)**

611 MBP-RHIM fusion proteins at concentrations of 1–4 mg/mL in 20 mM Tris.HCl, 200 mM NaCl,  
612 1 mM EDTA, 2.5 mM  $\text{CaCl}_2$ , 10 mM maltose pH 8.0 were incubated with 5 U Thrombin for 2  
613 hours at 37 °C. Samples were centrifuged at 16 000g for 10 min to separate insoluble and soluble



614 material and both fractions were analysed by SDS PAGE. His<sub>6</sub>-Ub-RHIM constructs in 8 M urea-  
615 containing buffer were dialysed overnight at room temperature, against 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 50 mM  
616 NaCl pH 7.4 and then incubated with deubiquitylating enzyme UBP41 (produced in-house  
617 according to Catanzariti et al) at 37°C for 3 hours. The RHIM-containing fragments self-assembled  
618 to form insoluble material during dialysis and incubation with UBP41. Samples were centrifuged to  
619 separate the supernatant, containing the cleaved His<sub>6</sub>-Ubiquitin, from the pellet. The pellet  
620 containing the RHIM fragment was washed twice with 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 50 mM NaCl pH 7.4,  
621 followed by addition of 6 M GuHCl, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 20 mM Tris.HCl pH 8.0 to solubilise the  
622 pellet and maintain the RHIM fragments in a monomeric form for storage.

### 623 **Size exclusion chromatography**

624 MBP-RHIM fusion proteins, before and after treatment with enzyme to remove the fusion partner,  
625 were analysed by size exclusion chromatography using a Superdex 75 10/30 GL column (GE  
626 Healthcare Life Sciences), running in 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl pH 7.4 with a flow rate of  
627 0.4 mL/min. Protein elution was monitored by absorbance at 280 nm and peaks were analysed by  
628 SDS-PAGE and negative stain transmission electron microscopy.

### 629 **Congo red assays**

630 Insulin (1 mg/mL) was incubated in 20 mM glycine pH 2.0, with shaking at 700 rpm for 4 hours at  
631 60 °C to convert it into amyloid fibril form. Samples of monomeric insulin, insulin fibrils or MBP-  
632 RHIM fusion proteins, at a concentration of 100 µM, were prepared with 2 µM Congo red in  
633 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl pH 7.4 in a 1 mL cuvette. The absorbance of each protein-  
634 containing solution was measured over the wavelength range 400 to 650 nm and compared to the  
635 absorbance profile of a solution containing only Congo red in buffer.

### 636 **M45 RHIM peptide**

637 A synthetic peptide encompassing the RHIM of M45 was purchased from Genscript with the  
638 sequence VRIMNGVSGIQIGNHNAMS. The peptide was solubilised in DMSO and then diluted  
639 with water to a final peptide concentration of 300 µM with addition of a trace amount of ammonium

640 hydroxide to aid solubilisation. Addition of Thioflavin T indicated the development of a ThT-  
641 positive structure. Samples were analysed by transmission electron microscopy.

#### 642 **Transmission Electron Microscopy (TEM)**

643 A droplet of protein or peptide-containing solution (20  $\mu$ L) was placed onto Parafilm<sup>TM</sup> and a  
644 carbon/formvar-coated copper grid (200 mesh, ProSciTech) was floated on the droplet surface for 1  
645 minute. Excess solution was removed by touching the edge of the grid to filter paper and the grid  
646 was washed with filtered water three times and then stained by floating on a 20- $\mu$ L droplet of 2%  
647 uranyl acetate for 2 minutes, followed by removal of excess solution by blotting. The grid was air-  
648 dried overnight at room temperature and then imaged using a Philips CM12 microscope operating at  
649 120 kV. Digital images were recorded using a Morada 11 MegaPixel CCD camera camera and  
650 iTEM digital imaging system.

#### 651 **ThT Kinetic Assays**

652 His-Ub or fluorescent RHIM-containing fusion proteins in 8 M urea-containing buffer were diluted  
653 into phosphate buffer (25 mM  $\text{NaH}_2\text{PO}_4$ , 150 mM NaCl, 0.5 mM DTT, pH 7.4) containing 40  $\mu$ M  
654 Thioflavin T to a final volume of 200  $\mu$ L, protein concentration 10  $\mu$ M His-Ub-M45, 2.5  $\mu$ M  
655 mCHERRY-RIPK3<sub>387-518</sub> or 2.5  $\mu$ M YPet-RIPK1<sub>497-583</sub> and residual urea concentration of 0.2 M.  
656 Three replicates of each protein sample were analysed simultaneously in a 96-well black  
657 fluorescence plate sealed with optically clear film (Corning®). Samples were incubated at 25 °C  
658 and fluorescence was measured at 480 nm, with excitation at 440 nm, every 60 s in a POLARstar  
659 Omega microplate reader (BMG Labtech).

#### 660 **Fibril co-assembly for TEM**

661 His<sub>6</sub>-Ub-RIPK3<sub>387-518</sub> and RIPK1<sub>497-583</sub> and M45<sub>1-90</sub> were mixed together in 1:1 molar ratios or with  
662 an appropriate volume of urea-containing buffer and then dialysed against 25 mM  $\text{NaH}_2\text{PO}_4$ ,  
663 150 mM NaCl, 0.5 mM DTT pH 7.4 for a minimum of 4 hours. Samples were removed and used to  
664 prepare negatively stained grids for TEM, as above.

665

666 **Fluorescence anisotropy assays**

667 Covalent labelling of RIPK1<sub>497-583</sub> with the Alexa 488 fluorescence tag was performed in the  
668 amyloid state. Samples of 1–4 mL of 100  $\mu$ M RHIM fragment in 6 M GuHCl, obtained from the  
669 cleavage of His<sub>6</sub>-Ub-RIPK1<sub>497-583</sub>, were dialysed against phosphate buffer at pH 8.0 with 0.5 mM  
670 DTT at room temperature for 3h, followed by further dialysis against 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 150 mM  
671 NaCl pH 7.4 at pH 8.0 with two buffer changes. After the dialysis process, RIPK1-RHIM protein  
672 was in an amyloid state and the sample was centrifuged at 16 000g for 10 min. The supernatant was  
673 discarded and the amyloid pellet was resuspended in 1mL of 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, pH  
674 8.0. A 50– $\mu$ L volume of 10 mg/mL Alexa 488 (10 mg/mL in DMSO) was added to the amyloid  
675 sample. The sample was incubated on a rocker for 1 h at room temperature and then unbound dye  
676 was removed by centrifugation at 16000g for 10 min. The pellet was washed 3 times with 1 mL of  
677 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, pH 8.0 and then resuspended in 1 mL of 8 M urea, 20 mM  
678 NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, pH 7.4 for storage. The degree of labelling was calculated to be 5%.  
679 For polarization experiments, samples containing a molar ratio of 1:1999 of Alexa 488-RIPK1<sub>497-583</sub>  
680 to unlabelled RIPK1<sub>497-583</sub>, RIPK3<sub>387-518</sub> or M45<sub>1-90</sub> in 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, pH 7.4  
681 were prepared to a final volume of 300  $\mu$ L. Total final protein concentrations of unlabelled protein  
682 were 0.85  $\mu$ M for RIPK1, 0.69  $\mu$ M RIPK3 and 1.05  $\mu$ M for M45 and the residual urea  
683 concentration was 0.052 M. Three replicates of each protein sample were analysed simultaneously  
684 in a 96-well black fluorescence plate sealed with optically clear film (Corning®). Samples were  
685 incubated at 25 °C in a POLARstar Omega microplate reader (BMG Labtech) and excited at  
686 480 nm. The parallel and perpendicular fluorescence emission was recorded at 520 nm, every 60 s.  
687 These fluorescence intensities were used to calculate the fluorescence polarization.

688 **X-ray fibre diffraction**

689 His<sub>6</sub>-Ub-M45<sub>1-90</sub> protein was allowed to assemble into fibrils by dialysis out of 8 M urea-containing  
690 buffer into water overnight. The insoluble fibrils were pelleted by centrifugation at 16000g for  
691 10 min, resuspended in a small volume of water and allowed to air-dry from a droplet suspended

692 between the wax-filled ends of two glass capillaries. X-ray fibre diffraction images were obtained  
693 using an in-house Cu K $\alpha$  Rigaku rotating anode source (wavelength 1.5418 Å) and MARresearch  
694 image plate detector.

#### 695 **SDS-stability assays**

696 Protein samples (0.5–1 mL) of individual fluorescent RHIM-containing constructs as well as  
697 mixtures of these proteins were diluted to final individual protein concentrations of 20  $\mu$ M with 8 M  
698 urea, 25 mM NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, pH 7.4. Approximately 50  $\mu$ L of each sample was removed  
699 for analysis as the monomeric, non-assembled control. The remaining solutions were dialysed  
700 overnight at room temperature against 25 mM NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, 0.5 mM DTT, pH 7.4.  
701 Samples were removed and prepared with 4% glycerol and 0.0008% bromophenol blue and 0 or 2%  
702 SDS, incubated at room temperature for 10 min and then analysed by electrophoresis using a 1%  
703 agarose gel running in TAE buffer containing 0.1% SDS. Gels were imaged on a Bio-Rad Chemi-  
704 Doc imaging system with 605/50nm and 695/55nm emission filters. In order to study the stability of  
705 the amyloid fibrils by SDS-PAGE, mCHERRY RHIM-containing constructs, individually or in  
706 mixtures, were dialysed against phosphate buffer overnight, at room temperature. After dialysis,  
707 two 50- $\mu$ L samples were centrifuged at 16,000g for 10 min and the resulting supernatants removed  
708 and pooled (soluble sample). One pellet was resuspended with 50  $\mu$ L of 8 M urea, 25 mM  
709 NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, pH 7.4 to allow visualisation of the insoluble material produced during  
710 dialysis (insoluble sample). The other pellet was resuspended in 50  $\mu$ L of 2% SDS in 25 mM  
711 NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, pH 7.4 and incubated at 37 °C for 10 minutes. SDS-containing samples  
712 were then centrifuged at 16,000g for 10 min. The supernatant was removed (SDS-soluble sample)  
713 and the pellet (SDS-insoluble sample) resuspended in 50  $\mu$ L urea-containing buffer. All samples  
714 were boiled with NuPAGE LDS sample buffer and NuPAGE reducing agent (50 mM DTT, Thermo  
715 Scientific) and then analysed by SDS PAGE.

716

717

718 **Confocal microscopy**

719 Protein samples (0.5 mL) in 8 M urea containing mixtures of monomeric, purified YPET, ECFP  
720 and mCHERRY RHIM-containing constructs at final individual protein concentrations of either 2.5  
721 or 5  $\mu$ M were dialysed overnight at room temperature against 25 mM  $\text{NaH}_2\text{PO}_4$ , 150 mM NaCl,  
722 0.5 mM DTT, pH 7.4. Samples (15  $\mu$ L) were placed on glass slides, protected with cover slips and  
723 imaged on a Zeiss LSM 510 Meta confocal microscope.

724 **Single molecule fluorescence spectroscopy**

725 Mixtures of fluorescent fusion proteins with YPet or mCHERRY fluorophores were prepared at  
726 70  $\mu$ M in 8 M urea, 25 mM  $\text{NaH}_2\text{PO}_4$ , 150 mM NaCl, 0.5 mM DTT, pH 7.4 and then diluted 100-  
727 fold with 25 mM  $\text{NaH}_2\text{PO}_4$ , 150 mM NaCl, 0.5 mM DTT, pH 7.4 in a 192-well silicone plate for  
728 single molecule measurement before monitoring. Plates were analyzed at room temperature on a  
729 Zeiss Axio Observer microscope with a custom-built data acquisition setup. Fluorescence signal  
730 was collected and separated using a 565  $\square$  nm dichroic mirror. For detection of YPET-tagged  
731 proteins, signal was passed through a 525/20  $\square$  nm band pass filter; for detection of mCHERRY-  
732 tagged proteins a 580  $\square$  nm long pass filter was used. For experiments with all three RHIM-  
733 containing proteins, protein mixtures containing YPet-RIPK<sub>387-518</sub>, mCHERRY-RIPK<sub>1497-583</sub> and  
734 His<sub>6</sub>-Ub-M45<sub>1-90</sub> were prepared in 8 M urea-containing buffer, to provide samples containing the  
735 RIPK3:RIPK1:M45 RHIMs in the range ~4:4:1 to ~1:1:4. Samples were diluted 100-fold into  
736 25 mM  $\text{NaH}_2\text{PO}_4$ , 150 mM NaCl, 0.5 mM DTT, pH 7.4 and 20  $\mu$ L pipetted into a 192-well silicone  
737 plate for single molecule measurement. For experiments with multiple fluorophores, the signal from  
738 the two channels was recorded simultaneously in 1  $\square$  ms time bins.

739 **Generation of M45-expressing HT-29 cells and cell death assays**

740 M45<sub>1-90</sub> and M45<sub>1-277</sub> fragments were amplified from MCMV (Smith) and cloned upstream of the  
741 E.Coli DNA gyrase subunit B (residues 2-220) in the pCDH-MCS-EF1-neo lentivector. All  
742 insertions were validated by sequencing (Garvan Institute, Sydney, Australia). Lentivirus for M45<sub>1-90</sub>-  
743 gyrase, M45<sub>1-277</sub>-gyrase or empty vector control were made in 293T cells by co-transfecting the

744 lentivectors with psPAX2 and pMD2G using Fugene HD (Promega). HT-29s were then transduced  
745 with the lentivirus particles in the presence of polybrene (Santa Cruz), and after 3 days G418  
746 (Roche) was added for the subsequent 10 days to select for successfully transduced cells. M45<sub>1-90</sub>-  
747 gyrase, M45<sub>1-277</sub>-gyrase or empty-vector-control transduced HT-29s were seeded in 96 well plates  
748 ( $10^4$  cells/well) and allowed to adhere for 6 h before treating with 900 nM coumermycin A1 (Enzo)  
749 or media (no treatment control), followed 1 hour later with combinations of TNF (30 ng/ml, R and  
750 D), BV-6 (1  $\mu$ M, Selleckchem) and z-VAD-fmk (25  $\mu$ M, R and D), or DMSO only (Sigma-  
751 Aldrich) as control. Cell viability was then assessed 17-18 hour post-treatment by measuring levels  
752 of intracellular ATP, using the CellTiter-Glo2 Assay (Promega). Data was expressed as percentage  
753 of cell survival relative to the DMSO only control. Luminescence was measured using an Infinite  
754 M1000 Pro plate reader (TECAN).

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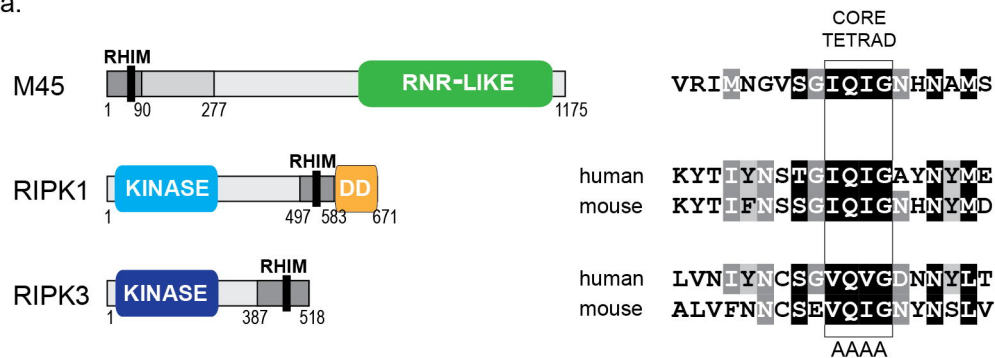
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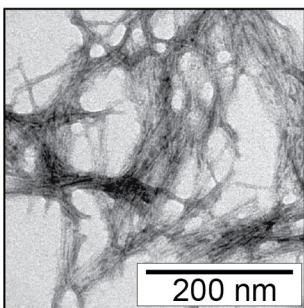
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- 890

Figure 1.

a.



b.



c.

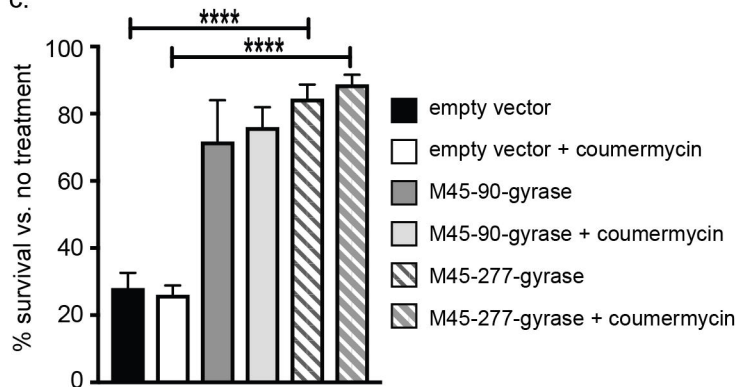


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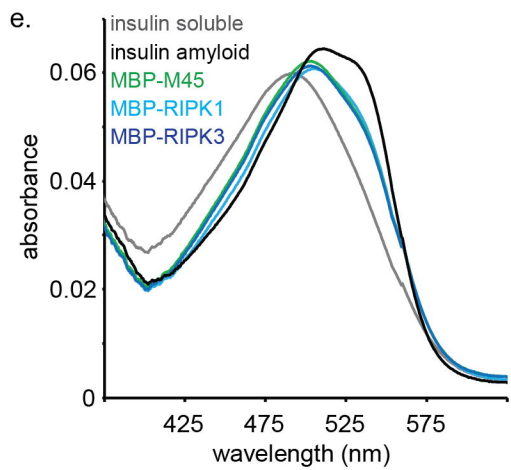
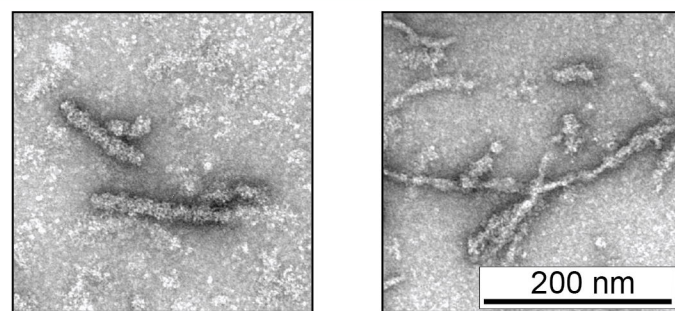
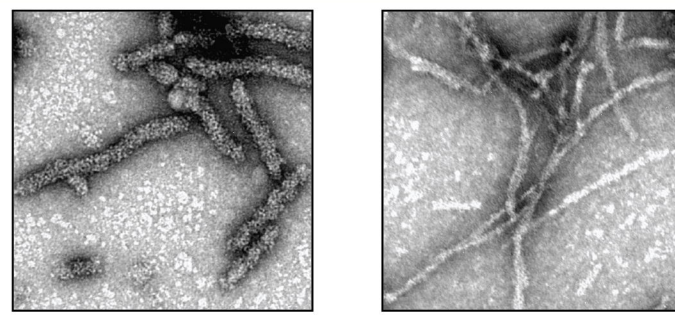
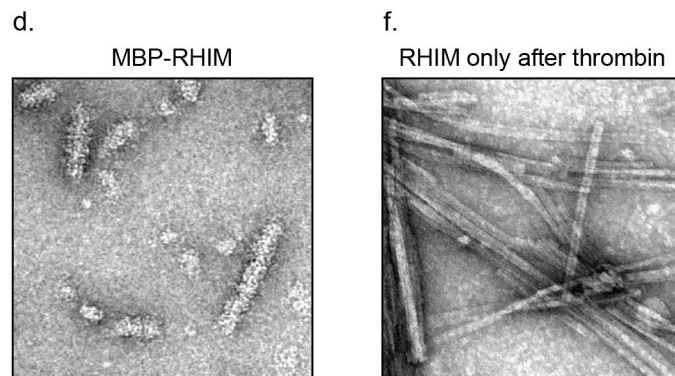
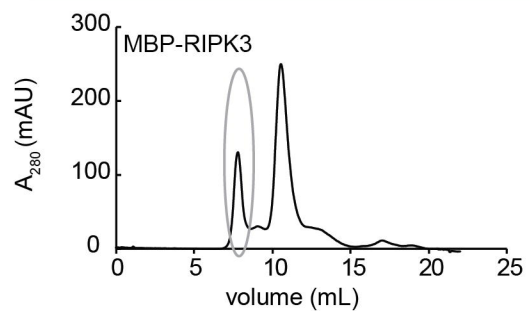
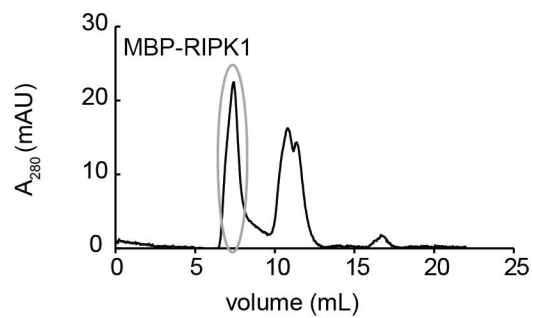
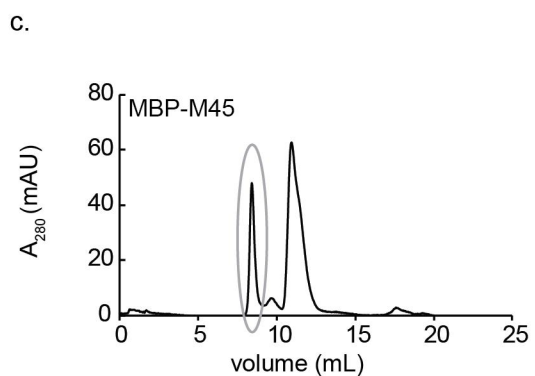
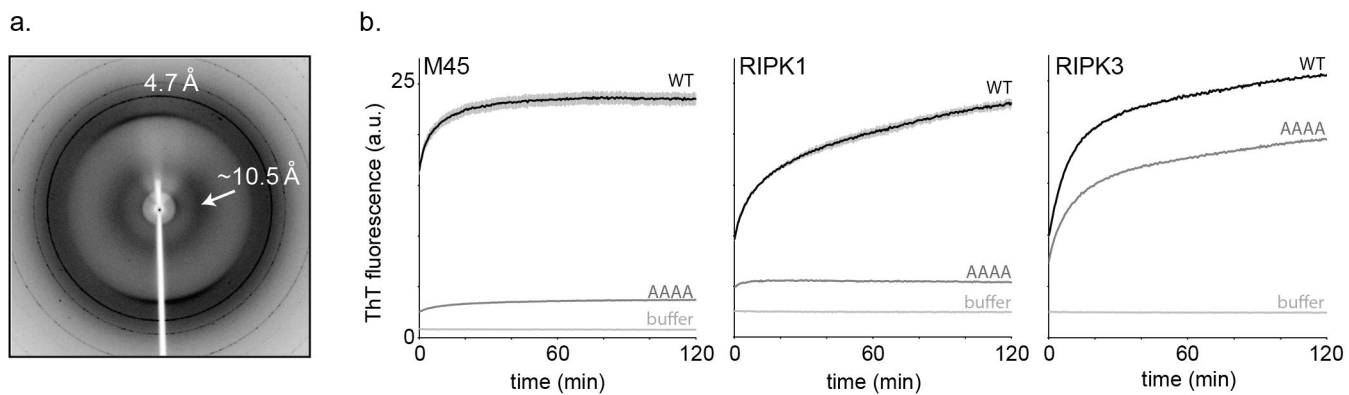


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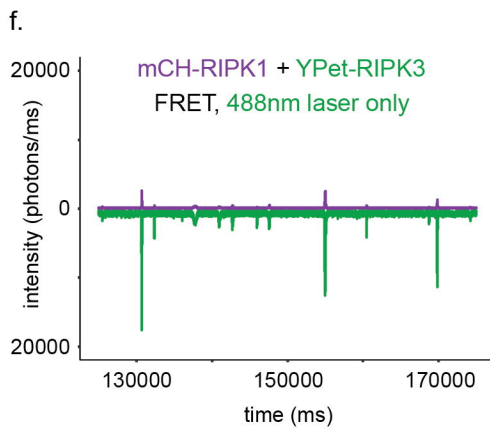
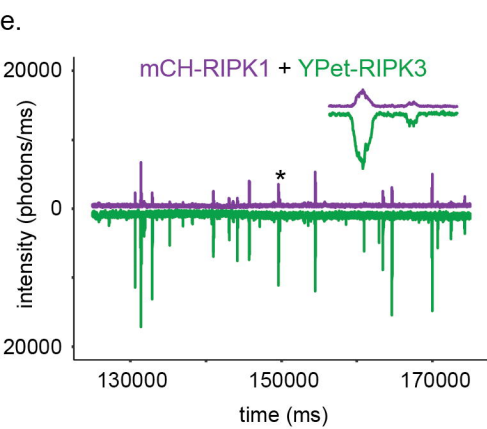
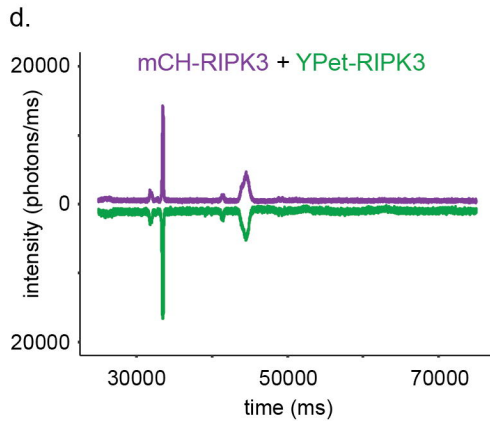
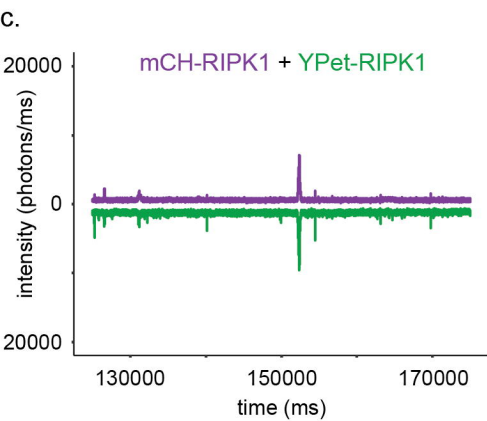
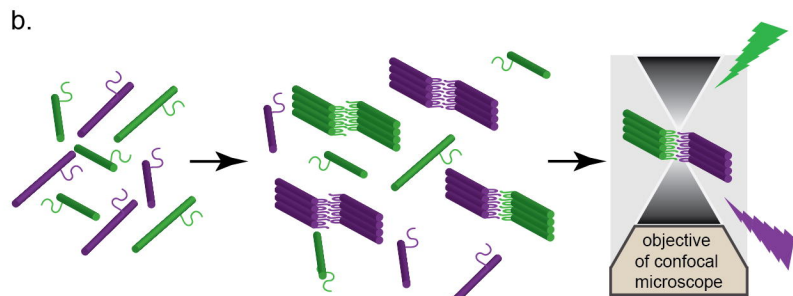
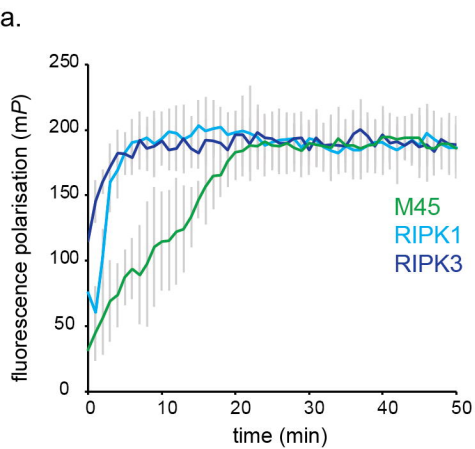


Figure 4.

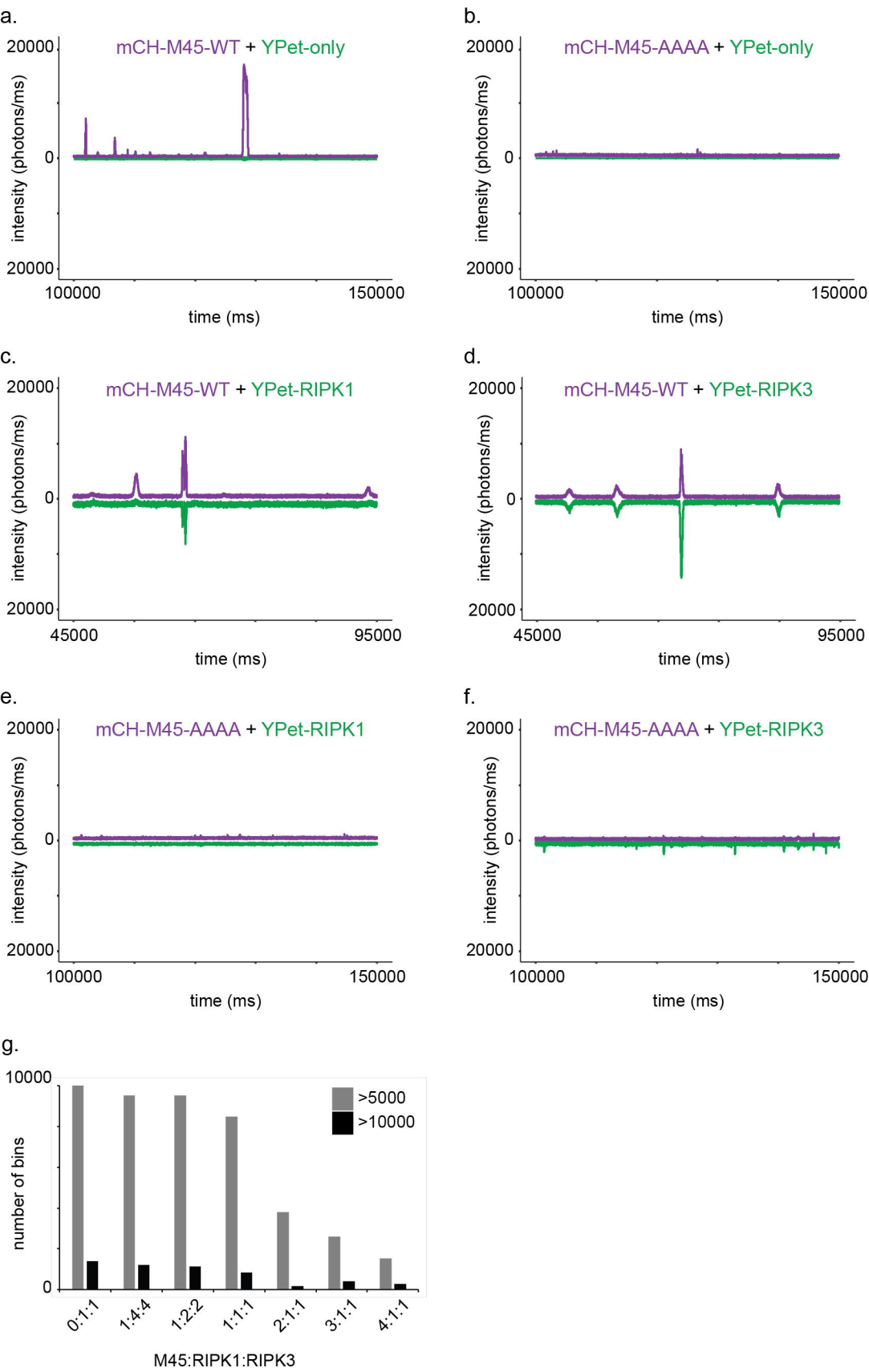
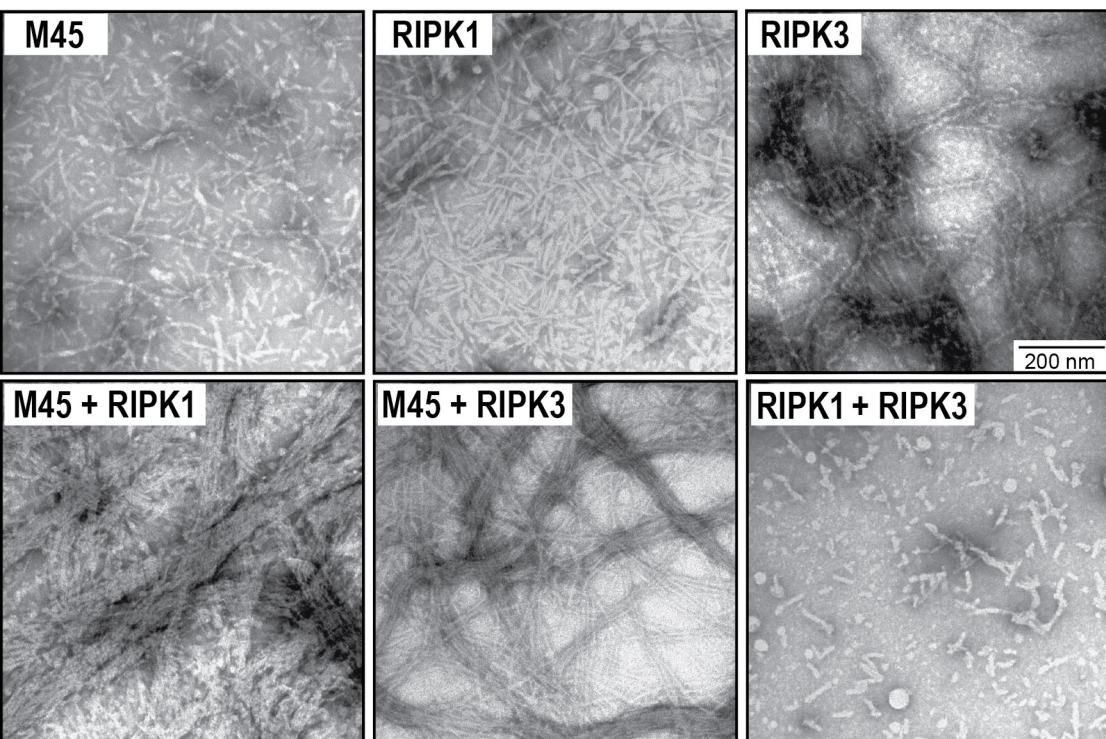
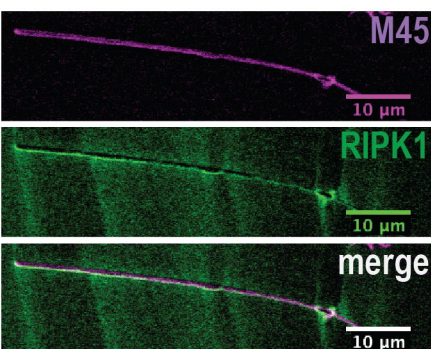


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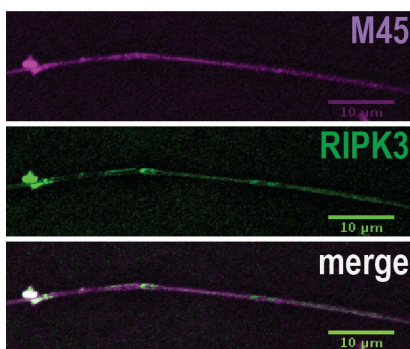
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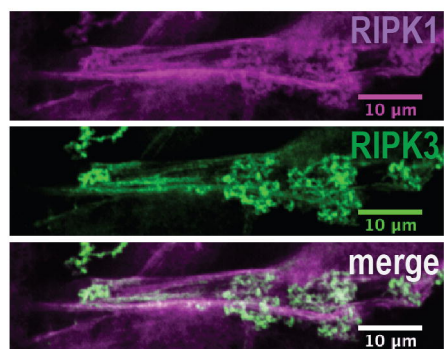
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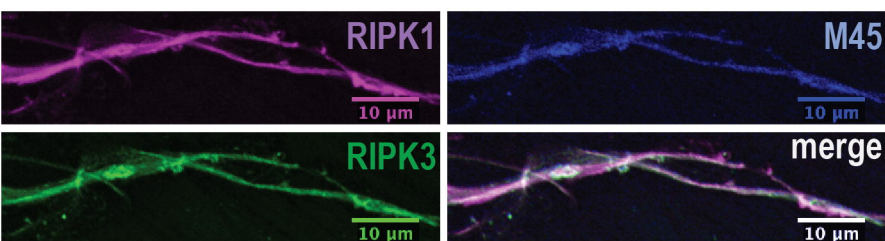
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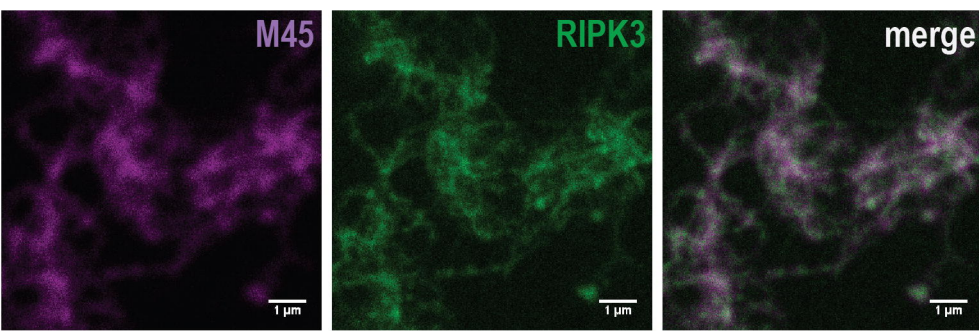
d.



e.



f.



g.

