

1 **Membrane permeabilization is mediated by distinct epitopes in mouse and human**
2 **orthologs of the necroptosis effector, MLKL**

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20 **ABSTRACT**

21 Necroptosis is a lytic programmed cell death pathway with origins in innate immunity that is
22 frequently dysregulated in inflammatory diseases. The terminal effector of the pathway,
23 MLKL, is licensed to kill following phosphorylation of its pseudokinase domain by the
24 upstream regulator, RIPK3 kinase. Phosphorylation provokes the unleashing of MLKL's N-
25 terminal four-helix bundle (4HB or HeLo) domain, which binds and permeabilizes the plasma
26 membrane to cause cell death. The precise mechanism by which the 4HB domain permeabilizes
27 membranes, and how the mechanism differs between species, remains unclear. Here, we
28 identify the membrane binding epitope of mouse MLKL using NMR spectroscopy. Using
29 liposome permeabilization and cell death assays, we validate K69 in the α 3 helix, W108 in the
30 α 4 helix, and R137/Q138 in the first brace helix as crucial residues for necroptotic signaling.
31 This epitope differs from the phospholipid binding site reported for human MLKL, which
32 comprises basic residues primarily located in the α 1 and α 2 helices. In further contrast to
33 human and plant MLKL orthologs, in which the α 3- α 4 loop forms a helix, this loop is
34 unstructured in mouse MLKL in solution. Together, these findings illustrate the versatility of
35 the 4HB domain fold, whose lytic function can be mediated by distinct epitopes in different
36 orthologs.

37 **INTRODUCTION**

38 Necroptosis is a caspase-independent, lytic cell death mode with ancestral origins in host
39 defense^{1, 2, 3, 4, 5, 6, 7, 8, 9}, which is frequently dysregulated in disease^{10, 11, 12, 13, 14, 15, 16}. The
40 inflammatory nature of necroptosis has led to its implication in a range of human pathologies,
41 including renal^{11, 13} and gastrointestinal diseases^{14, 17}. Necroptotic signaling is instigated by
42 ligation of death receptors, such as TNF Receptor 1, or pathogen detectors, such as ZBP1/DAI,
43 in cellular contexts where the IAP E3 Ubiquitin ligase family and the proteolytic enzyme,
44 Caspase-8, are downregulated or their catalytic activities compromised (reviewed in ref.¹⁸).
45 Downstream of receptor activation, a cytoplasmic platform termed the necrosome is assembled
46 in which the Receptor-interacting serine/threonine protein kinase (RIPK)-1 recruits RIPK3,
47 leading to its activation by autophosphorylation^{1, 19, 20, 21, 22}. Subsequently, RIPK3
48 phosphorylates the pseudokinase domain of the necroptotic executioner, Mixed lineage kinase
49 domain-like (MLKL)^{23, 24}, to induce its dissociation from the necrosome^{25, 26, 27}, assembly into
50 high molecular weight complexes^{27, 28, 29}, and trafficking to the plasma membrane^{18, 27, 30}. When
51 a threshold level of activated MLKL accumulates at the plasma membrane^{27, 30}, MLKL perturbs
52 the lipid bilayer to cause cell death via an incompletely understood mechanism³¹. This mode
53 of cell death involves the leakage of cellular contents, including DAMPs, into the extracellular
54 milieu to provoke an inflammatory response³².

55

56 While the core principles of necroptotic signaling and MLKL activation are preserved between
57 species, the precise molecular mechanisms appear to differ. Detailed studies of the
58 pseudokinase domains of MLKL orthologs have revealed their propensity to adopt distinct
59 conformations^{23, 29, 33, 34, 35}, which governs recognition by RIPK3 and results in very strict
60 species specificity^{36, 37}. Additionally, the role of activation loop phosphorylation in triggering
61 MLKL activation appears to vary between orthologs. Phosphorylation serves as a trigger for
62 release of the killer 4HB domain in mouse MLKL^{23, 28, 38, 39}, as a cue for MLKL release from
63 the necrosome and interconversion to the closed, active form in human MLKL^{25, 26, 29, 34}, and a
64 likely role in negating occupation of the pseudoactive site in horse MLKL³³. Consistent with
65 the diverse regulatory mechanisms governing the MLKL pseudokinase domain molecular
66 switch, the N-terminal executioner 4HB domain exhibits heterogeneous membrane
67 permeabilization between species³⁷. However, the molecular basis for how and why
68 recombinant mouse MLKL 4HB domain more efficiently permeabilizes lipid bilayers than the
69 human and chicken 4HB domains has remained unclear.

70

71 NMR studies of human MLKL's 4HB domain over the past 7 years have provided important
72 insights into the residues involved in phospholipid headgroup and inositol phosphate
73 recognition^{40, 41, 42, 43}. These studies have implicated basic residues located principally within
74 the α 1 and α 2 helices in negatively-charged phospholipid binding^{42, 43}, while inositol
75 phosphate recognition relies on an epitope centred on the loop connecting the α 2 and α 3
76 helices, and the α 1 helix, including lipid binding residues^{40, 41}. Considering the low sequence
77 identity between human and mouse MLKL 4HB domains^{33, 44}, we employed NMR
78 spectroscopy to define the residues that mediate lipid recognition in mouse MLKL and to
79 identify structural differences from its human counterpart. Remarkably, in NMR relaxation
80 experiments, we identified residues on the opposing face of the mouse MLKL 4HB domain,
81 relative to those implicated in human MLKL lipid and inositol phosphate recognition, as
82 mediators of liposome binding. Mutation of these residues compromised liposome
83 permeabilization *in vitro*, with a subset of these sites found to be functionally crucial for mouse
84 MLKL necroptotic signaling in cells. Collectively, these data illustrate that mouse and human
85 MLKL rely on distinct lipid-binding residues to enact cell death and support the idea that the
86 4HB (also known as HeLo) domain can serve as a versatile scaffold for lipid recognition and
87 permeabilization.

88 **RESULTS**

89

90 **Mouse MLKL 4HB domain adopts a folded helical structure**

91 To characterize the structure of N-terminal four-helix bundle (4HB) domain and the first brace
92 helix of mouse MLKL (residues 1-158; termed mouse MLKL₍₁₋₁₅₈₎ herein) in solution, we
93 subjected ²H,¹⁵N,¹³C-labelled protein to non-uniformly sampled three-dimensional NMR
94 methodology. As previously, mouse MLKL₍₁₋₁₅₈₎ purified as a monomer, owing to the absence
95 of the second brace helix that is required for trimerization³⁶. From these 3D NMR experiments,
96 we could successfully assign 95% of the backbone resonances corresponding to residues 1-158
97 of mouse MLKL (**Figure 1a**), with additional backbone amides corresponding to the vector-
98 encoded remnant sequence (GAMGS) also observed (numbered as residues -4 to 0 in **Figure**
99 **1b-c**). As anticipated from the crystal structure of full-length mouse MLKL, the 4HB domain
100 (residues 1-125) and the adjacent brace helix 1 exhibited predominantly positive $\Delta\text{C}\alpha-\Delta\text{C}\beta$
101 smoothed values (**Figure 1b**), which is consistent with the expected helical structure. In
102 contrast, the region corresponding to the S82 to G91 backbone amides exhibited trends to both
103 negative and positive $\Delta\text{C}\alpha-\Delta\text{C}\beta$ values ($\Delta\text{C}\alpha-\Delta\text{C}\beta < \pm 1.0$), indicating a lack of regular
104 secondary structure in this region. Interestingly, the overlapping region encompassing S79 to
105 K94 could not be modelled in the mouse MLKL crystal structure due to lack of electron
106 density^{23, 45}, consistent with it occurring as an unstructured loop. To further investigate the
107 internal dynamics within the mouse MLKL 4HB domain structure in solution, we recorded a
108 steady state ¹⁵N{¹H}-NOE experiment⁴⁶ on ¹⁵N-labelled mouse MLKL₍₁₋₁₅₈₎ (**Figure 1b**). The
109 average ¹⁵N{¹H}-NOE values (0.82 ± 0.07) for mouse MLKL (residues D2-V150) support the
110 existence of the mouse MLKL 4HB domain and adjacent brace helix occurring in solution as
111 a structured protein with flexible termini (vector encoded residues -4 to 0 and residues 151-
112 158, average ¹⁵N{¹H}-NOE values < 0.5). For the region S79 to K94, which corresponds to
113 the loop connecting the α 3 and α 4 helices in the mouse MLKL crystal structure, an average
114 ¹⁵N{¹H}-NOE value of (0.62 ± 0.05) was recorded. These data are consistent with this loop
115 exhibiting higher flexibility than the remaining mouse MLKL 4HB+brace core structure.

116

117 **Two clusters mediate mouse MLKL 4HB+brace binding to liposomes**

118 Studies of the human MLKL 4HB domain by NMR spectroscopy have identified principally
119 basic residues as the mediators of lipid binding, lipid permeabilization and cell death^{42, 43}.
120 While analogous studies have not been performed to date on mouse MLKL 4HB domain, very

121 few of the key residues within human MLKL are conserved in the mouse ortholog.
122 Accordingly, we sought to deduce which mouse MLKL 4HB domain residues mediate lipid
123 binding, and whether they spatially differ to the reported lipid-binding residues in the human
124 MLKL 4HB domain, by performing a 2D ^1H - ^{15}N HSQC monitored titration of uniformly ^{15}N -
125 labeled mouse MLKL₍₁₋₁₅₈₎ with liposomes of a plasma membrane-like composition. Using this
126 approach, we identified two clusters of residues in mouse MLKL that exhibited marked
127 attenuation of peak intensity (**Figure 2a-b**). Diminished peak intensity is a sensitive means of
128 detecting the engagement of different sites within the mouse MLKL 4HB+brace protein with
129 liposomes, which enables each individual backbone amide resonance to serve as a probe to
130 report changes in their solvent exposure, motions and interactions. Among these two clusters,
131 cluster I comprised R34-Q40 in the α 2 helix and D106-E110 in the neighboring α 4 helix; and
132 cluster II was composed of V67-A71 in the α 3 helix, N92-N101 in the α 4 helix and preceding
133 region, and D136-D139 in brace helix 1 (**Figure 2b**). We further validated clusters I and II as
134 liposome interacting sites in mouse MLKL using a ^{15}N amide spin transverse relaxation (^{15}N -
135 R_2) experiment at 70.9 MHz for mouse MLKL₍₁₋₁₅₈₎ in the presence and absence of liposome
136 in the ratio 1:0.5 (mouse MLKL:liposome) at 25 °C (**Figure 2c**). As expected, in the presence
137 of liposomes, the ^{15}N - R_2 values for regions clusters I and II within mouse MLKL₍₁₋₁₅₈₎ (**Figure**
138 **2c**) showed a marked increase, reflecting the chemical exchange on a fast timescale with
139 liposomes. Collectively, these data confirm roles for sites clustered on the centre of α 2 and α 4
140 helices (cluster I) and the N-terminal ends of the α 4 helix and the flanking α 3 and brace helices
141 (cluster II) in liposome binding, suggesting liposome engagement is mediated via an extended
142 interface (**Figure 2b**).
143

144 **Membrane binding residues contribute to liposome permeabilization**

145 We next sought to examine whether individual substitutions of sites identified as liposome
146 interactors in NMR spectroscopy experiments would impact liposome permeabilization. To
147 this end, we introduced Ala substitutions to H36 (α 2 helix), K69 (α 3 helix), N92, H98, W108
148 (α 4 helix and preceding region) and R137/Q138 (brace helix 1) (**Figure 3a**) in mouse MLKL₍₁₋
149 ₁₅₈₎ (**Figure 3b-c**) and prepared recombinant proteins for *in vitro* dye release assays. These
150 residues were selected because they comprise the solvent-exposed sites in clusters I and II
151 when mapped to the mouse MLKL crystal structure²³. We reasoned that shifts observed for
152 adjacent hydrophobic core residues were likely a secondary effect of their proximity to lipid-
153 binding residues and therefore did not mutate core residues to avoid disrupting 4HB domain

154 folding. In these assays, liposomes loaded with the self-quenching dye, 5(6)-
155 carboxyfluorescein, were incubated with 8 μ M recombinant mouse MLKL₍₁₋₁₅₈₎ and dye
156 release measured spectrophotometrically. While wild-type mouse MLKL₍₁₋₁₅₈₎ permeabilized
157 liposomes with comparable kinetics to previous studies^{36, 37}, alanine substitutions of sites
158 identified as liposome binding residues in NMR experiments led to dampened permeabilization
159 of liposomes in all cases except N92A (**Figure 3d-e**). Notably, alanine substitution of the
160 neighbouring residues, H98 and R137/Q138, within cluster II compromised liposome
161 permeabilization most severely. These data validate residues located on the α 2, α 3 and α 4
162 helices and the first brace helix as liposome interactors, which individually likely contribute to
163 membrane permeabilization. It is noteworthy that the introduction of individual mutations into
164 mouse MLKL₍₁₋₁₅₈₎ did not lead to complete abrogation of liposome permeabilization, as
165 expected based on earlier studies of the human MLKL ortholog^{43, 47}. As in human MLKL, we
166 expect that residues on mouse MLKL₍₁₋₁₅₈₎ act collectively to mediate lipid binding and bilayer
167 permeabilization, and as such, there is some redundancy between lipid-interacting residues
168 within the domain.

169

170 **Residues in the α 3 helix, α 4 helix, and in the first brace mediate necroptotic signaling**

171 Having identified residues that compromise liposome permeabilization by recombinant mouse
172 MLKL₍₁₋₁₅₈₎, we next introduced Ala substitutions of each liposome binding residue into
173 constructs encoding full-length mouse MLKL (**Figure 4a**). We stably introduced wild-type
174 and mutant MLKL into *Mlk1^{-/-}* Mouse Dermal Fibroblast (MDF) cells via a doxycycline-
175 inducible lentiviral system. Following doxycycline treatment to induce expression (**Supp. Fig.**
176 **1**), we examined the cellular response to the necroptotic stimulus, TSI (Tumor necrosis factor
177 (TNF, T); Smac mimetic, Compound A (S); and the pan-caspase inhibitor, emricasan/IDN-
178 6556 (I)^{25, 36, 48}) using IncuCyte live cell imaging. The capacity of each MLKL construct to
179 reconstitute the necroptotic signaling pathway was measured by quantifying SYTOX Green
180 uptake, as a measure of cell death, relative to the total number of cells stained by cell-permeable
181 DNA probe, SPY620. Importantly, expression of full-length wild-type mouse MLKL
182 successfully restored sensitivity to the necroptotic stimulus, TSI, resulting in ~80% cell death
183 at 5 h post-TSI stimulation (**Figure 4b**). Comparable necroptotic cell death kinetics were also
184 observed for the mutant MLKL constructs, H36A, N92A and H98A, indicating that,
185 individually, these residues do not impact necroptotic signaling (**Figure 4b; Supplementary**
186 **Fig. 2a-c**). In contrast, alanine substitution of K69, W108 and R137/Q138 markedly reduced

187 cell death relative to wild-type MLKL, demonstrating that substitution of these residues
188 attenuates necroptotic signaling (**Figure 4b-c; Supplementary Fig. 2d-f**). We further
189 validated these differences in cell death between MLKL constructs in an orthogonal assay by
190 quantifying the release of lactate dehydrogenase (LDH) that arises from plasma membrane
191 lysis following TSI stimulation (**Figure 4d**). Consistent with necroptotic cell death monitored
192 by IncuCyte imaging, the LDH release values identified K69, W108 and R137/Q138 located
193 on the α 3, α 4 helices and the first brace helix, respectively, as functionally crucial for mouse
194 MLKL necroptotic signaling.

195
196 MLKL binds to membranes once it has been phosphorylated by RIPK3^{28, 39, 49, 50}. Consistent
197 with this notion, mutation of the surface-exposed residues in clusters I and II did not have a
198 major influence on the phosphorylation of RIPK3 or MLKL upon TSI-stimulation (**Figure 4e**).
199 Importantly, we observed the presence of phosphorylated MLKL and RIPK3 in wild-type and
200 all mutant MLKL constructs after TSI-treatment (1.5 or 3 h), indicating that our cellular
201 findings are not attributable to compromise of an upstream necroptosis pathway checkpoint.
202 We noted that while RIPK1 was detected under basal conditions for all cell lines in our
203 immunoblots, RIPK1 was not detectable following TSI-stimulation in all conditions, which we
204 attribute to compromised detection following the post-translational modifications that
205 accompany necroptotic stimulation²¹, as previously reported⁴⁹. It is notable that K69A, W108A
206 and R137A/Q138A MLKL were expressed at lower levels than most constructs, following
207 doxycycline treatment (**Figure 4e**). However, because these mutant MLKL proteins were
208 expressed at an equivalent level to H36A MLKL, which exhibited comparable cell death
209 kinetics to wild-type MLKL, any deficits in necroptotic signaling are not a consequence of
210 lower protein expression. We used a mouse MLKL pseudokinase domain-specific antibody to
211 detect MLKL expression (WEHI clone 5A6⁴⁹), which ensures any differences in detection
212 reflect levels, rather than altered reactivity that might arise from using the brace region-directed
213 antibody (WEHI clone 3H1²³).

214 **DISCUSSION**

215 Over the past five years, it has emerged that MLKL orthologs exhibit differing propensities to
216 permeabilize lipid bilayers and thus to enact cell death. While our understanding of the
217 divergent activation and regulatory mechanisms among the pseudokinase domains of MLKL
218 orthologs has been greatly enhanced by detailed structural studies^{23, 29, 34, 35}, knowledge of
219 differences between their membrane-permeabilizing executioner domain, the N-terminal four-
220 helix bundle (4HB) domain, is limited. Distinctions between human and mouse MLKL 4HB
221 domains are evident from their sequences, with only 52% identity at the amino acid level, and
222 here we sought to further understand differences at the mechanistic level using a combination
223 of NMR spectroscopy, biochemical and cellular assays.

224

225 Our NMR spectroscopy experiments validated that the mouse MLKL 4HB and first brace helix
226 adopts a folded helical structure in solution, consistent with the fold observed in the crystal
227 structure of full-length mouse MLKL²³. In keeping with the full-length mouse MLKL crystal
228 structure, we did not observe consistent positive $\Delta C\alpha - \Delta C\beta$ values for residues within the loop
229 connecting the $\alpha 3$ and $\alpha 4$ helices, indicating that this loop does not form a helix. Indeed, the
230 reduced $^{15}\text{N}\{^1\text{H}\}$ -NOE values in this loop (relative to the domain overall) supports the assertion
231 that the loop is flexible, as originally proposed based on the lack of density for this region in
232 the full-length mouse MLKL crystal structure²³. This contrasts the human MLKL 4HB domain
233 NMR^{41, 43, 51} and crystal structures^{30, 51}, where the loop connecting the $\alpha 3$ and $\alpha 4$ helices forms
234 a helix, which is the target of the covalent MLKL inhibitor, NSA²⁴. Interestingly, like human,
235 but in contrast to mouse, MLKL 4HB domain structures, the recent cryo-EM structure of a
236 plant MLKL ortholog, which is believed to have arisen via convergent evolution, revealed a
237 helix in the loop connecting the $\alpha 3$ and $\alpha 4$ helices of the 4HB domain⁵².

238

239 Using NMR relaxation experiments, we then implicated two clusters of residues as lipid
240 interactors by titrating the N-terminal helical region of mouse MLKL with liposomes that
241 emulated a plasma membrane composition. Importantly, we chose to examine a monomeric
242 form of the mouse MLKL (residues 1-158; 4HB domain and first brace helix)³⁶ in our NMR
243 experiments, which allows us to attribute any resonance broadening in titrations to liposome
244 binding, and not oligomerization events. Broadly, the identified sites are spatially-proximal to
245 those identified as key mediators of necroptotic signaling in our earlier cellular studies of
246 mouse MLKL^{28, 37} (**Figure 5a-b; Supplementary Table 1**). However, whether the arising

247 defects in cell signaling were attributable to deficits in lipid recognition, or other impacts on
248 necroptotic checkpoints, had not been formally examined. Here, we add to current knowledge
249 by establishing roles for mouse MLKL K69 (α 3 helix), W108 (α 4 helix) and R137/Q138 (first
250 brace helix) in liposome permeabilization in dye release assays and necroptotic signaling in
251 reconstituted *Mlk1*^{-/-} MDF cells. Our finding that, despite deficits in signaling, these MLKL
252 constructs and the upstream regulator, RIPK3, were phosphorylated following necroptotic
253 stimulation indicates that mouse MLKL can still undergo RIPK3-mediated phosphorylation
254 via the proposed transient “kiss and run” mechanism^{23, 26, 28, 45}. These data support the notion
255 that the loss-of-function mutations identified here arise as a consequence of compromised lipid
256 recognition and membrane permeabilization downstream of RIPK3 interaction.

257
258 By mapping the crucial residues for necroptotic signaling identified herein, and from earlier
259 studies^{28, 37}, on to the mouse MLKL 4HB domain+brace structure, it emerges that the lipid-
260 binding epitope is centered on the α 3- α 4 helical face of mouse MLKL (**Figure 5b**).
261 Importantly, this differs from the epitope in human MLKL, which is located on the opposing
262 α 1- α 2 helical face of the 4HB domain (**Figure 5a, c**), as deduced from a combination of NMR
263 spectroscopy, liposome permeabilization assays and cell death assays^{29, 42, 43, 47}. In contrast to
264 mouse MLKL, the implicated residues in human MLKL lipid interaction and membrane
265 permeabilization are typically positively-charged (**Figure 5a, c; Supplementary Table 2**).
266 While no studies have been performed on the capacity of mouse MLKL 4HB domain to engage
267 inositol phosphates to date, it is notable that, again, positively charged residues within the
268 human MLKL 4HB domain have been attributed functions in inositol phosphate binding.
269 Although the sites of lipid engagement employed by the 4HB domain of plant MLKL are
270 currently unknown⁵², it is notable that basic residues are largely absent from regions
271 corresponding to binding residues within human MLKL. In our study of mouse MLKL, we
272 used a plasma membrane-like lipid cocktail (**Supplementary Table 4**) to prepare unilamellar
273 vesicles termed liposomes for our NMR titrations. On the other hand, studies of human MLKL
274 have typically used isolated, highly negatively-charged phospholipid headgroups or inositol
275 phosphates^{40, 41, 42}, which may favour binding to positive sites on the human MLKL 4HB
276 domain. Additionally, the inositol phosphate binding epitope overlaps that of the lipid-binding
277 epitope and, as a result, this poses challenges for ascribing clear functions for inositol
278 phosphates in regulating necroptotic signaling in cells. The precise function of inositol

279 phosphates as modulators of necroptosis, and whether a similar regulatory function is conferred
280 upon mouse MLKL, remains of outstanding interest.

281

282 Collectively, our findings uncover distinct species-dependent differences in lipid recognition
283 between mouse and human MLKL. This plasticity illustrates the role of the HeLo/4HB domain
284 as a scaffold for lipid engagement and permeabilization. Importantly, this work provides
285 invaluable insight into how MLKL mediates necroptotic cell death and establishes a platform
286 for future high-resolution structural studies in membranes to address the precise mechanism by
287 which MLKL permeabilizes membranes.

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298

299 **AUTHOR CONTRIBUTIONS**

300 AS and CRH designed and performed experiments, analysed data and co-wrote the paper with
301 JMM; KW carried out the biodeuteration of the recombinant protein for NMR; CF, KAD, SEG,
302 AVJ, ALS, JMH and AW performed experiments and analysed data; PEC, EJP, PRG and JMM
303 supervised the project and contributed to experimental design and data analysis. All authors
304 commented on the manuscript.

305

306 **COMPETING INTERESTS**

307 CF, KAD, SEG, ALS, JMH, PEC, EJP and JMM contribute to, or have contributed to, a project
308 with Anaxis Pharma to develop necroptosis inhibitors. The remaining authors declare no
309 conflicts of interest.

310 **METHODS**

311 **Expression constructs.** For expression in mammalian cells, wild-type full-length MLKL was
312 amplified by PCR from a mouse MLKL template (synthesised by DNA2.0, CA) and ligated
313 into the doxycycline-inducible, puromycin-selectable mammalian expression vector, pF
314 TRE3G PGK puro (Amp^r) using BamHI and EcoRI restriction sites, as before²³. Mutant mouse
315 MLKL cDNAs were synthesized and subcloned into pF TRE3G PGK puro as BamHI-EcoRI
316 fragments by ATUM (CA). Vector DNA was co-transfected into HEK293T cells with pVSVg
317 and pCMV ΔR8.2 helper plasmids to generate lentiviral particles, which were transduced into
318 three biologically independent Mouse Dermal Fibroblast (MDF) cell lines (*Mlkl*^{-/-}, derived
319 from different mice using a previously described method²³) and selected for genomic
320 integration using puromycin (2.5 µg mL⁻¹; StemCell Technologies) using established
321 procedures^{28, 36}. For recombinant protein constructs, wild-type mouse MLKL₍₁₋₁₅₈₎ was
322 amplified by PCR from the mouse MLKL template and subcloned into the bacterial expression
323 vector pETNusH Htb (Kan^r) (derived from pETM60)^{53, 54} as an in-frame fusion with a TEV
324 (tobacco etch virus) protease-cleavable NusA-His₆ tag. Mutant mouse MLKL₍₁₋₁₅₈₎ constructs
325 were amplified by PCR from the respective pF TRE3G PGK puro constructs (ATUM, CA) and
326 subcloned into pETNusH Htb as BamHI-EcoRI fragments. All insert sequences were verified
327 by Sanger sequencing (AGRF, VIC, Australia). All primers used in this study are listed in
328 **Supplementary Table 3.**

329

330 **Biodeuteration and protein expression of mouse MLKL₍₁₋₁₅₈₎.** Uniformly ¹⁵N-labelled ([U-
331 ¹⁵N]), ¹³C¹⁵N-labelled [U-¹³C, ¹⁵N] and fractional deuterated (f-²H) [U-¹³C, ¹⁵N]-labelled
332 recombinant mouse MLKL₍₁₋₁₅₈₎ was expressed via the pETNusH Htb vector at the National
333 Deuteration Facility (NDF), Australian Nuclear Science and Technology Organization
334 (ANSTO) in 1 L batch cultures using an established high cell density protocol⁵⁵.

335

336 Briefly, for the [U-¹⁵N]- and [U-¹³C, ¹⁵N]-labelled mouse MLKL₍₁₋₁₅₈₎ constructs, 300 µL of
337 freshly transformed *E. coli* BL21StarTM(DE3) cells were inoculated into 10 mL of H₂O ModC1
338 minimal medium, supplemented with kanamycin (40 µg L⁻¹) and incubated overnight at 30 °C
339 shaking at 220 rpm. The cell suspensions were diluted 5x in ¹H, ¹⁵N-ModC1 medium (40 g/L
340 glycerol, 5.16 g/L ¹⁵NH₄Cl ≥ 98 atom % ¹⁵N) or ¹H, ¹³C, ¹⁵N-ModC1 medium (20 g/L glycerol-
341 ¹³C₃ 99 atom % ¹³C, 5.16 g/L ¹⁵NH₄Cl ≥ 98 atom % ¹⁵N) and grown at 37 °C for two OD₆₀₀
342 doublings, respectively for the [U-¹⁵N]) and [U-¹³C, ¹⁵N] labelled constructs. Finally, cells were
343 inoculated into fresh ¹H, ¹⁵N-ModC1 to a volume of 100 mL and grown to an OD₆₀₀ of 0.9-1.2

344 before inoculation into 900 mL of labelled expression medium (as described above) in a 1 L
345 working volume bioreactor. *E. coli* cells were grown at 25 °C until OD₆₀₀ of ~9.5 and
346 expression was induced by addition of isopropylthio-β-D-galactopyranoside (IPTG) at a final
347 concentration of 1 mM. After ~24 h induction at 20 °C, during which a further 5.16 g of
348 ¹⁵NH₄Cl was added to the culture, each labelled cell suspension was pelleted by centrifugation
349 at 8000×g for 20 min and each biomass stored at -80 °C.

350

351 Briefly, a three step deuterated minimal medium adaptation process was followed for the (f-
352 ²H) [^{U-13}C, ¹⁵N]-labelled mouse MLKL₍₁₋₁₅₈₎ construct starting with 300 μL of freshly
353 transformed *E. coli* BL21StarTM(DE3) cells inoculated into 10 mL of 50% deuterium oxide
354 (D₂O) (v/v) ModC1 minimal medium (20 g/L glycerol) with 40 μg L⁻¹ kanamycin and
355 incubated overnight at 37 °C with shaking at 220 rpm. The resulting cell suspension was diluted
356 5-fold in ²H, ¹³C, ¹⁵N-ModC1 medium (D₂O 99.8 atom % D, 20 g/L glycerol-¹³C₃ 99 atom %
357 ¹³C, 5.16 g/L ¹⁵NH₄Cl ≥ 98 atom % ¹⁵N) and grown at 37 °C for approximately one OD₆₀₀
358 doubling. Finally, cells were inoculated into fresh ²H, ¹³C, ¹⁵N-ModC1 to a volume of 100 mL
359 and grown to an OD₆₀₀ of 1.1 before inoculation into 900 mL of labelled expression medium
360 as described in a 1 L working volume bioreactor. *E. coli* cells were grown at 37 °C until OD₆₀₀
361 reached 8.2 and expression induced by addition of IPTG at a final concentration of 1 mM. After
362 26 h induction at 20 °C, during which a further 5.16 g of ¹⁵NH₄Cl was added to the culture, the
363 labelled cell suspension was pelleted by centrifugation at 8000×g for 20 min and biomass
364 stored at -80 °C.

365

366 **Protein expression of unlabelled mouse MLKL (1-158).** Mouse MLKL₍₁₋₁₅₈₎ constructs with
367 in-frame TEV protease cleavable N-terminal NusA-His₆ tags (pETNusH Htb) were expressed
368 in *E. coli* BL21-Codon Plus (DE3)-RIL cells cultured in Super Broth supplemented with
369 kanamycin (50 μg mL⁻¹) at 37°C with shaking at 220 rpm to an OD₆₀₀ of ~0.6-0.8. Protein
370 expression was induced by the addition of IPTG (final concentration of 1 mM) and the
371 temperature was lowered to 18 °C for incubation overnight. Following protein expression, the
372 cell suspension was pelleted by centrifugation at 8000×g for 20 min and biomass stored at -80
373 °C.

374

375 **Recombinant protein purification.** For liposome permeabilization assays, cell pellets of mouse
376 MLKL₍₁₋₁₅₈₎ were resuspended in wash buffer [20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 5
377 mM imidazole (pH 8.0), 20% glycerol, 1 mM TCEP [Tris-(2-carboxyethyl)phosphine],

378 supplemented with Complete protease inhibitor cocktail (Roche), and lysed by sonication. The
379 whole cell lysate was clarified by centrifugation (45,000×g, 1 h, 4 °C), filtered (0.2 µM) and
380 the supernatant was incubated with pre-equilibrated Ni-NTA agarose (HisTag, Roche) at 4 °C
381 for 1 h with gentle agitation. Ni-NTA beads were then pelleted via centrifugation and washed
382 thoroughly with wash buffer. Bound protein was eluted from the beads using elution buffer [20
383 mM Tris-HCl (pH 8.0), 500 mM NaCl, 250 mM imidazole (pH 8.0), 20% glycerol, 1 mM
384 TCEP], filtered through a 0.45-µm filter, mixed with 300 µg of recombinant His₆-TEV and
385 dialysed overnight in size exclusion buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM
386 TCEP], supplemented with 5% glycerol at 4 °C. Following protease cleavage, the dialysate
387 was further purified using Ni-NTA chromatography to eliminate uncut material, cleaved NusA
388 and the TEV protease. The flowthrough containing the mouse MLKL₍₁₋₁₅₈₎ construct was
389 concentrated via centrifugal ultrafiltration (10 kDa molecular weight cut-off; Millipore) and
390 loaded onto a Superdex 75 Increase 10/300 GL size exclusion column (Cytiva) equilibrated in
391 size exclusion buffer. Protein purity was assessed by SDS-PAGE (Fig. 3c). Protein that was
392 not immediately used in experiments was aliquoted, flash-frozen in liquid nitrogen and stored
393 at –80 °C.

394

395 For NMR experiments, cell pellets of [¹⁵N]-, [¹³C, ¹⁵N]- and (f-²H) [¹³C, ¹⁵N]-labelled
396 mouse MLKL₍₁₋₁₅₈₎ were resuspended in low imidazole buffer [20 mM HEPES (pH 7.5), 200
397 mM NaCl, 5% v/v glycerol, 35 mM imidazole (pH 7.5), 1 mM TCEP], supplemented with
398 Complete protease cocktail inhibitor (Roche), and lysed by sonication. The whole cell lysate
399 was clarified by centrifugation (45,000×g, 1 h, 4 °C), filtered (0.2 µM) and the supernatant was
400 loaded onto a HisTrap FF 5 ml column (Cytiva) pre-equilibrated with low imidazole buffer at
401 4 °C. After washing in low imidazole buffer, the bound protein was eluted using high imidazole
402 buffer [20 mM HEPES (pH 7.5), 200 mM NaCl, 5% v/v glycerol, 375 mM imidazole (pH 7.5),
403 1 mM TCEP]. The eluant was further purified by cleaving the NusA-His₆ tag by incubating
404 with TEV protease, dialysis overnight in size exclusion buffer [20 mM HEPES (pH 7.5), 200
405 mM NaCl, 1 mM TCEP] and a second round of HisTrap-chromatography to eliminate uncut
406 material, cleaved NusA and the TEV protease. The flowthrough was concentrated via
407 centrifugal ultrafiltration (10 kDa molecular weight cut-off; Millipore) and loaded onto a
408 Superdex 75 Increase 10/300 GL size exclusion column (Cytiva) equilibrated in NMR size
409 exclusion buffer [20 mM HEPES (pH 6.8), 100 mM NaCl, 1 mM TCEP]. Protein purity was
410 assessed by SDS-PAGE and then used fresh for each NMR experiment.

411

412 **NMR samples and NMR Spectroscopy.** NMR experiments were all performed at 25 °C on a
413 700-MHz Bruker Avance HDIII spectrometer equipped with triple resonance cryoprobe.
414 Proteins samples were prepared in a buffer containing 20 mM HEPES, 100 mM NaCl and 1
415 mM TCEP at pH 6.8 supplemented with 10% ²H₂O. Backbone resonances (¹³C α , ¹³C β , ¹³C',
416 ¹⁵N and NH) of residues were assigned from 3D HNCACB, HN(CO)CACB, HNCO and
417 HNCA experiments using non-uniform sampling (NUS). For NUS, sampling schedules were
418 generated using Poisson gap sampler with 10% of the total number of points collected for all
419 the 3D NMR experiments⁵⁶. Spectra were reconstructed with compressed sensing algorithm
420 using qMDD⁵⁷ and processed using NMRPipe⁵⁸ and data analyzed in NMRFAM-SPARKY⁵⁹.
421 The ¹H chemical shifts were referenced directly to DSS at 0 ppm and the ¹³C and ¹⁵N chemical
422 shifts were subsequently referenced using the ¹³C/¹H and ¹⁵N/¹H ratios as described
423 previously⁶⁰.

424 **NMR relaxation experiments.** Protein was used at ~180 μ M with 2.5 mM liposomes (100 nm
425 diameter). TCEP was added fresh (to 1mM) to sample prior to data collection. NMR size
426 exclusion buffer [20 mM HEPES (pH 6.8), 100 mM NaCl, 1 mM TCEP]. ¹⁵N-R₂ experiments
427 were collected with a recycle time of 2.6 s and 16 scans per FID and ¹⁵N{¹H}-NOE experiments
428 were collected with a saturation pulse of 4 s and an additional relaxation delay of 5 s and 32
429 scans per FID. ¹⁵N-R₂ relaxation delays of 16.96 ($\times 2$), 33.92, 67.84, 101.76 ($\times 2$), 135.68, 169.6
430 ($\times 2$), 203.52 and 237.44 ms were used. The repeated spectra were used to estimate instrumental
431 error. ¹⁵N relaxation parameters were determined using the program *relax* (version 3.3.4)⁶¹. For
432 R₂ rate constants, errors were estimated using 500 Monte Carlo Simulations. The steady state
433 ¹⁵N{¹H}-NOE values for mouse MLKL₍₁₋₁₅₈₎ were estimated from the ratios of peak intensities
434 obtained from spectra acquired with and without proton saturation using *relax*. Errors for
435 ¹⁵N{¹H}-NOE experiment were calculated based on noise level in the spectrum.
436

437 **Reagents and antibodies.** Primary antibodies used in this study for immunoblotting were: rat
438 anti-mouse MLKL (WEHI clone 5A6; produced in-house and soon available from Millipore
439 as MABC1634; 1:2000)⁴⁹; rabbit anti-phospho-S345 mouse MLKL (Cell Signaling
440 Technology; clone D6E3G; 1:2000); rat anti-mouse RIPK3 (WEHI clone 8G7; produced in-
441 house⁷ and soon available from Millipore as MABC1595; 1:2000); rabbit anti-phospho-
442 T231/S232 mouse RIPK3 (Genentech; clone GEN135-35-9⁶²; lot PUR73907; 1:2000); rabbit
443 anti-mouse or human RIPK1 (Cell Signaling Technology; clone D94C12; 1:2000); and mouse
444 anti-Actin (A1978, Sigma-Aldrich, St Louis, MO, USA; 1:5000). Secondary antibodies used

445 in this study were: horseradish peroxidase (HRP)-conjugated goat anti-rat IgG (Southern
446 Biotech 3010-05), HRP-conjugated goat anti-mouse IgG (Southern Biotech 1010-05), and
447 HRP-conjugated goat anti-rabbit IgG (Southern Biotech 4010-05). All secondary antibodies
448 were used at a dilution of 1:10000. Recombinant hTNF-Fc, produced in-house, and the Smac-
449 mimetic, Compound A, have been previously described^{63, 64}. The pan-caspase inhibitor, IDN-
450 6556/emricasan, was provided by Tetralogic Pharmaceuticals.

451
452 **Cell culture.** *Mlkl*^{-/-} MDF cells were cultured in Dulbecco's Modified Eagle Medium (DMEM;
453 Gibco) supplemented with 8% (v/v) Fetal Calf Serum (FCS; Sigma), penicillin (100 U mL⁻¹),
454 streptomycin (100 µg mL⁻¹). Puromycin (2.5 µg mL⁻¹; StemCell Technologies) was added for
455 lines stably transduced with inducible mouse MLKL constructs. Routine testing confirmed cell
456 lines to be mycoplasma-negative.

457
458 **IncuCyte cell death assays.** *Mlkl*^{-/-} MDF cells were seeded into 96-well plates at 8 x10⁴
459 cells/well and left to adhere for 4-5 h prior to treatment with doxycycline (20 ng mL⁻¹)
460 overnight to induce expression of the relevant full-length mouse MLKL constructs. Cells were
461 then treated with necroptotic stimulus comprising, TNF (100 ng mL⁻¹), the Smac-mimetic
462 compound A (500 nM) and the pan-caspase inhibitor IDN-6556 (10 µM) (TSI) to induce
463 necroptosis in FluoroBrite DMEM media (ThermoFisher Scientific) supplemented with 1%
464 FCS, 1 mM Na pyruvate (ThermoFisher Scientific), 1 mM L-GlutaMAX (ThermoFisher
465 Scientific), SYTOX Green nucleic acid stain (ThermoFisher Scientific, 1:20000) and SPY620
466 live cell DNA stain (Spirochrome, 1:1000). Cells were then imaged using the IncuCyte S3
467 System (Essen Bioscience) with default bright-field, red and green channel settings on 10x
468 objective. Scans were obtained every 30 min for 5 h, where percent cell death was quantified
469 based upon the number of SYTOX Green-positive cells per image over the number of SPY620-
470 positive cells per image using IncuCyte S3 v2018A software (Essen Bioscience). Data were
471 plotted as mean ± SEM from three biologically independent *Mlkl*^{-/-} MDF cell lines (*n* = 6 to 9).

472
473 **Immunoblot.** *Mlkl*^{-/-} MDF cells were seeded into 24-well plates at 7 x10⁴ cells/well and left to
474 adhere for 4-5 h. Cells were induced overnight with doxycycline (20 ng mL⁻¹) and then treated
475 with TNF (100 ng mL⁻¹), Smac-mimetic (Compound A; 500 nM) and pan-caspase inhibitor,
476 IDN-6556 (5 µM) (TSI) for 1.5 or 3.0 h. Cells were lysed in ice-cold RIPA buffer [10 mM Tris-
477 HCl pH 8.0, 1 mM EGTA, 2 mM MgCl₂, 0.5% v/v Triton X100, 0.1% w/v Na deoxycholate,

478 0.5% w/v SDS and 90 mM NaCl] supplemented with 1x Protease & Phosphatase Inhibitor
479 Cocktail (Roche) and 100 U/mL Denarase (c-LEcta). Whole-cell lysates were boiled at 100 °C
480 for 10-15 min in 1 × SDS Laemmli lysis buffer (126 mM Tris-HCl, pH 8, 20% v/v glycerol,
481 4% w/v SDS, 0.02% w/v bromophenol blue, 5% v/v 2-mercaptoethanol), and then resolved by
482 4 to 15% Tris-Glycine gel (Bio-Rad). Proteins were transferred to PVDF membrane, blocked
483 with 5% w/v skim milk powder in TBST and then probed overnight with primary antibodies
484 (as per *Reagents and antibodies* above). The signals were revealed by enhanced
485 chemiluminescence on a ChemiDoc Touch Imaging System (BioRad) using an appropriate
486 HRP-conjugated secondary antibody (as per *Reagents and antibodies* above). Before probing
487 different proteins with primary antibody, membranes were incubated in mild stripping buffer
488 [200 mM glycine pH 2.9, 1% w/v SDS, 0.5 mM TCEP] for 30 min at room temperature then
489 re-blocked.

490

491 **Lactate Dehydrogenase (LDH) release.** Colorimetric LDH release assay kit (Promega G1780)
492 was performed according to manufacturer's instructions. Data are plotted as mean ± SD of
493 three independent replicates.

494

495 **Liposome preparation.** Large Unilamellar Vesicles (LUVs) were prepared using a plasma
496 membrane-like lipid mix ([Supplementary Table 4](#)) and resuspended in chloroform as a 20 mg
497 mL⁻¹ (~25 mM for most lipids) stock as previously reported^{29,37}. Dried lipids were resuspended
498 in either 500 µL of LUV buffer [10 mM HEPES pH 7.5, 135 mM KCl] with 50 mM 5(6)-
499 Carboxyfluorescein dye (Sigma) to form dye filled liposomes for permeabilization assays or
500 NMR size exclusion buffer [20 mM HEPES (pH 6.8), 100 mM NaCl, 1 mM TCEP] for NMR
501 studies. The mixture was then freeze-thawed at least 5×, by immersion in liquid nitrogen until
502 fully frozen, followed by immersion in a 37 °C water bath until contents had thawed. The lipid
503 mixture was extruded through polycarbonate membranes of 100 nm size cut-off (Avanti Polar
504 Lipids, AL, USA), a minimum of 21 times to form liposomes, using a pre-warmed mini
505 extruder (Avanti Polar Lipids, AL, USA). Liposomes stocks were at approximately 2.5 mM
506 lipid concentration and were stored at 4 °C in the dark.

507

508 **Liposome dye release assays.** Recombinant mouse MLKL₍₁₋₁₅₈₎ protein was diluted to 16 µM
509 (2× desired final concentration) in LUV buffer, and 50 µL aliquoted into adjacent wells of a
510 96 well Flat-bottom plate (ThermoFisher Scientific). Prior to use, the liposomes (100 nm
511 diameter filled with 5(6)-Carboxyfluorescein dye) were purified from excess dye using a PD-

512 10 desalting column (Cytiva) and diluted to 20 μ M in LUV buffer. At the plate reader (Hidex
513 Chameleon Multilabel Microplate Reader; Lab Logic), the protocol was pre-programmed,
514 before 50 μ L of liposomes was promptly added to each well of the 96 well plate using a multi-
515 channel pipette. The plate was then immediately placed in the plate reader and measurements
516 started. Fluorescence was measured every 2 min for 60 minutes (31 measurements) at 20 °C
517 with excitation wavelength of 485 nm and emission wavelength of 535 nm. 100% dye release
518 was determined by the incubation of liposomes with 50 μ L of 1% CHAPS detergent in LUV
519 buffer, while a baseline was determined by the incubation of liposomes with 50 μ L of LUV
520 buffer alone. All assays were performed in triplicate. Data were plotted as mean \pm SEM of
521 three independent assays and data is presented as a percentage of maximum dye release.

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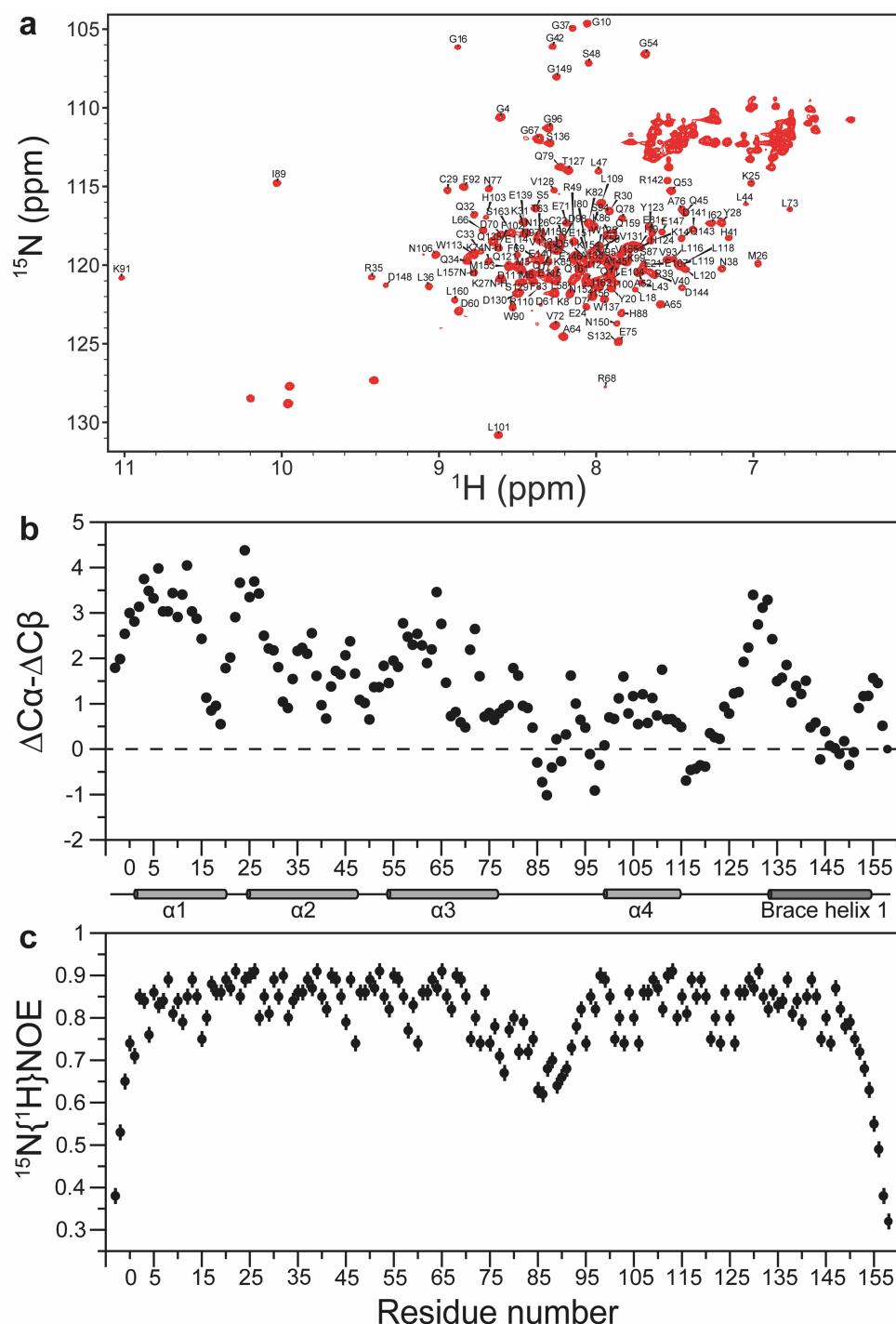
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771 **FIGURE LEGENDS**

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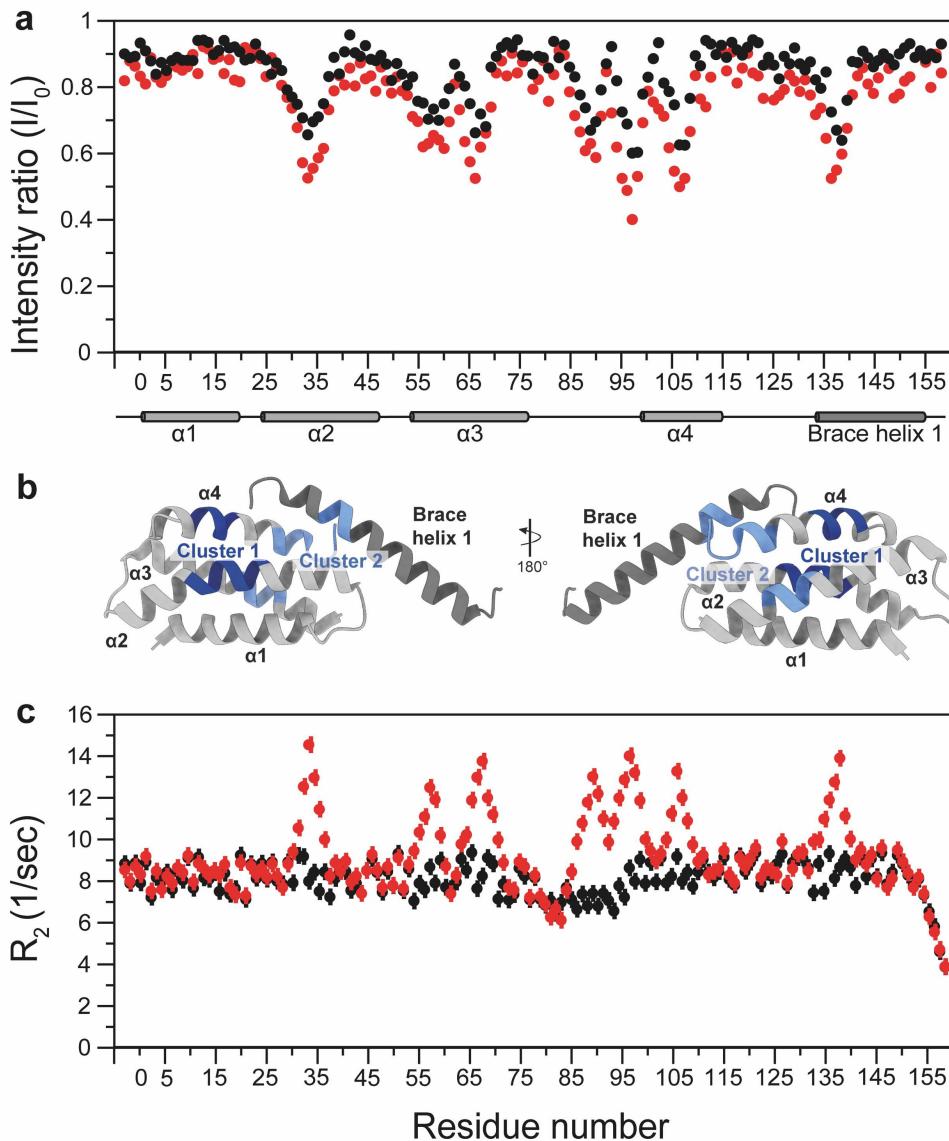


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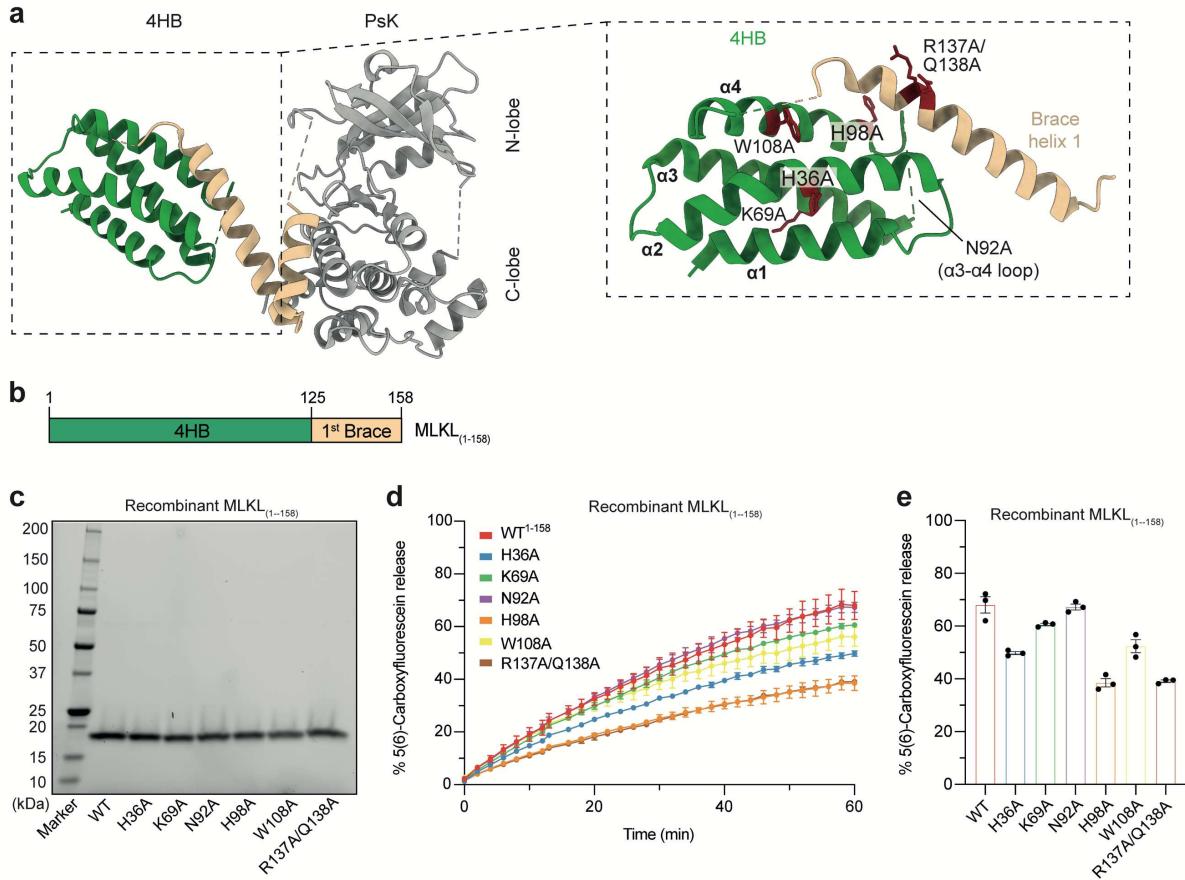
774 **Figure 1 | Mouse MLKL adopts a folded helical structure. a)** Full ^{1}H , ^{15}N HSQC spectrum
775 of mouse MLKL₍₁₋₁₅₈₎ with residue assignments. Unmarked resonances in the upper right
776 corner belong to Asn and Gln sidechains and in the lower left corner belong to Trp-indole
777 sidechains have not been assigned. The residues, GAMGS, are a cloning artifact and numbered
778 -4 to 0, while residues M1-S158 are from mouse MLKL. **b)** Plots of $^{13}\text{C}\alpha\beta$ secondary chemical

779 shifts and **c)** Steady state $^{15}\text{N}\{^1\text{H}\}$ -NOEs for mouse MLKL₍₁₋₁₅₈₎ with error bars calculated
780 based on average estimated noise level for $^{15}\text{N}\{^1\text{H}\}$ -NOE. Experiments were conducted at pH
781 6.8 and 25 °C. The helical secondary structure shown between panels **(b)** and **(c)** reflects that
782 from the full-length mouse MLKL crystal structure (PDB, 4BTF)²³.

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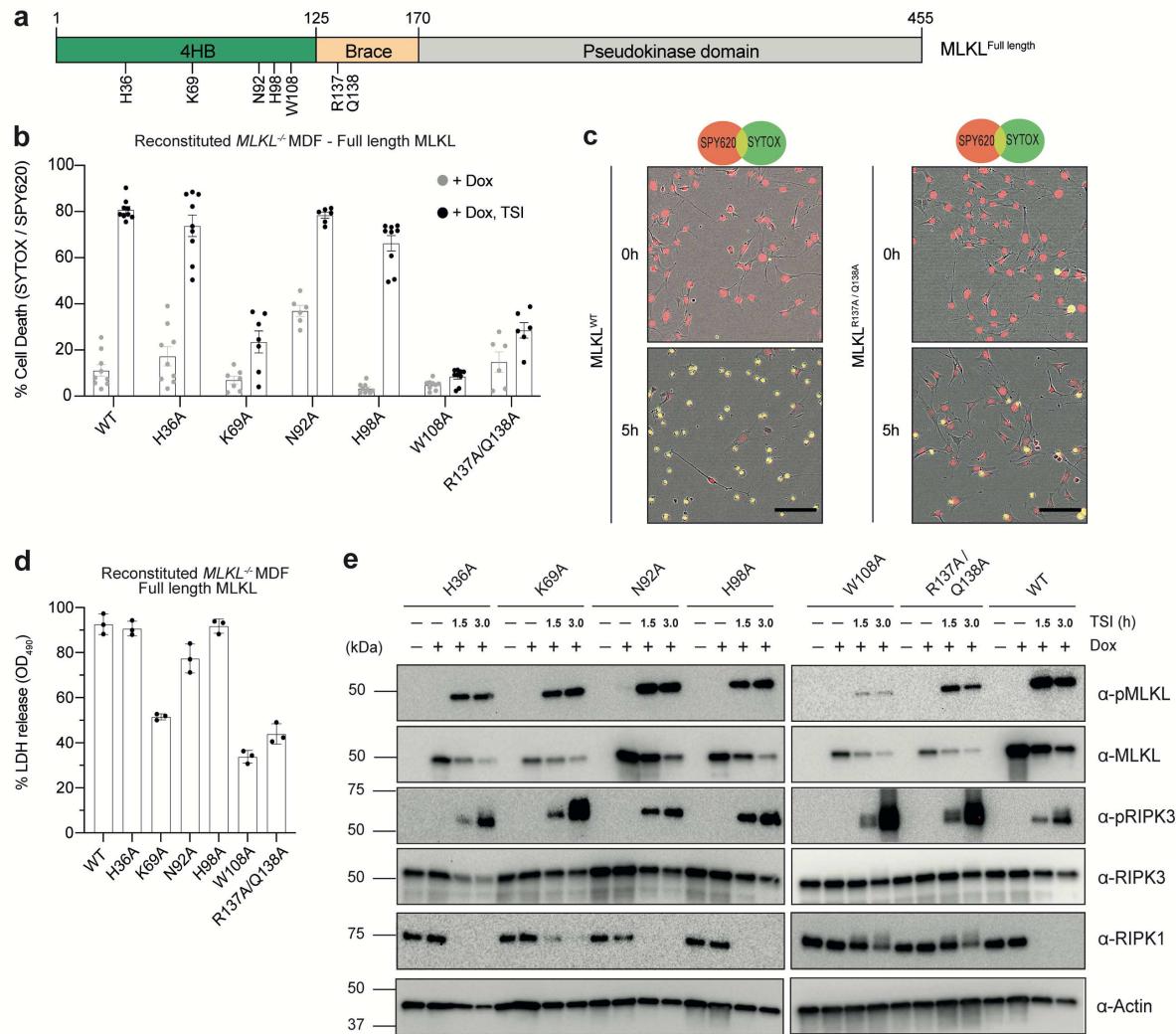
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785 **Figure 2 | Two clusters of residues in mouse MLKL₍₁₋₁₅₈₎ mediate lipid binding.** Mouse
786 MLKL₍₁₋₁₅₈₎ binds to liposomes shown by titrating 100 μM ^{15}N -labelled mouse MLKL₍₁₋₁₅₈₎
787 with liposomes in the 1:0.5 (black circles) and 1:1 (red circles) ratio. **a**) Plot of change in ^1H N
788 and ^{15}N peak intensity, where I is the intensity of the peak in the presence of liposome and I_0
789 in the absence. The helical secondary structure shown between panels **(a)** and **(b)** reflects that
790 from the full-length mouse MLKL crystal structure (PDB, 4BTF)²³. **b**) Clusters I (light blue)
791 and II (dark blue) of residues in mouse MLKL₍₁₋₁₅₈₎ that exhibited marked attenuation of peak
792 intensity are mapped onto the mouse MLKL crystal structure (PDB, 4BTF). **c**) Backbone ^{15}N
793 transverse relaxation rates (R_2) measured at ^{15}N frequency of 70.9 MHz, with liposomes in the
794 1:0.5 (black circles) and 1:1 (red circles) ratio. Error bars were calculated using Monte Carlo
795 Simulations for R_2 measurement. Experiments in **(a)** and **(c)** were conducted at pH 6.8 and 25
796 °C.



797

798 **Figure 3 | Liposome permeabilization assays validate lipid-interacting residues.** **a)** Full-
799 length mouse MLKL crystal structure (PDB, 4BTF)²³, comprising a 4HB (green), two-brace
800 helices (beige) and a bilobal pseudokinase (PsK) domain (grey). The surface-exposed residues
801 implicated to interact with lipids using NMR spectroscopy are shown as sticks and coloured
802 red (inset). Ala substitutions were introduced to each of these residues in MLKL₍₁₋₁₅₈₎. **b)**
803 Architecture of mouse MLKL₍₁₋₁₅₈₎. **c)** Reducing SDS-PAGE of all mouse MLKL₍₁₋₁₅₈₎ purified
804 constructs. Each purified recombinant protein construct was resolved by reducing SDS-PAGE
805 to assess purity. **d)** Liposome dye release assay using recombinant wild-type and mutant mouse
806 MLKL₍₁₋₁₅₈₎ at 8 μ M. Release of 5(6)-Carboxyfluorescein was monitored at 485 nm over 60
807 min. **e)** Evaluation of total dye release from wild-type and alanine substitution mutants of
808 mouse MLKL₍₁₋₁₅₈₎. Data in (d-e) represent mean \pm SEM of three independent assays.

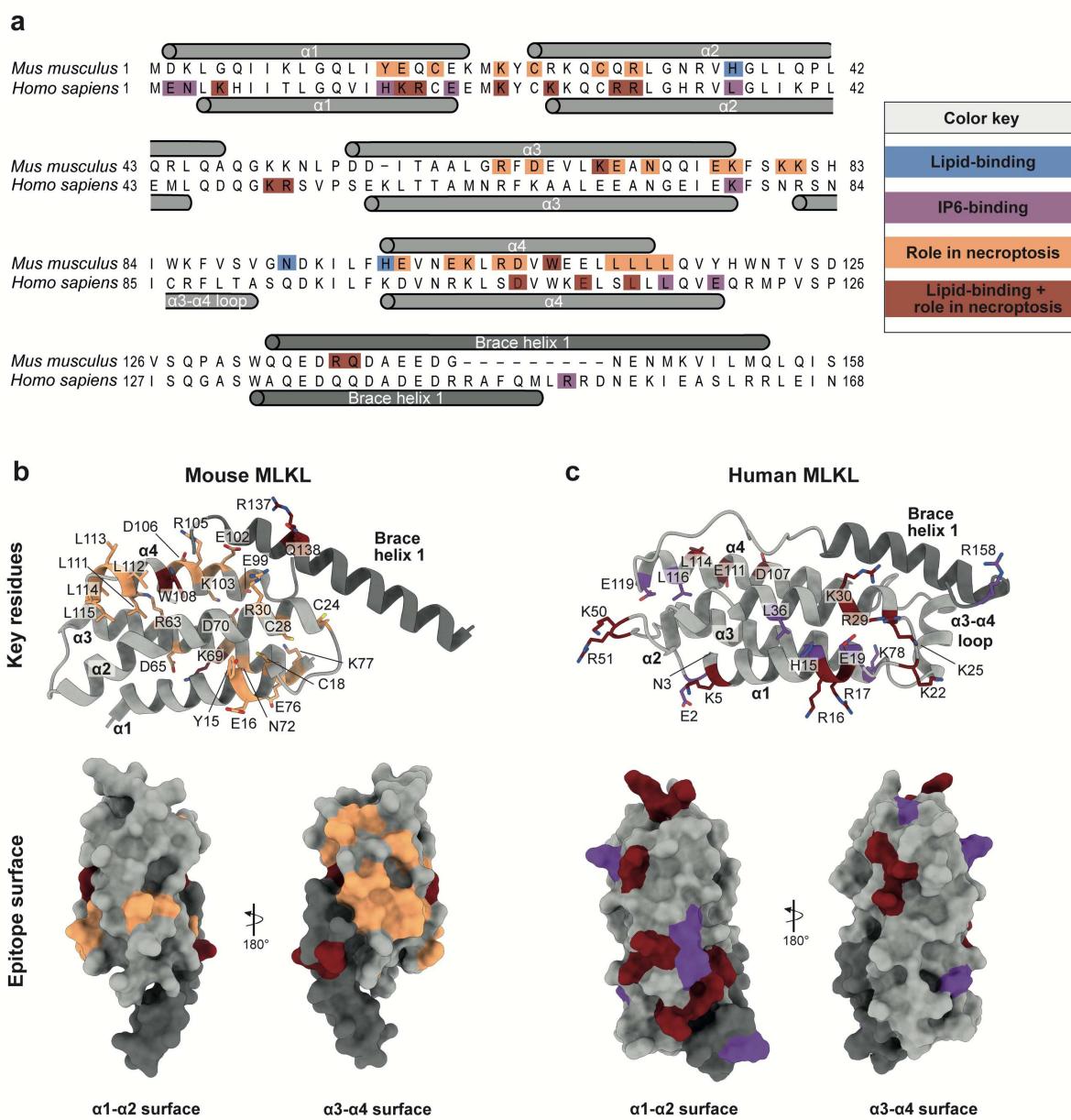
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811 **Figure 4 | Mutation of lipid-binding residues in the 4HB and in the first brace compromise
812 necroptotic signaling. a)** Architecture of full-length mouse MLKL, with each Ala substitution
813 highlighted. **b)** Evaluation of necroptotic signaling for wild-type (WT) and alanine substitution
814 mutants of full-length mouse MLKL in MDF *Mlk1*^{-/-} cells. WT or mutant mouse MLKL
815 expression was induced with doxycycline (Dox) and cell death was quantified using IncuCyte
816 S3 live cell imaging in the presence or absence of the necroptotic stimulus, TNF and Smac-
817 mimetic Compound A and pan-caspase inhibitor, IDN-6556, (TSI) for 5 h, by determining the
818 number SYTOX Green-positive cells (dead cells) relative to the number of SPY620-positive
819 cells (total cell confluency). Data represent mean \pm SEM from three biologically independent
820 MDF *Mlk1*^{-/-} cell lines ($n = 6$ to 9). **c)** Time-lapse micrographs of SPY620 uptake (SPY; red)
821 and SYTOX Green uptake (SYTOX; green) as indices of total cell confluency and cell death,
822 respectively. Both 0 and 5 h timepoints are shown post Dox induction and TSI treatment.
823 Micrographs are representative of $n = 6$ -9 independent experiments using IncuCyte S3 live cell
824 imaging. Scale bar (black) represents 100 μ m. **d)** Following induction with doxycycline and 3

825 h of TSI treatment, the extracellular release of lactate dehydrogenase (LDH) as an index of
826 plasma membrane lysis (relative to detergent-treated cells) was measured by
827 spectrophotometry at 490 nm. Data represent mean \pm SD of three independent experiments. e)
828 *Mlkl*^{-/-} MDF cells expressing WT and mutant full-length mouse MLKL following Dox
829 induction were treated with the necroptotic stimulus, TSI, for 1.5 or 3 h. Whole cell-lysates
830 were then fractioned by SDS-PAGE and probed by immunoblot for RIPK1, RIPK3, pRIPK3,
831 MLKL and pMLKL with anti-actin as a loading control. Immunoblots are representative of
832 $n = 2$ independent experiments.

833



835 **Figure 5 | Membrane permeabilization is mediated by distinct epitopes in mouse and**
836 **human MLKL. a)** Sequence alignment of MLKL four-helix-bundle (4HB) + first brace helix
837 from mouse and human MLKL. Secondary structure from experimental structures of mouse²³
838 and human⁴³ MLKL 4HB + brace helices are annotated above and below the sequences,
839 respectively. Residues in blue have been validated to bind lipids; residues in purple have been
840 validated to bind inositol hexaphosphate (IP6); residues in orange when mutated to alanine
841 exhibited deficits in cellular necroptotic signaling; and mutation of lipid-binding residues in
842 dark red exhibited deficits in cellular necroptotic signaling. **b)** Cartoon representation of mouse
843 MLKL 4HB (grey) + first brace (dark grey, Top panel) (PDB, 4BTF)²³. Key residues (orange)
844 and lipid-binding residues (dark red) that exhibit deficits in cellular necroptotic signaling are
845 shown as sticks. The lower panel shows a representation of the mouse MLKL α1-α2 helix and

846 α 3- α 4 helix molecular surface, where each residue is color-coded as above. **c)** Cartoon
847 representation of human MLKL 4HB (grey) + first brace (dark grey, Top panel) (PDB,
848 2MSV)⁴³. Key IP6-binding residues (purple) and lipid-binding residues (dark red) that exhibit
849 deficits in cellular necroptotic signaling are shown as sticks. The lower panel shows a
850 representation of the human MLKL α 1- α 2 helix and α 3- α 4 helix molecular surface, where
851 each residue is color-coded as above. Between the surface representations in **(b)** and **(c)**, the
852 different lipid-binding epitope for mouse and human MLKL can be observed.

853