1　**A toolbox for imaging RIPK1, RIPK3 and MLKL in mouse and human cells**

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13　Running title: Imaging necroptosis in mouse and human cells

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15　Keywords: kinase, pseudokinase, cell death, programmed necrosis, fixative

## ABSTRACT

Necroptosis is a lytic, inflammatory cell death pathway that is dysregulated in many human pathologies. The pathway is executed by a core machinery comprising the RIPK1 and RIPK3 kinases, which assemble into necrosomes in the cytoplasm, and the terminal effector pseudokinase, MLKL. RIPK3-mediated phosphorylation of MLKL induces oligomerization and translocation to the plasma membrane where MLKL accumulates as hotspots and perturbs the lipid bilayer to cause death. The precise choreography of events in the pathway, where they occur within cells, and pathway differences between species, are of immense interest. However, they have been poorly characterized due to a dearth of validated antibodies for microscopy studies. Here, we describe a toolbox of antibodies for immunofluorescent detection of the core necroptosis effectors, RIPK1, RIPK3 and MLKL, and their phosphorylated forms, in human and mouse cells. By comparing reactivity with endogenous proteins in wild-type cells and knockout controls in basal and necroptosis-inducing conditions, we characterise the specificity of frequently-used commercial and recently-developed antibodies for detection of necroptosis signaling events. Importantly, our findings demonstrate that not all frequently-used antibodies are suitable for monitoring necroptosis by immunofluorescence microscopy, and methanol- is preferable to paraformaldehyde-fixation for robust detection of specific RIPK1, RIPK3 and MLKL signals.

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## INTRODUCTION

Cell death by necroptosis is thought to have originated as an ancestral host defence mechanism, which is reflected in the breadth of pathogen-encoded proteins that inhibit the pathway[1, 2, 3, 4, 5, 6]. In addition to reported innate immunity functions[7, 8, 9], the dysregulation of necroptosis has been implicated in a range of pathologies, including ischemic-reperfusion injuries, such as in the kidney[10, 11] and heart[12], and inflammatory diseases[13, 14, 15, 16], including inflammatory bowel disease[17]. Accordingly, there is widespread interest in therapeutically-targeting the pathway to counter human disease. Owing to the recent identification of the terminal effectors of the pathway, RIPK3 (in 2009)[1, 18, 19] and MLKL (in 2012)[20, 21], however, the extent of indications attributable to necroptotic cell death is poorly understood. Precisely defining pathologies impacted by necroptosis has posed a challenge owing to the dearth of antibodies validated to specifically detect members of the pathway and their activated (phosphorylated) forms in fixed cells and tissues. As a result, the contribution of necroptosis to many pathologies remains a subject of ongoing debate[22, 23, 24, 25].

Necroptotic cell death signaling is initiated by ligation of death receptors, such as the TNF receptor 1, or pathogen detectors, such as Toll-like receptors 3 and 4 and the ZBP1/DAI intracellular viral RNA sensor protein. In cellular contexts where the activities of the Inhibitors of Apoptosis proteins (IAPs) E3 Ubiquitin ligase family and the proteolytic apoptotic effector, Caspase-8, are depleted or compromised, necroptosis ensues. The precise choreography of necroptotic signaling is still emerging, although recent studies have defined key events and checkpoints in the pathway[26, 27, 28, 29]. Following pathway induction, RIPK1 autophosphorylation prompts hetero-oligomerization with RIPK3 via an amyloid-forming motif in the region C-terminal to their kinase domains termed the RHIM (RIP Homotypic Interaction Motif) to form a cytoplasmic platform known as the necrosome[30, 31, 32]. Upon RIPK3 activation by autophosphorylation within necrosomes[33, 34], RIPK3 is primed to phosphorylate the activation loop of the MLKL pseudokinase domain to activate MLKL's killing function[14, 20, 27, 28, 35, 36, 37, 38, 39]. In the case of human MLKL, dormant MLKL appears to be at least in

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58    part pre-associated with RIPK3 in the cytoplasm[20, 40], and stable recruitment to necrosomes appears

59    to be an essential checkpoint in MLKL activation[28]. In the case of mouse MLKL, a transient

60    interaction between RIPK3 and MLKL appears to be sufficient for MLKL activation in mouse cells[35,

61    36, 37]. Regardless of species, MLKL phosphorylation is thought to provoke a conformational change

62    in the pseudokinase domain that leads to oligomerization and unmasking of the killer N-terminal

63    four-helix bundle (4HB) domain[37, 40, 41, 42, 43]. The human MLKL 4HB domain likely engages

64    chaperones to facilitate translocation to the plasma membrane via an actin-, Golgi- and microtubule-

65    dependent mechanism, where MLKL accumulates in hotpots. When a threshold is surpassed, the 4HB

66    domains of MLKL permeabilize the membrane to induce cell death[26, 29].

67         While biochemical studies have defined these steps and checkpoints, visualizing the

68    spatiotemporal dynamics of endogenous proteins during in necroptosis using microscopy-based

69    approaches has proven challenging in the absence of antibodies that have been validated for target

70    specificity. Similarly, the lack of validated reagents poses a challenge to immunohistochemical

71    staining of patient tissue sections, and therefore attribution of a role for necroptosis in pathologies,

72    because knockout tissue controls are not available. Here, we have established procedures for staining

73    endogenous RIPK1, RIPK3 and MLKL, and their phosphorylated forms, in fixed mouse and human

74    cells. While several frequently-used antibodies were found to be suitable for selectively staining these

75    proteins, as validated by comparisons with cells deficient for each protein, many exhibited non-

76    specific staining and are therefore unsuitable for immunofluorescence and immunohistochemistry.

77    Our studies also highlight the importance of validating antibody compatibility with fixation methods.

78    In most cases, paraformaldehyde fixation ablated epitopes, presumably because RIPK3 and MLKL

79    are lysine-rich and prone to modification by crosslinking, whereas methanol fixation enabled specific

80    detection of these proteins. Due to the sequence divergence between mouse and human RIPK3 and

81    MLKL[41, 43, 44, 45, 46], it was not possible to specifically-detect proteins across species using a single

82    reagent, which necessitated the development of a new antibody that specifically detected mouse

83   MLKL. Collectively, our studies present a toolbox of selective antibodies that will enable critical

84   analysis of the chronology, checkpoints and kinetics of necroptotic signaling in mouse and human

85   cells.

86

87   **RESULTS**

88   **Immunofluorescent detection of human MLKL**

89   We recently described a monoclonal antibody, clone 10C2 (source: WEHI Antibody Facility, in-

90   house), that recognises an epitope centred on residues 413-471 of human MLKL[29] (Fig. 1a). The

91   initial screen that identified this clone suggested it yielded specific immunosignals when cells were

92   fixed with methanol, but not when cells were fixed with paraformaldehyde. Organic solvents such as

93   methanol preserve cells by precipitating proteins, whereas aldehyde-based agents such as

94   paraformaldehyde fix cells by crosslinking lysines and other primary amines. Because human MLKL

95   is lysine-rich (93rd percentile in the cytoplasmic and membrane-associated proteome),

96   paraformaldehyde likely causes widespread crosslinking of MLKL, which in turn masks epitopes and

97   reduces immunoreactivity. As several other necroptotic proteins are lysine-rich, we considered

98   whether the choice of fixative was a critical variable for robust immunodetection of MLKL, RIPK3

99   and RIPK1 in human and mouse cells. Accordingly, we compared the performance of 22 antibodies

100   for immunofluorescent staining of human HT29 cells and mouse dermal fibroblasts (MDFs) - two

101   cellular models that are well-characterized to undergo necroptosis when treated with TNF, Smac-

102   mimetic and IDN-6556 (herein referred to as TSI)[5, 28, 29, 44, 47]. To quantitatively gauge the

103   performance of each antibody, their immunosignals were characterized in four ways: 1) A ratio of

104   the immunofluorescent signals between a positive and negative control. This produces a signal-to-

105   noise curve that estimates 'specific signal abundance' (Fig. 1b). For testing phospho-specific

106   antibodies, wild-type cells undergoing necroptosis were used as a positive control and untreated wild-

107   type cells as a negative control (Fig. S1). For testing all other antibodies, untreated wild-type cells

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108      were used as a positive control and relevant knockout cells as a negative control. 2) Micrographs

109      were gated so that the minimum/maximum immunosignals corresponded to the $5^{th}/95^{th}$ percentiles of

110      the signal-to-noise curve (Fig. 1b). 3) The percentage of total signals that fall within this gate were

111      determined to show how much signal from the positive control was considered specific (Fig. 1c). 4)

112      Antibodies were immunoblotted against positive and negative controls to independently assess their

113      specificity.

114      Using this approach, we confirmed that clone 10C2 specifically detects endogenous human

115      MLKL in methanol-fixed, but not paraformaldehyde-fixed cells (Fig. 1d-f). The 10C2 clone produces

116      a favourable immunostaining profile, where the specific signals are abundant (Fig. 1e) and these

117      specific signals represent the majority of all detectable signals (Fig.1d). We recently developed

118      another MLKL-specific antibody, clone 7G2 (source: in-house), that recognises an epitope centred

119      on αF-αG loop the C-lobe of the human MLKL pseudokinase domain, which differs to the site

120      recognised by the 10C2 clone[29]. Despite binding a distinct site in human MLKL to 10C2, the 7G2

121      clone shows similar specificity for human MLKL in immunoblot analyses and only yields specific

122      immunofluorescent signals on methanol-fixed cells (Fig. 1d-f). Notably, clone 7G2 is inferior to clone

123      10C2 for immunofluorescence studies, because specific signals were less abundant (Fig. 1e) and were

124      a minor fraction of all detectable signals (Fig.1d).

125      We observed that clone EPR9514 (source: Abcam), an antibody raised against phospho-S358

126      of human MLKL[48], which is a hallmark of MLKL activation during necroptosis[20], produced specific

127      and comparable immunofluorescent signals in both methanol- and paraformaldehyde-fixed cells

128      undergoing necroptosis (Fig. 1e). As MLKL can be disulfide-crosslinked during necroptosis[49, 50] (Fig.

129      S2A-B), we tested whether fixation in the presence of N-ethylmaleimide (NEM) to prevent further

130      disulfide bonding, or post-fixative treatment with Tris(2-carboxyethyl)phosphine (TCEP) to reduce

131      disulfide bonds, altered the immunofluorescent detection of phospho-MLKL by clone EPR9514.

132      However, neither preventing nor reducing disulfide bonds in fixed cells precluded the

133    immunodetection of MLKL by clone EPR9514 (Fig. S2C-D). Indeed, disulfide-crosslinking may be

134    a by-product of MLKL activation given that recombinant human MLKL oligomerizes in the absence

135    of disulfide bonds (Fig. S2E).

136        Two other anti-human MLKL antibodies were found to be non-specific (Novus Biological

137    clone 954702; and Sigma-Aldrich M6697, a polyclonal antibody raised against residues 58-70; Fig.

138    1a and 1e), with equivalent signal intensity and diffuse staining observed in wild-type and $MLKL^{-/-}$

139    cells. Lastly, immunoblotting confirmed that clones 10C2, 7G2 (both in-house) and EPR9514

140    (Abcam) were highly-specific with bands corresponding to human MLKL's molecular weight of

141    54kDa observed in wild-type but not $MLKL^{-/-}$ HT29 cell lysates. In contrast, clone 954702 (Novus

142    Biological MAB9187) and the Sigma-Aldrich M6697 polyclonal antibody were non-specific, with

143    multiple bands not corresponding to the molecular weight of MLKL observed in both wild-type and

144    $MLKL^{-/-}$ HT29 cell lysates (Fig. 1g).

145        In summary, three of the five antibodies that were tested selectively recognised human MLKL

146    in methanol-fixed cells (clones 10C2, 7G2 and EPR9514) and, accordingly, we recommend methanol

147    fixation for detecting endogenous human MLKL by immunofluorescence. Under these conditions,

148    cytoplasmic forms of MLKL could be detected using the 10C2 and 7G2 clones, and phospho-MLKL

149    accumulating at the cell periphery with the EPR9514 clone (Fig. 1f). These data underscore the

150    importance of careful consideration of fixation method when using MLKL as a histological marker

151    of necroptosis.

152

153    **Immunofluorescent detection of human RIPK3**

154    We next assessed five anti-human RIPK3 antibodies (Fig. 2a). Two antibodies raised against the C-

155    terminus (source: ProSci 2283, Novus Biological NBP2-24588) and one antibody raised against the

156    N-terminus of RIPK3 (source: Novus Biological MAB7604, clone 780115) yielded non-specific

157    signals via both immunofluorescence and immunoblotting (Fig. 2b-e). Our recently described

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158   antibody, clone 1H2 (source: in-house)[5], detected RIPK3 via immunofluorescence in methanol-fixed,

159   but not paraformaldehyde-fixed cells. However, most of the immunofluorescent signals produced by

160   1H2 were non-specific (Fig. 2b), and the remaining signals are specific but of very low abundance

161   (Fig. 2c). Thus, although clone 1H2 is highly-selective when used as an immunoblotting reagent (Fig.

162   2d[5]), it should only be used for the immunofluorescent detection of human RIPK3 under carefully

163   controlled conditions. Our data also showed that clone D6W2T (source: Cell Signaling Technology),

164   an antibody raised against a mark of human RIPK3 activation in necroptosis, phospho-S227,

165   produced specific immunofluorescent signals in paraformaldehyde-fixed cells undergoing

166   necroptosis, but not in methanol-fixed cells (Fig. 2d). Again, the low abundance of these specific

167   immunosignals suggests that clone D6W2T should only be used for immunofluorescence under

168   carefully controlled conditions, such as where $RIPK3^{-/-}$ control cells are available for direct

169   comparison. Another caveat is that immunoblotting showed that clone D6W2T not only detected

170   RIPK3 in cells undergoing necroptosis, but also in untreated cells when RIPK3 was presumably not

171   phosphorylated at S227 (Fig. 2d). Therefore, while two of the five antibodies that were tested

172   specifically recognised human RIPK3, the low abundance of their specific signals complicates use of

173   these antibodies for studying endogenous human RIPK3 via immunofluorescence. At present, more

174   specific antibodies are needed to enable the immunofluorescent detection of human RIPK3.

175

176   **Immunofluorescent detection of human RIPK1**

177   We next tested three monoclonal anti-RIPK1 antibodies: one against the N-terminal domain (source:

178   Cell Signaling Technology clone D94C12), one against the C-terminal region (source: BD

179   Transduction Laboratories clone 38/RIP) and one raised against phospho-S166 (source: Cell

180   Signaling Technology clone D8I3A; Fig. 3a). All three clones yielded specific immunofluorescent

181   signals in methanol- and paraformaldehyde-fixed cells, however, these signals were more abundant

182   in methanol-fixed samples (Fig. 3b-d). All three clones were also specific via immunoblot (Fig. 3e).

183   Importantly, the immunosignals from clones D94C12 and 38/RIP were markedly lower in cells

184   undergoing necroptosis than in unstimulated wild-type cells (Fig. 3d-e). This observation is likely

185   due to RIPK1 undergoing widespread post-translational modification and/or proteasomal degradation

186   during TNF-induced cell death[51, 52]. Thus, despite clones D94C12 and 38/RIP exhibiting a favourable

187   immunostaining profile in unstimulated cells, their decreased reactivity in cells undergoing

188   necroptosis indicates that they should be used judiciously for immunofluorescent labelling of samples

189   for accurate analysis of necroptosis. Conversely, while clone D813A preferentially stains RIPK1 in

190   cells undergoing necroptosis, specific signals that were detected also had low intensity (Fig. 3b) and

191   low abundance (Fig. 3c). In summary, three highly-specific antibodies exist for human RIPK1.

192   However, because their specific signals have very low intensity and abundance in necroptotic cells,

193   their use for the immunofluorescent detection of RIPK1 during necroptosis should be carefully

194   controlled, including side-by-side examination of wild-type and *RIPK1*^{-/-} control cells.

195

196   **Immunofluorescent detection of mouse MLKL**

197   To our knowledge, there are currently no validated monoclonal antibodies for the immunofluorescent

198   detection for mouse MLKL in unstimulated cells. Accordingly, we raised a monoclonal antibody,

199   clone 5A6 (source: in-house), against full-length mouse MLKL (Fig. 4a). The epitope for clone 5A6

200   resides in the C-terminal domain of mouse MLKL (Fig. S3). As shown in Fig. 4a-d, clone 5A6

201   produced specific and abundant signals for endogenous mouse MLKL in methanol-fixed, but not

202   paraformaldehyde-fixed cells. Immunoblotting confirmed that clone 5A6 was highly-specific (Fig.

203   4e). Notably, compared to human MLKL which translocates en masse into cytoplasmic clusters

204   during necroptosis, the relocation of mouse MLKL into cytoplasmic clusters during necroptosis was

205   more subtle. This observation may relate to the notion that human MLKL is recruited to the

206   necrosome in a more stable manner than mouse MLKL[28].

207    Phosphorylation of mouse MLKL at S345 is essential for necroptosis in murine cells[37], and

208    therefore many antibodies have been raised against this phosphosite. We tested three commonly-used

209    antibodies (Fig. 4a-e), namely clones: EPR9515(2) (source: Abcam), 7C6.1[39] (source: Millipore

210    MABC1158) and D6E3G (source: Cell Signaling Technology). All three clones yielded specific

211    immunosignals in methanol-fixed cells (Fig. 4c), whereas only clone 7C6.1 was compatible with

212    paraformaldehyde fixation. However, the performance of clone D6E3G was superior to the other anti-

213    phospho-MLKL antibodies, because specific signals were more abundant (Fig. 4c) and represented a

214    higher percentage of the total detectable signal (Fig. 4b). This trend was also evident via immunoblot,

215    with clone 7C6.1 being largely non-specific, clone EPR9515(2) being moderately specific and clone

216    D6E3G exhibiting a high degree of specificity towards activated MLKL in necroptotic cells (Fig. 4e).

217    In summary, clones 5A6 and D6E3G allow the immunofluorescent detection of mouse MLKL

218    under resting and necroptotic conditions, respectively. Moreover, as with human MLKL, methanol is

219    the fixative of choice for robust immunofluorescent detection of endogenous mouse MLKL.

220

**Immunofluorescent detection of mouse RIPK3**

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222    There are numerous well-validated antibodies raised against mouse RIPK3, including a polyclonal

223    antibody against the C-terminus (source: ProSci 2283), and monoclonal antibodies recognising

224    phospho-T231/phospho-S232 (source: Genentech clone GEN135-35-9[53]) and the extended kinase

225    domain (residues 2-353) of mouse RIPK3 (clone 8G7[5], in-house; Fig. 5a). These antibodies yielded

226    specific signals via both immunofluorescence and immunoblot analyses (Fig. 5b-e). Interestingly,

227    while phospho-RIPK3 concentrates into cytoplasmic clusters during necroptosis, this relocation event

228    was not detected by other, anti-total RIPK3 antibodies (Fig. 5d). This finding suggests that the

229    epitopes for RIPK3 detection are obscured via protein-protein interactions during the chronology of

230    necroptotic signaling events. To further probe the steps in the pathway, we generated another mouse

231    RIPK3 antibody, clone 1H12 (source: in-house), which has a favourable staining profile (Fig. 5b-c)

232  and which recognises the translocation of mouse RIPK3 into cytoplasmic clusters during necroptosis

233  (Fig. 5d). Notably, monoclonal antibodies raised against the N-terminal kinase domain of RIPK3,

234  8G7 and 1H12, detected several species of lower molecular weight than full length RIPK3 by

235  immunoblot. These signals are likely to be spliced isoforms of mouse RIPK3 of varying lengths, each

236  of which harbour the kinase domain antigen (residues 2-353), which are therefore not detected by

237  antibodies directed towards the C-terminus of the full-length mouse RIPK3 isoform, such as ProSci

238  2283. In summary, there are several excellent options for the immunofluorescent detection of

239  endogenous mouse RIPK3. Notably, unlike MLKL and RIPK1, the choice of fixative has no major

240  bearing on the ability to detect mouse RIPK3 by immunofluorescence (Fig. 5c).

241

242  **Immunofluorescent detection of mouse RIPK1**

243  RIPK1 exhibits greater sequence identity between mouse and human orthologs than either RIPK3 or

244  MLKL[41]. In keeping with this, both clone D94C12 (source: Cell Signaling Technology) and clone

245  38/RIP (source: BD Transduction Laboratories), which were raised against human RIPK1, also

246  specifically detected mouse RIPK1 via immunofluorescence and immunoblotting (Fig. 6a-e). A

247  polyclonal antibody raised against phospho-S166 of mouse RIPK1 (31122; source: Cell Signaling

248  Technology) was also found to specifically detect RIPK1 via immunoblot (Fig. 6e) and in methanol-

249  fixed, but not paraformaldehyde-fixed, cells undergoing necroptosis (Fig. 6b-d).

250  In summary, three highly-specific antibodies exist for mouse RIPK1. However, as was

251  observed in human cells, necroptosis in mouse cells causes a marked reduction in RIPK1 levels (Fig.

252  6d-e). Thus, the specific immunosignals from the antibodies tested are of low intensity and abundance

253  in necroptotic cells. Therefore, careful gating of signals is recommended for the study of mouse

254  RIPK1 in necroptotic cells.

255

256  **Antibody cocktails for imaging endogenous necroptotic signaling**

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257   Having validated and compared the performance of numerous reagents, we now propose different

258   antibody combinations that can be used to study endogenous necroptotic signaling (Fig. 7a-b).

259   Because these recommendations are made to facilitate the investigation of endogenous necroptotic

260   signaling in fixed cells, we also provide an ImageJ macro to generate signal-to-noise curves for the

261   gating of specific immunosignals (Supplementary File 1). To exemplify the advantages of visualising

262   endogenous necroptotic events *in situ*, we co-stained MDFs for non-phosphorylated MLKL (clone

263   5A6; source: in-house) and phosphorylated MLKL (clone D6E3G; source: Cell Signaling

264   Technology). Similar to what was described for human MLKL[29], mouse MLKL was observed to

265   concentrate into cytoplasmic clusters during necroptosis. However, this compartmentalisation event

266   is less pronounced than those observed for human MLKL in necroptotic HT29 cells (arrowhead; Fig.

267   7c). Also similar to what was described for human MLKL[29], phosphorylated MLKL was observed to

268   form focal structures at the plasma membrane, rather than docking uniformly with the cell periphery

269   (Fig. 7c). Remarkably, although smaller than the hotspots of phosphorylated human MLKL that form

270   in necroptotic HT29 cells, junctional accumulations of phosphorylated mouse MLKL were also

271   apparent in necroptotic MDFs (Fig. 7d and Supplementary Video 1). These data suggest that the

272   assembly of both mouse and human MLKL into macromolecular structures at the plasma membrane,

273   and preferentially at sites of intercellular contact, may be an intrinsic and penultimate feature of

274   necroptotic signaling.

275

276   **DISCUSSION**

277   Here, we profiled the immunofluorescent staining of MLKL, RIPK3 and RIPK1 in fixed human and

278   mouse cells with a panel of 22 antibodies. While 17 of 22 antibodies were capable of detecting their

279   respective necroptotic protein target in fixed wild-type cells and not their knockout counterparts, only

280   14 of 22 antibodies exhibited favourable signal-to-noise for robust immunostaining (as summarized

281   in Fig. 7a).

The choice of fixative was a critical variable for the immunofluorescent detection of necroptotic proteins. With the exception of phospho-S227 in human RIPK3 and pT231/pS232 in mouse RIPK3, all other epitopes exhibited increased immunoreactivity in methanol-fixed samples. Indeed, immunodetection of MLKL was often found to be entirely contingent upon the use of methanol as a fixative. This observation has ramifications for the experimental and clinical immunohistological detection of cell death, which is almost exclusively performed on aldehyde-fixed samples. Thus, stringent antigen retrieval steps to reverse crosslinks will likely be necessary for the development of a diagnostic assay for necroptosis in patient samples. High resolution microscopy is also mostly performed on aldehyde-fixed cell monolayers. However, as these cell monolayers cannot typically withstand antigen retrieval, we recommend the use of methanol fixation for high resolution immunofluorescence of necroptosis; even though methanol fixation can be sub-optimal for both the retention of certain biomolecules[54] and the preservation of cellular architecture[55, 56]. Indeed, all but one of the antibodies (source: Cell Signaling Technology clone D6W2T) in the suggested toolbox in Fig. 7a are compatible with methanol fixation.

Our data underscore the importance of using controlled experimental conditions to maximise the identification of specific immunosignals. To this end, we used wild-type cells that express high levels of MLKL, RIPK3 and RIPK1, and which have a well-characterised response to necroptotic stimuli. If possible, cell cultures should be fixed when low, but measurable, levels of necroptotic death are occurring (Fig. S1). This is because end-stage dead cells are often lost during/after methanol fixation, and because the immunosignals for phospho-RIPK1, phospho-RIPK3 and phospho-MLKL peak prior to cell death[29]. Notably, the use of knockout cells (for non-phospho-targets) or unstimulated wild-type cells (for phospho-sites) as a negative control is critical, as it provides the information needed to define the signal-to-noise profile of an antibody (Fig. 1b). Additionally, it is also helpful if different antibodies directed toward the same target yield concordant immunofluorescent signals, and if accompanying immunoblot data confirm their specificity. This

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307 combined approach was important for deducing that clone D6E3G (source: Cell Signaling

308 Technology) is the best option for detecting phospho-MLKL in mouse cells (Fig. 4).

309     It is concerning that the specificity of some antibodies could not be verified. For instance,

310 while numerous studies use the ProSci 2283 antibody to detect human RIPK3[57, 58, 59], we found that

311 it only recognised mouse RIPK3. Similarly, M6697 (source: Sigma-Aldrich) and clone 7C6.1

312 (source: Millipore) have been widely used to respectively detect human MLKL and mouse MLKL

313 phospho-S345 [39, 60, 61, 62, 63, 64, 65], however these reagents exhibited a low degree of specificity in our

314 hands. Nonetheless, as we did not exhaustively test the performance of these antibodies for

315 immunofluorescence using a wide variety of fixatives, blocking agents, and detergents, we cannot

316 categorically state that they are non-selective. Moreover, as ProSci 2283 (reported to detect mouse

317 and human RIPK3) and Sigma-Aldrich M6697 (reported to detect human MLKL) are polyclonal

318 antibodies, batch-to-batch variation may explain differences in their specificity between studies. It is

319 also noteworthy that the majority of monoclonal antibodies that yielded the most abundant and

320 specific signals were raised against recombinant full-length protein or component domains, rather

321 than unfolded partial domains or peptides. We reasoned that epitopes that are exposed in the cellular

322 forms of these proteins would be similarly presented within a folded protein for immune recognition

323 in the host. Phosphosite-specific antibodies differ in this regard; these were raised using linear

324 phosphopeptides and their specificity likely reflects the unstructured and solvent-exposed nature of

325 the sequences in which they reside.

326     Broadly, the specific immunosignals described in this study provide consensus about three

327 key compartmentalisation events that underlie necroptotic signaling[66]. First, the core necroptotic

328 proteins - MLKL, RIPK3 and RIPK1 - predominantly reside in the cytosol under basal conditions.

329 Interestingly, rather than being diffusely distributed across the cytosol, this necroptotic machinery

330 exists as small puncta that are below the resolution limit of conventional light microscopy. It may be

331 that these necroptotic proteins are preassembled into a membrane-less organelle under basal

14

332 conditions. Second, RIPK1, RIPK3 and MLKL then coalesce into large cytoplasmic clusters. These

333 clusters preferentially locate to the perinuclear space and are necrosome-related as they co-stain for

334 the presence of phospho-RIPK1, -RIPK3 and -MLKL (Fig. 1-7 and ref.[29]). It is important to note that

335 the formation of these necrosomal clusters in TSI-treated HT29 cells occurs subsequent to the

336 phosphorylation of MLKL[29]. While these clusters likely represent a higher-order form of the

337 necrosome, the mechanisms that govern cluster formation and limit their overall size are currently

338 unknown. Third, as necroptotic signaling continues, phospho-MLKL is actively trafficked away from

339 the clusters towards the plasma membrane[26] where it accumulates into supramolecular assemblies

340 called "hotspots"[29]. Recent findings suggest the release of phospho-MLKL from clusters arises from

341 MLKL undergoing a conformational change that accompanies disengagement from necrosomal

342 RIPK3[40]. Our data support such a disengagement step because neither RIPK1 nor RIPK3 were

343 observed to accumulate at the plasma membrane during necroptosis in mouse or human cells (Fig. 2-

344 3 and 5-6). This disengagement step, and formation of supramolecular assemblies by both mouse and

345 human MLKL at the plasma membrane, are likely to be two additional checkpoints in necroptotic

346 signaling.

347

348 **METHODS**

349 *Materials* – Primary antibodies and the dilutions used for immunoblotting/immunofluorescence

350 were: rat anti-human MLKL (clone 10C2; produced in-house[29]; 1:2000/1:400), rat anti-human

351 MLKL (clone 7G2; produced in-house[29]; 1:2000/1:400), rabbit anti-phospho-S358 human MLKL

352 (Abcam ab187091; clone EPR9514; 1:2000/1:200), rat anti-mouse, human, rat, horse MLKL (clone

353 3H1; produced in-house[37] and available as Millipore MABC604; 1:2000/ N/A), mouse anti-phospho-

354 T357 human MLKL (Novus Biological MAB9187; clone 954702; 1:1000/1:200), rabbit anti-human

355 MLKL (Sigma-Aldrich M6697; 1:1000/1:200), rabbit anti-RIPK3 (ProSci #2283; 1:1000/1:200),

356 rabbit anti-human RIPK3 (Novus Biological NBP2-24588; 1:1000/1:200), mouse anti-human RIPK3

357  (Novus Biological; clone 780115; 1:1000/1:200), rat anti-human RIPK3 (clone 1H2; produced in-

358  house[5] and available as Millipore MABC1640; 1:2000/1:400), rabbit anti-phospho-S227 human

359  RIPK3 (Cell Signaling Technology; clone D6W2T; 1:2000/1:200), rabbit anti-mouse or human

360  RIPK1 (Cell Signaling Technology; clone D94C12; 1:2000/1:200), mouse anti-mouse or human

361  RIPK1 (BD Biosciences; clone 38/RIP; 1:1000/1:100), rabbit anti-phospho-S166 human RIPK1 (Cell

362  Signaling Technology; clone D8I3A; 1:2000/1:200), rat anti-mouse MLKL (clone 5A6; produced in-

363  house), rabbit anti-phospho-S345 mouse MLKL (Abcam; clone EPR9515(2); 1:2000/1:200), mouse

364  anti-phospho-S345 mouse MLKL (Millipore MABC158; clone 7C6.1; 1:2000/1:200), rabbit anti-

365  phospho-S345 mouse MLKL (Cell Signaling Technology; clone D6E3G; 1:2000/1:200), rat anti-

366  mouse RIPK3 (clone 8G7; produced in-house[5] and available from Millipore as MABC1595;

367  1:2000/1:400), rat anti-mouse RIPK3 (clone 1H12; produced in-house; 1:2000/1:400), rabbit anti-

368  phospho-T231/S232 mouse RIPK3 (Genentech; clone GEN135-35-9[53]; 1:2000/1:400), rabbit anti-

369  phospho-S166 mouse RIPK1 (Cell Signaling Technology 31122; 1:2000/1:200), mouse anti-GAPDH

370  (Millipore MAB374; 1:2000/N/A). Secondary antibodies for immunoblotting were: horseradish

371  peroxidase (HRP)-conjugated goat anti-rat IgG (Southern Biotech 3010-05), HRP-conjugated goat

372  anti-mouse IgG (Southern Biotech 1010-05), and HRP-conjugated goat anti-rabbit IgG (Southern

373  Biotech 4010-05). All secondary antibodies for immunoblotting were used at a dilution of 1:10000.

374  Secondary immunofluorescence detection reagents were: AlexaFluor647-conjugated donkey anti-

375  rabbit IgG (ThermoFisher Scientific A31573), AlexaFluor568-conjugated donkey anti-rabbit IgG

376  (ThermoFisher Scientific A10042), AlexaFluor568-conjugated donkey anti-mouse IgG

377  (ThermoFisher Scientific A10037), AlexaFluor-594 donkey anti-rat IgG (ThermoFisher Scientific A-

378  21209), AlexaFluor488-conjugated donkey anti-rat IgG (ThermoFisher Scientific A21208). All

379  secondary antibodies for immunofluorescence were used at a dilution of 1:1000. Bond-Breaker TCEP

380  solution (ThermoFisher Scientific 77720). *N*-Ethylmaleimide (Sigma-Aldrich E3876).

381

382     ***5A6 and 1H12 antibody production*** – Antibodies were generated at the Walter and Eliza Hall

383     Institute Monoclonal Antibody Facility by immunizing Wistar rats with recombinant full-length

384     mouse MLKL[37] for clone 5A6 or recombinant mouse RIPK3 residues 2-353 for clone 1H12 and the

385     previously-reported clone 8G7[5] that was expressed and purified from Sf21 insect cells using the

386     baculovirus expression system, before splenocytes were fused with SP2/O mouse myeloid cells and

387     arising hybridoma lines cloned. The specificity of clone 5A6 for mouse MLKL was validated via

388     immunoblot analyses as exemplified in Fig. 4e and Fig. S3. The specificity of clone 1H12 for mouse

389     RIPK3 was validated via immunoblot analyses as exemplified in Fig. 5e.

390

391     ***Cell lines*** – HT29 cells were provided by Mark Hampton (University of Otago). *RIPK1^{-/-}*, *RIPK3^{-/-}*

392     and *MLKL^{-/-}* HT29 cells have been previously reported[28, 29, 67]. MDF lines were generated in-house

393     from the tails of wild-type C57BL/6J, *Mlkl[-/-][37]* and *Ripk3[-/-][68]* mice and immortalized by SV40 large T

394     antigen as reported previously[35, 37]. The sex and precise age of these animals were not recorded,

395     although our MDFs are routinely derived from tails from 8-week-old mice. *Mlkl^{-/-}Ripk1^{-/-}* MDFs were

396     derived from the belly/back dermis of *Mlkl^{-/-}Ripk1^{-/-}* E19.5 mice[16] and immortalized as described

397     above. MDF lines were generated in accordance with protocols approved by the Walter and Eliza

398     Hall Institute of Medical Research Animal Ethics Committee. Cell line identities were not further

399     validated, although their morphologies and responses to necroptotic stimuli were consistent with their

400     stated origins. Cell lines were routinely monitored to confirm they were mycoplasma-free.

401

402     ***Cell culture*** – HT29 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Life

403     Technologies) containing 8% v/v heat-inactivated fetal calf serum (FCS), 2 mM L-Glutamine/-

404     GlutaMAX (ThermoFisher Scientific 35050061), 50 U ml$^{-1}$ penicillin and 50 U ml$^{-1}$ streptomycin

405     (G/P/S) under humidified 10% $CO_2$ at 37°C.

406

407    *Cell Treatment* – Cells were seeded into Ibi-treated 8-well μ-slides (Ibidi 80826) in media containing

408    8% v/v FCS and G/P/S at $3.0 \times 10^4$ cells per well for HT29 and $0.25 \times 10^4$ cells per well for MDFs.

409    Cells were left to adhere overnight then treated in media containing 1% v/v FCS and G/P/S and

410    supplemented with the following stimuli: 100ng/mL recombinant human TNF-α-Fc (produced in-

411    house as in ref.[69]), 500nM Smac mimetic/Compound A (provided by Tetralogic Pharmaceuticals; as

412    in ref.[70]), 5μM IDN-6556 (provided by Tetralogic Pharmaceuticals). Unless stipulated, HT29 cells

413    were stimulated for 7.5 h with TSI and MDF cells were stimulated for 90 min with TSI.

414

415    *LDH release* – Colorimetric LDH release assay kit (Promega G1780) was performed according to

416    manufacturer's instructions.

417

418    *Whole cell lysate, SDS-PAGE, immunoblot & quantification* – Cells were lysed in ice-cold RIPA

419    buffer (10mM Tris-HCl pH 8.0, 1mM EGTA, 2mM $MgCl_2$, 0.5% v/v Triton X100, 0.1% w/v Na

420    deoxycholate, 0.5% w/v SDS and 90mM NaCl) supplemented with 1x Protease & Phosphatase

421    Inhibitor Cocktail (Cell Signaling Technology 5872S) and 100U/mL Benzonase (Sigma-Aldrich

422    E1014). Whole cell lysates were boiled for 10 minutes in 1× SDS Laemmli sample buffer (126 mM

423    Tris-HCl, pH 8, 20% v/v glycerol, 4% w/v SDS, 0.02% w/v bromophenol blue, 5% v/v 2-

424    mercaptoethanol), and resolved on 1.5mm NuPAGE 4–12% Bis-Tris gels (ThermoFisher Scientific

425    NP0335BOX) using MES Running buffer (ThermoFisher Scientific NP000202) or Bio-Rad Criterion

426    TGX 4-15% gels (Bio-Rad 5678085) using 1x TGS buffer (Bio-Rad 1610772). After transfer onto

427    nitrocellulose, membranes were blocked in 5% w/v skim milk powder in TBS-T, probed with primary

428    antibodies (see *Materials* above) then the appropriate HRP-conjugated secondary antibody (see

429    *Materials* above) and signals revealed by enhanced chemiluminescence (Merck P90720) on a

430    ChemiDoc Touch Imaging System (Bio-Rad). Between each probe, membranes were incubated in

431     stripping buffer (200mM glycine pH 2.9, 1% w/v SDS, 0.5mM TCEP) for 30 minutes at room

432     temperature then re-blocked.

433

434     ***Subcellular fractionation, BN-PAGE & immunoblot –*** HT29 cells or MDFs were seeded into 6-well

435     plates ($1.0 \times 10^6$ cells per well) in media containing 8% v/v FCS and G/P/S and equilibrated overnight

436     under humidified 10% $CO_2$ at 37 °C conditions. Cells were then treated in media containing 1% FCS

437     and G/P/S supplemented with the agonists (as indicated above). Cells were fractionated into

438     cytoplasmic and membrane fractions[35]. Cells were permeabilized in MELB buffer (20 mM HEPES

439     pH 7.5, 100 mM KCl, 2.5 mM $MgCl_2$ and 100 mM sucrose, 0.025% v/v digitonin, 2 μM N-ethyl

440     maleimide, phosphatase and protease inhibitors). Crude membrane and cytoplasmic fractions were

441     separated by centrifugation (5 minutes 11,000$g$), and fractions prepared in buffers to a final

442     concentration of 1% w/v digitonin. The samples were resolved on a 4–16% Bis-Tris Native PAGE

443     gel (ThermoFisher), transferred to polyvinylidene difluoride (Merck IPVH00010). After transfer,

444     membranes were destained (in 50% (v/v) methanol, 25% (v/v) acetic acid), denatured (in 6M

445     Guanidine hydrochloride, 10 mM Tris pH6.8, 5 mM β-mercaptoethanol), blocked in 5% skim milk

446     (Diploma), and probed in the same manner as above.

447

448     ***Protein production and purification*** – Full-length human and mouse MLKL and the C-terminal,

449     pseudokinase domain of mouse and human MLKL were expressed in Sf21 insect cells using the Bac-

450     to-Bac system (ThermoFisher Scientific) and purified using established procedures[28, 37, 42]. Briefly,

451     proteins were expressed with an N-terminal, TEV protease-cleavable GST (full length human MLKL)

452     or $His_6$ (full length mouse MLKL and pseudokinase domains) tags and captured from lysates using

453     glutathione resin (UBP Bio) or HisTag Ni-NTA resin (Roche) respectively. Proteins were cleaved

454     on-resin from the GST tag or off-resin for $His_6$ tags using His-tagged TEV protease, before protease

455     was removed by Ni-NTA chromatography (HisTag resin, Roche). Protein was concentrated by

456    centrifugal ultrafiltration and applied to a Superdex-200 (GE Healthcare) size exclusion

457    chromatography column; protein was eluted in 20 mM HEPES pH 7.5, 200 mM NaCl, 5% v/v

458    glycerol. Protein containing fractions were spin concentrated to 5-10 mg/mL, aliquoted, snap frozen

459    in liquid $N_2$ and stored at –80ºC until required.

460

461    ***Immunofluorescence*** – Cells in 8-well μ-Slides (Ibidi 80826) were ice-chilled for 3 minutes, then

462    washed in ice-cold Dulbecco's PBS (dPBS; ThermoFisher Scientific 14190144), then fixed for 30

463    minutes in either ice-cold methanol or ice-cold 4% w/v PFA. Cells were washed twice in ice-cold

464    dPBS, then blocked in ice-cold Tris-balanced salt solution with 0.05% v/v Triton-X100 (TBS-T)

465    supplemented with 10% v/v donkey serum (Sigma-Aldrich D9663) for >1 hour. Cells were incubated

466    in primary antibodies (see ***Materials*** above) overnight at 4 ºC in TBS-T with 10% v/v donkey serum.

467    Cells were washed twice in TBS-T then incubated in the appropriate secondary antibodies

468    supplemented with 0.1μg/mL Hoechst 33342 (ThermoFisher Scientific H3570) for 3 hours at room

469    temperature with gentle rocking. Cells were washed four times in ice-cold TBS-T then stored at 4 ºC

470    until imaged. Where indicated, to demarcate the plasma membrane, 2μL of biotinylated wheat germ

471    agglutinin (Sigma-Aldrich L5142) was added to each well 10 minutes before fixation. The fixed

472    wheat germ agglutinin was then detected via the addition of 1:1000 dilution of DyLight650-

473    conjugated streptavidin (ThermoFisher Scientific 84547) during the secondary antibody incubation

474    step.

475

476    ***Two-dimensional epifluorescence microscopy*** - Samples in TBS-T were imaged on an Inverted Axio

477    Observer.Z1 microscope (Zeiss) with the following specifications: C-Apochromat 40x/1.20 W

478    autocorr UV VIS IR lens, HXP 120V excitation source, AlexaFluor647 and DyLight650 imaged with

479    a $\lambda_{Excitation}$=625-655nm; $\lambda_{beamsplitter}$=660nm; $\lambda_{Emission}$=665-715nm filter, AlexaFluor568 imaged with a

480    $\lambda_{Excitation}$=532-544nm; $\lambda_{beamsplitter}$=560nm; $\lambda_{Emission}$=573-613nm, AlexaFluor488 imaged with a

481  $\lambda_{Excitation}$=450-490nm; $\lambda_{beamsplitter}$=495nm; $\lambda_{Emission}$=500-550nm, Hoechst 33342 imaged with a

482  $\lambda_{Excitation}$=359-371nm; $\lambda_{beamsplitter}$=395nm; $\lambda_{Emission}$=397-$\infty$ nm, a sCMOS PCO.edge 4.2 camera, ZEN

483  blue 2.5 pro capture software and ImageJ 1.53c post-acquisition processing software[71]. Typically, for

484  each independent experiment, 5-10 randomly selected fields were captured per treatment group,

485  whereby only the Hoechst 33342 signal was visualised prior to multi-channel acquisition. To ensure

486  consistent signal intensities across independent experiments, the same excitation, emission and

487  camera settings were used throughout this study.

488

489  *Airyscan microscopy* – Fixed immunostained cells in 8-well µ-Slides (Ibidi 80827) were subjected

490  to super-resolution 3-dimensional Airyscan microscopy on an Inverted LSM 880 platform (Zeiss)

491  equipped with the following specifications: a 63x/1.4 N.A. PlanApo DIC M27 oil immersion

492  objective (Zeiss), 405-, 488-, 568- and 640-nm laser lines, and radially-stacked Airyscan GaASP

493  detectors set to SR-mode, 405-, 488-, 561-, 633-nm laser lines and ZEN black 2.3 SP1 FP3 v14.0

494  capture software. Image stacks were acquired with a z-step size of 159nm. Super-resolution

495  deconvolution was performed using the automated '3D AiryScan Processing' function of ZEN blue

496  software.

497

498  *Hotspot quantitation* – Micrographs (captured as in ***two-dimensional epifluorescence microscopy***)

499  were opened in ImageJ 1.53c[71]. A rolling ball filter of 7 was applied and phospho-S358 MLKL

500  immunosignals thresholded ($\geq$7000 units) and objects segmented using the 'Analyze>Analyze

501  Particles' tool. Segmented objects with size 0.5-100$\mu m^2$ and feret diameter>2 (i.e. elliptical objects)

502  were considered hotspots. The number of segmented objects per 100 cells was taken as an index of

503  hotspot occurrence. The mean size of the segmented objects was taken as an index of hotspot size.

504  The fluorescence intensity of each segmented object divided by its size was taken as an index of

505  hotspot intensity.

506

## ACKNOWLEDGEMENTS

516

## AUTHOR CONTRIBUTIONS

518 Investigation: A.L.S., K.P., C.F., J.M.H., L.W., J.R., A.V.J., C.R.H., X.G., S.N.Y. Supervision and

519 Methodology: A.L.S., J.M.H., K.L.R., E.D.H. and J.M.M.; Conceptualization: A.L.S., E.D.H. and

520 J.M.M.; Writing – Original Draft: A.L.S. and J.M.M.; Writing – Review & Editing: all authors.

521

## COMPETING INTERESTS

523 A.L.S., K.P. C.F., J.M.H., S.N.Y. and J.M.M. contribute to a project developing necroptosis inhibitors

524 in collaboration with Anaxis Pharma. The other authors declare no competing interests.

525

## MATERIALS REQUESTS

527 All materials will be provided under Materials Transfer Agreement upon request to James Murphy

528 (jamesm@wehi.edu.au).

529

## REFERENCES

531

532 1.    Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, *et al.* Phosphorylation-driven
533       assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced
534       inflammation. *Cell* 2009, **137**(6)**:** 1112-1123.

536 2.    Guo H, Omoto S, Harris PA, Finger JN, Bertin J, Gough PJ, *et al.* Herpes simplex virus
537       suppresses necroptosis in human cells. *Cell Host Microbe* 2015, **17**(2)**:** 243-251.

539 3.    Pearson JS, Giogha C, Muhlen S, Nachbur U, Pham CL, Zhang Y, *et al.* EspL is a bacterial
540       cysteine protease effector that cleaves RHIM proteins to block necroptosis and inflammation.
541       *Nat Microbiol* 2017, **2:** 16258.

543 4.    Pearson JS, Murphy JM. Down the rabbit hole: Is necroptosis truly an innate response to
544       infection? *Cell Microbiol* 2017, **19**(8)**:** e12750.

546 5.    Petrie EJ, Sandow JJ, Lehmann WIL, Liang LY, Coursier D, Young SN, *et al.* Viral MLKL
547       Homologs Subvert Necroptotic Cell Death by Sequestering Cellular RIPK3. *Cell reports*
548       2019, **28**(13)**:** 3309-3319 e3305.

550 6.    Fletcher-Etherington A, Nobre L, Nightingale K, Antrobus R, Nichols J, Davison AJ, *et al.*
551       Human cytomegalovirus protein pUL36: A dual cell death pathway inhibitor. *Proc Natl Acad*
552       *Sci U S A* 2020, **117**(31)**:** 18771-18779.

554 7.    Kitur K, Wachtel S, Brown A, Wickersham M, Paulino F, Penaloza HF, *et al.* Necroptosis
555       Promotes Staphylococcus aureus Clearance by Inhibiting Excessive Inflammatory Signaling.
556       *Cell reports* 2016, **16**(8)**:** 2219-2230.

558 8.    Shubina M, Tummers B, Boyd DF, Zhang T, Yin C, Gautam A, *et al.* Necroptosis restricts
559       influenza A virus as a stand-alone cell death mechanism. *J Exp Med* 2020, **217**(11).

561 9.    Simpson J, Loh Z, Ullah MA, Lynch JP, Werder RB, Collinson N, *et al.* Respiratory Syncytial
562       Virus Infection Promotes Necroptosis and HMGB1 Release by Airway Epithelial Cells. *Am*
563       *J Respir Crit Care Med* 2020, **201**(11)**:** 1358-1371.

565 10. Muller T, Dewitz C, Schmitz J, Schroder AS, Brasen JH, Stockwell BR, *et al.* Necroptosis
566  and ferroptosis are alternative cell death pathways that operate in acute kidney failure. *Cell*
567  *Mol Life Sci* 2017, **74**(19)**:** 3631-3645.

569 11. Pefanis A, Ierino FL, Murphy JM, Cowan PJ. Regulated necrosis in kidney ischemia-
570  reperfusion injury. *Kidney Int* 2019, **96**(2)**:** 291-301.

572 12. Luedde M, Lutz M, Carter N, Sosna J, Jacoby C, Vucur M, *et al.* RIP3, a kinase promoting
573  necroptotic cell death, mediates adverse remodelling after myocardial infarction. *Cardiovasc*
574  *Res* 2014, **103**(2)**:** 206-216.

576 13. Hildebrand JM, Kauppi M, Majewski IJ, Liu Z, Cox AJ, Miyake S, *et al.* A missense mutation
577  in the MLKL brace region promotes lethal neonatal inflammation and hematopoietic
578  dysfunction. *Nat Commun* 2020, **11**(1)**:** 3150.

580 14. Wang H, Sun L, Su L, Rizo J, Liu L, Wang LF, *et al.* Mixed Lineage Kinase Domain-like
581  Protein MLKL Causes Necrotic Membrane Disruption upon Phosphorylation by RIP3. *Mol*
582  *Cell* 2014, **54**(1)**:** 133-146.

584 15. Rickard JA, Anderton H, Etemadi N, Nachbur U, Darding M, Peltzer N, *et al.* TNFR1-
585  dependent cell death drives inflammation in Sharpin-deficient mice. *Elife* 2014, **3:** e03464.

587 16. Rickard JA, O'Donnell JA, Evans JM, Lalaoui N, Poh AR, Rogers TW, *et al.* RIPK1 regulates
588  RIPK3-MLKL driven systemic inflammation and emergency hematopoiesis. *Cell* 2014,
589  **157**(2)**:** 1175-1188.

591 17. Pierdomenico M, Negroni A, Stronati L, Vitali R, Prete E, Bertin J, *et al.* Necroptosis is active
592  in children with inflammatory bowel disease and contributes to heighten intestinal
593  inflammation. *Am J Gastroenterol* 2014, **109**(2)**:** 279-287.

595 18. He S, Wang L, Miao L, Wang T, Du F, Zhao L, *et al.* Receptor interacting protein kinase-3
596  determines cellular necrotic response to TNF-alpha. *Cell* 2009, **137**(6)**:** 1100-1111.

598    19.    Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC*, et al.* RIP3, an energy metabolism
599          regulator that switches TNF-induced cell death from apoptosis to necrosis. *Science* 2009,
600          **325**(5938)**:** 332-336.

602    20.    Sun L, Wang H, Wang Z, He S, Chen S, Liao D*, et al.* Mixed lineage kinase domain-like
603          protein mediates necrosis signaling downstream of RIP3 kinase. *Cell* 2012, **148**(1-2)**:** 213-
604          227.

606    21.    Zhao J, Jitkaew S, Cai Z, Choksi S, Li Q, Luo J*, et al.* Mixed lineage kinase domain-like is a
607          key receptor interacting protein 3 downstream component of TNF-induced necrosis. *Proc*
608          *Natl Acad Sci U S A* 2012, **109**(14)**:** 5322-5327.

610    22.    Wang T, Perera ND, Chiam MDF, Cuic B, Wanniarachchillage N, Tomas D*, et al.*
611          Necroptosis is dispensable for motor neuron degeneration in a mouse model of ALS. *Cell*
612          *Death Differ* 2019.

614    23.    Dermentzaki G, Politi KA, Lu L, Mishra V, Perez-Torres EJ, Sosunov AA*, et al.* Deletion of
615          Ripk3 Prevents Motor Neuron Death In Vitro but not In Vivo. *eNeuro* 2019, **6**(1).

617    24.    Dominguez S, Varfolomeev E, Brendza R, Stark K, Tea J, Imperio J*, et al.* Genetic
618          inactivation of RIP1 kinase does not ameliorate disease in a mouse model of ALS. *Cell Death*
619          *Differ* 2020.

621    25.    Dara L, Johnson H, Suda J, Win S, Gaarde W, Han D*, et al.* Receptor interacting protein
622          kinase 1 mediates murine acetaminophen toxicity independent of the necrosome and not
623          through necroptosis. *Hepatology* 2015, **62**(6)**:** 1847-1857.

625    26.    Petrie EJ, Birkinshaw RW, Koide A, Denbaum E, Hildebrand JM, Garnish SE*, et al.*
626          Identification of MLKL membrane translocation as a checkpoint in necroptotic cell death
627          using Monobodies. *Proc Natl Acad Sci U S A* 2020, **117**(15)**:** 8468-8475.

629    27.    Petrie EJ, Czabotar PE, Murphy JM. The Structural Basis of Necroptotic Cell Death
630          Signaling. *Trends Biochem Sci* 2019, **44**(1)**:** 53-63.

28.  Petrie EJ, Sandow JJ, Jacobsen AV, Smith BJ, Griffin MDW, Lucet IS, *et al.* Conformational switching of the pseudokinase domain promotes human MLKL tetramerization and cell death by necroptosis. *Nat Commun* 2018, **9**(1): 2422.

29.  Samson AL, Zhang Y, Geoghegan ND, Gavin XJ, Davies KA, Mlodzianoski MJ, *et al.* MLKL trafficking and accumulation at the plasma membrane control the kinetics and threshold for necroptosis. *Nat Commun* 2020, **11**(1): 3151.

30.  Li J, McQuade T, Siemer AB, Napetschnig J, Moriwaki K, Hsiao YS, *et al.* The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis. *Cell* 2012, **150**(2): 339-350.

31.  Mompean M, Li W, Li J, Laage S, Siemer AB, Bozkurt G, *et al.* The Structure of the Necrosome RIPK1-RIPK3 Core, a Human Hetero-Amyloid Signaling Complex. *Cell* 2018, **173**(5): 1244-1253 e1210.

32.  Sun X, Yin J, Starovasnik MA, Fairbrother WJ, Dixit VM. Identification of a novel homotypic interaction motif required for the phosphorylation of receptor-interacting protein (RIP) by RIP3. *J Biol Chem* 2002, **277**(11): 9505-9511.

33.  Cook WD, Moujalled DM, Ralph TJ, Lock P, Young SN, Murphy JM, *et al.* RIPK1- and RIPK3-induced cell death mode is determined by target availability. *Cell Death Differ* 2014, **21**(10): 1600-1612.

34.  Orozco S, Yatim N, Werner MR, Tran H, Gunja SY, Tait SW, *et al.* RIPK1 both positively and negatively regulates RIPK3 oligomerization and necroptosis. *Cell Death Differ* 2014, **21**(10): 1511-1521.

35.  Hildebrand JM, Tanzer MC, Lucet IS, Young SN, Spall SK, Sharma P, *et al.* Activation of the pseudokinase MLKL unleashes the four-helix bundle domain to induce membrane localization and necroptotic cell death. *Proc Natl Acad Sci U S A* 2014, **111**(42): 15072-15077.

665  36.  Murphy JM. The Killer Pseudokinase Mixed Lineage Kinase Domain-Like Protein (MLKL).
666       *Cold Spring Harb Perspect Biol* 2020, **12**(8).
667

668  37.  Murphy JM, Czabotar PE, Hildebrand JM, Lucet IS, Zhang JG, Alvarez-Diaz S*, et al.* The
669       pseudokinase MLKL mediates necroptosis via a molecular switch mechanism. *Immunity*
670       2013, **39**(3)**:** 443-453.
671

672  38.  Tanzer MC, Tripaydonis A, Webb AI, Young SN, Varghese LN, Hall C*, et al.* Necroptosis
673       signalling is tuned by phosphorylation of MLKL residues outside the pseudokinase domain
674       activation loop. *The Biochemical journal* 2015, **471**(2)**:** 255-265.
675

676  39.  Rodriguez DA, Weinlich R, Brown S, Guy C, Fitzgerald P, Dillon CP*, et al.* Characterization
677       of RIPK3-mediated phosphorylation of the activation loop of MLKL during necroptosis. *Cell*
678       *Death Differ* 2016, **23**(1)**:** 76-88.
679

680  40.  Garnish SE, Meng Y, Koide A, Sandow JJ, Denbaum E, Jacobsen AV*, et al.* Conformational
681       interconversion of MLKL and disengagement from RIPK3 precedes cell death by necroptosis.
682       Under review.
683

684  41.  Davies KA, Fitzgibbon C, Young SN, Garnish SE, Yeung W, Coursier D*, et al.* Distinct
685       pseudokinase domain conformations underlie divergent activation mechanisms among
686       vertebrate MLKL orthologues. *Nat Commun* 2020, **11**(1)**:** 3060.
687

688  42.  Murphy JM, Lucet IS, Hildebrand JM, Tanzer MC, Young SN, Sharma P*, et al.* Insights into
689       the evolution of divergent nucleotide-binding mechanisms among pseudokinases revealed by
690       crystal structures of human and mouse MLKL. *The Biochemical journal* 2014, **457**(3)**:** 369-
691       377.
692

693  43.  Tanzer MC, Matti I, Hildebrand JM, Young SN, Wardak A, Tripaydonis A*, et al.*
694       Evolutionary divergence of the necroptosis effector MLKL. *Cell Death Differ* 2016, **23**(7)**:**
695       1185-1197.
696

697    44.    Davies KA, Tanzer MC, Griffin MDW, Mok YF, Young SN, Qin R, *et al.* The brace helices
698          of MLKL mediate interdomain communication and oligomerisation to regulate cell death by
699          necroptosis. *Cell Death Differ* 2018, **25**(9)**:** 1567-1580.

701    45.    Dondelinger Y, Hulpiau P, Saeys Y, Bertrand MJM, Vandenabeele P. An evolutionary
702          perspective on the necroptotic pathway. *Trends Cell Biol* 2016, **26**(10)**:** 721-732.

704    46.    Newton K, Manning G. Necroptosis and Inflammation. *Annu Rev Biochem* 2016, **85:** 743-
705          763.

707    47.    Petrie EJ, Birkinshaw RW, Koide A, Denbaum E, Hildebrand JM, Garnish SE, *et al.*
708          Identification of MLKL membrane translocation as a checkpoint in necroptotic cell death
709          using Monobodies. *Proc Natl Acad Sci U S A* 2020.

711    48.    Wang H, Sun L, Su L, Rizo J, Liu L, Wang LF, *et al.* Mixed lineage kinase domain-like
712          protein MLKL causes necrotic membrane disruption upon phosphorylation by RIP3. *Mol Cell*
713          2014, **54**(1)**:** 133-146.

715    49.    Cai Z, Jitkaew S, Zhao J, Chiang HC, Choksi S, Liu J, *et al.* Plasma membrane translocation
716          of trimerized MLKL protein is required for TNF-induced necroptosis. *Nature cell biology*
717          2014, **16**(1)**:** 55-65.

719    50.    Chen X, Li W, Ren J, Huang D, He WT, Song Y, *et al.* Translocation of mixed lineage kinase
720          domain-like protein to plasma membrane leads to necrotic cell death. *Cell research* 2014,
721          **24**(1)**:** 105-121.

723    51.    Alturki NA, McComb S, Ariana A, Rijal D, Korneluk RG, Sun SC, *et al.* Triad3a induces the
724          degradation of early necrosome to limit RipK1-dependent cytokine production and
725          necroptosis. *Cell Death Dis* 2018, **9**(6)**:** 592.

727    52.    Simpson DS, Gabrielyan A, Feltham R. RIPK1 ubiquitination: Evidence, correlations and the
728          undefined. *Semin Cell Dev Biol* 2020.

53. Newton K, Wickliffe KE, Maltzman A, Dugger DL, Strasser A, Pham VC*, et al.* RIPK1 inhibits ZBP1-driven necroptosis during development. *Nature* 2016, **540**(7631)**:** 129-133.

54. Hoetelmans RW, Prins FA, Cornelese-ten Velde I, van der Meer J, van de Velde CJ, van Dierendonck JH. Effects of acetone, methanol, or paraformaldehyde on cellular structure, visualized by reflection contrast microscopy and transmission and scanning electron microscopy. *Appl Immunohistochem Mol Morphol* 2001, **9**(4)**:** 346-351.

55. Moloney M, McDonnell L, O'Shea H. Atomic force microscopy of BHK-21 cells: an investigation of cell fixation techniques. *Ultramicroscopy* 2004, **100**(3-4)**:** 153-161.

56. Whelan DR, Bell TD. Correlative Synchrotron Fourier Transform Infrared Spectroscopy and Single Molecule Super Resolution Microscopy for the Detection of Composition and Ultrastructure Alterations in Single Cells. *ACS Chem Biol* 2015, **10**(12)**:** 2874-2883.

57. Xu X, Kalac M, Markson M, Chan M, Brody JD, Bhagat G*, et al.* Reversal of CYLD phosphorylation as a novel therapeutic approach for adult T-cell leukemia/lymphoma (ATLL). *Cell Death Dis* 2020, **11**(2)**:** 94.

58. Yang C, Li J, Yu L, Zhang Z, Xu F, Jiang L*, et al.* Regulation of RIP3 by the transcription factor Sp1 and the epigenetic regulator UHRF1 modulates cancer cell necroptosis. *Cell Death Dis* 2017, **8**(10)**:** e3084.

59. Wang S, Ni HM, Dorko K, Kumer SC, Schmitt TM, Nawabi A*, et al.* Increased hepatic receptor interacting protein kinase 3 expression due to impaired proteasomal functions contributes to alcohol-induced steatosis and liver injury. *Oncotarget* 2016, **7**(14)**:** 17681-17698.

60. Zhang S, Che L, He C, Huang J, Guo N, Shi J*, et al.* Drp1 and RB interaction to mediate mitochondria-dependent necroptosis induced by cadmium in hepatocytes. *Cell Death Dis* 2019, **10**(7)**:** 523.

61. Ali M, Mocarski ES. Proteasome inhibition blocks necroptosis by attenuating death complex aggregation. *Cell Death Dis* 2018, **9**(3)**:** 346.

29

764

765 62. Gong YN, Guy C, Olauson H, Becker JU, Yang M, Fitzgerald P, *et al.* ESCRT-III Acts
766 Downstream of MLKL to Regulate Necroptotic Cell Death and Its Consequences. *Cell* 2017,
767 **169**(2)**:** 286-300 e216.

768

769 63. Yoon S, Bogdanov K, Kovalenko A, Wallach D. Necroptosis is preceded by nuclear
770 translocation of the signaling proteins that induce it. *Cell Death Differ* 2016, **23**(2)**:** 253-260.

771

772 64. Wang Z, Jiang H, Chen S, Du F, Wang X. The mitochondrial phosphatase PGAM5 functions
773 at the convergence point of multiple necrotic death pathways. *Cell* 2012, **148**(1-2)**:** 228-243.

774

775 65. Mizumura K, Cloonan SM, Nakahira K, Bhashyam AR, Cervo M, Kitada T, *et al.* Mitophagy-
776 dependent necroptosis contributes to the pathogenesis of COPD. *J Clin Invest* 2014, **124**(9)**:**
777 3987-4003.

778

779 66. Samson AL, Garnish SE, Hildebrand JM, Murphy JM. Location, location, location: a
780 compartmentalized view of necroptotic signaling. Under review.

781

782 67. Tanzer MC, Khan N, Rickard JA, Etemadi N, Lalaoui N, Spall SK, *et al.* Combination of IAP
783 antagonist and IFNgamma activates novel caspase-10- and RIPK1-dependent cell death
784 pathways. *Cell Death Differ* 2017, **24**(3)**:** 481-491.

785

786 68. Newton K, Sun X, Dixit VM. Kinase RIP3 is dispensable for normal NF-kappa Bs, signaling
787 by the B-cell and T-cell receptors, tumor necrosis factor receptor 1, and Toll-like receptors 2
788 and 4. *Mol Cell Biol* 2004, **24**(4)**:** 1464-1469.

789

790 69. Bossen C, Ingold K, Tardivel A, Bodmer JL, Gaide O, Hertig S, *et al.* Interactions of tumor
791 necrosis factor (TNF) and TNF receptor family members in the mouse and human. *J Biol*
792 *Chem* 2006, **281**(20)**:** 13964-13971.

793

794 70. Vince JE, Wong WW, Khan N, Feltham R, Chau D, Ahmed AU, *et al.* IAP antagonists target
795 cIAP1 to induce TNFalpha-dependent apoptosis. *Cell* 2007, **131**(4)**:** 682-693.

796

797    71.    Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis.

798            *Nat Methods* 2012, **9**(7)**:** 671-675.

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800

801     **FIGURES LEGENDS**

**Figure 1**
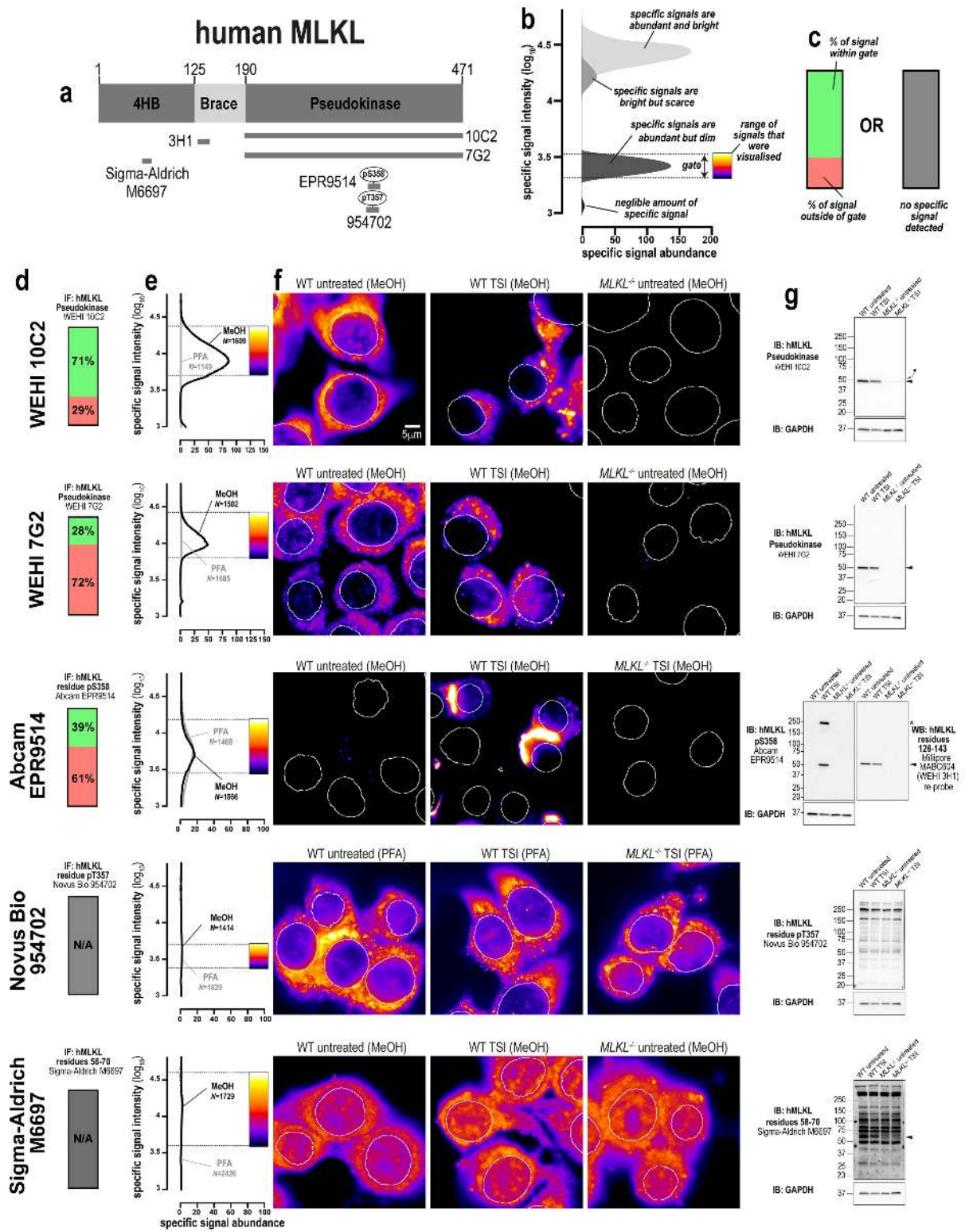


802

803 **Figure 1: Methanol fixation is optimal for the immunofluorescent detection of human MLKL**

804 **a** Human MLKL domain architecture showing the immunogens used to raise the tested anti-MLKL

805 antibodies. **b** Demonstration of how signal-to-noise ratios were used to quantify the abundance and

806 brightness of specific immunofluorescent signals generated by different antibodies. The 5th and 95th

807 percentile of each signal-to-noise curve defines the gate where specific immunosignals were

808 observed. As indicated by the pseudocolour look-up-table, only immunosignals within this gate were

809 visualised. **c** Chart exemplifying how the amount of signal within the gate, relative to the total amount

810 of detectable signal, provides another gauge of antibody specificity for immunofluorescence. **d**

811 Quantitation of the percentage of gated signals for the tested MLKL antibodies. **e** Quantitation of

812 specific signal abundance produced by the tested MLKL antibodies on methanol-fixed (MeOH) or

813 paraformaldehyde-fixed (PFA) HT29 cells. The number of cells imaged (N) to generate each signal-

814 to-noise curve is shown. **f** Micrographs of immunofluorescent signals for the tested MLKL antibodies

815 on HT29 cells. As indicated by each pseudocolour look-up-table, only immunosignals within the

816 respective gate in panel **e** were visualised. Data are representative of n=3 (clones 10C2, 7G2) and

817 n=2 (Abcam clone EPR9514, Novus Biological MAB9187/clone 954702 and Sigma-Aldrich M6697)

818 independent experiments. Nuclei were detected by Hoechst 33342 staining and are demarked by

819 white outlines in micrographs. **g** Immunoblot using the tested MLKL antibodies against wild-type

820 and *MLKL*$^{-/-}$ HT29 cell lysates. Closed arrowheads indicate the main specific band. Asterisks indicate

821 non-specific bands that could otherwise confound data interpretation. Immunoblots were re-probed

822 for GAPDH as loading control.

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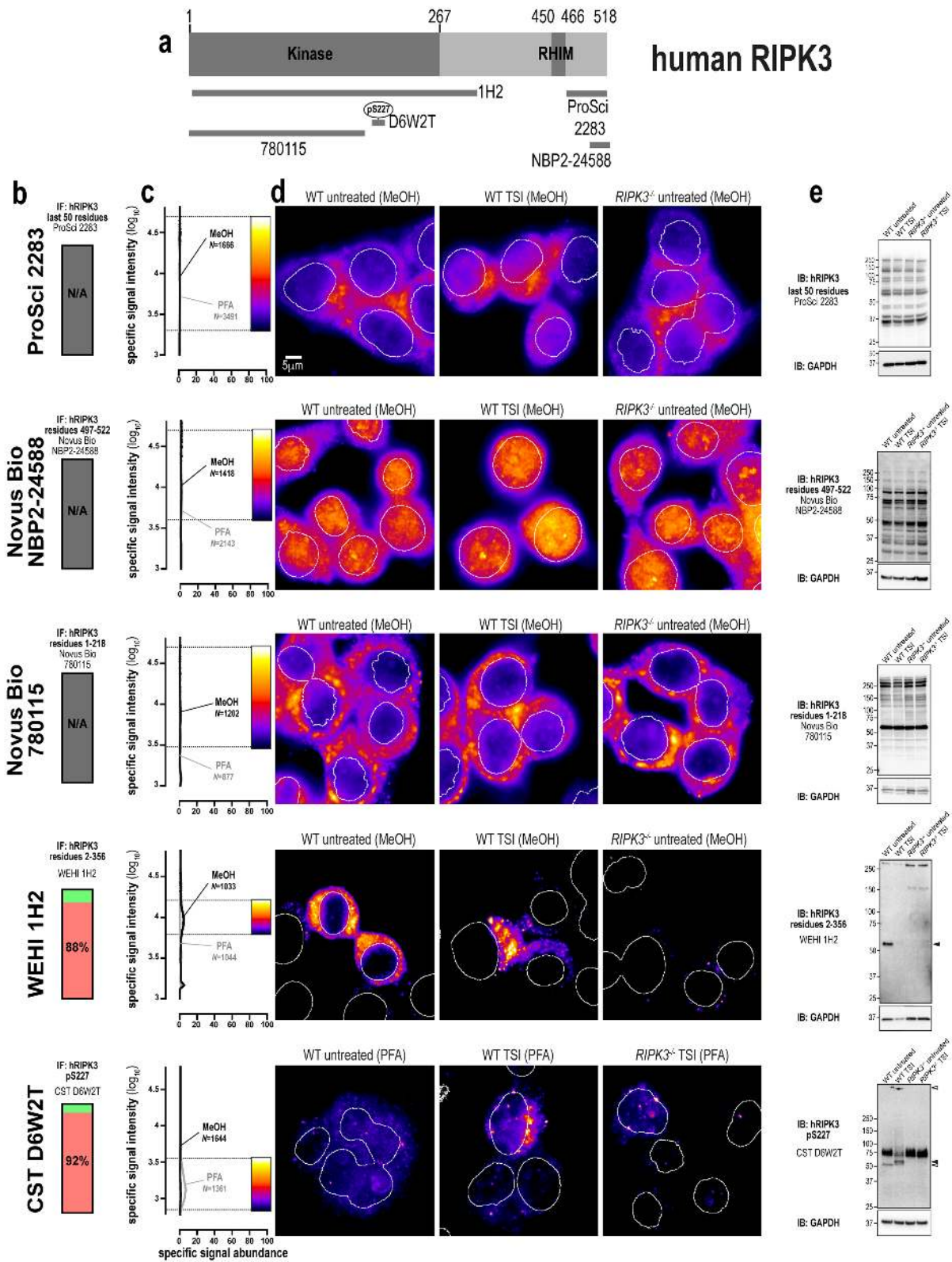
**Figure 2**



824

825

826 **Figure 2: Better antibodies are needed for imaging human RIPK3.**

827 **a** Human RIPK3 domain architecture showing the immunogens or epitopes for the tested anti-RIPK3

828 antibodies. **b** Quantitation of the percentage of gated signals for the tested RIPK3 antibodies. **c**

829 Quantitation of specific signal abundance produced by the tested RIPK3 antibodies on methanol-

830 fixed (MeOH) or paraformaldehyde-fixed (PFA) HT29 cells. The number of cells imaged (N) to

831 generate each signal-to-noise curve is shown. **d** Micrographs of immunofluorescent signals for the

832 tested RIPK3 antibodies on HT29 cells. As indicated by each pseudocolour look-up-table, only

833 immunosignals within the respective gate in panel **c** were visualised. Data are representative of n=3

834 (Cell Signaling Technology clone D6W2T and in-house clone 1H2) and n=2 (ProSci 2283, Novus

835 Biological NBP2-24588 and MAB7604/clone 780115) independent experiments. Nuclei were

836 detected by Hoechst 33342 staining and are demarked by white outlines in micrographs. **e**

837 Immunoblot using the tested RIPK3 antibodies against wild-type and *RIPK3*$^{-/-}$ HT29 cell lysates.

838 Closed arrowheads indicate the main specific band. Open arrowheads indicate other specific bands

839 of interest. Immunoblots were re-probed for GAPDH as loading control.

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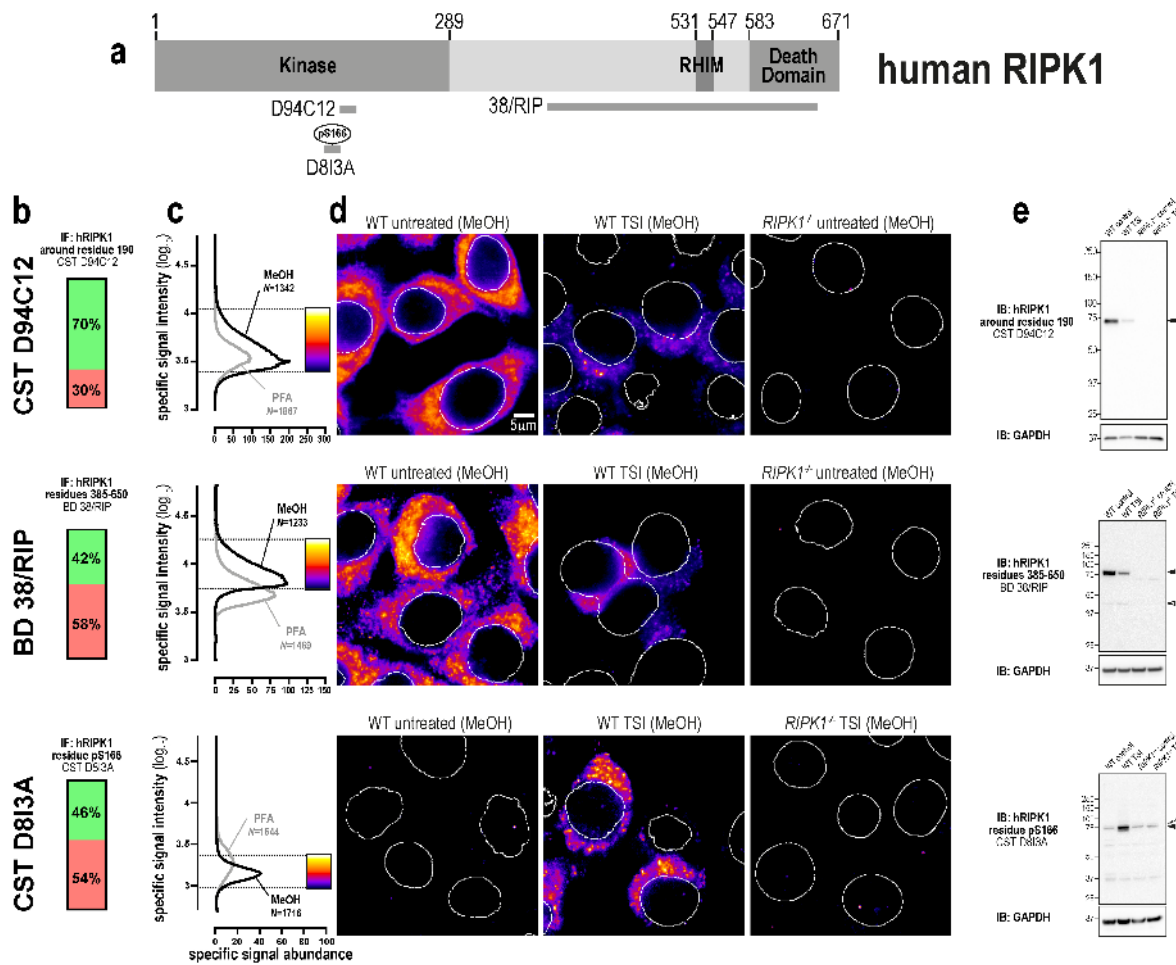
## Figure 3



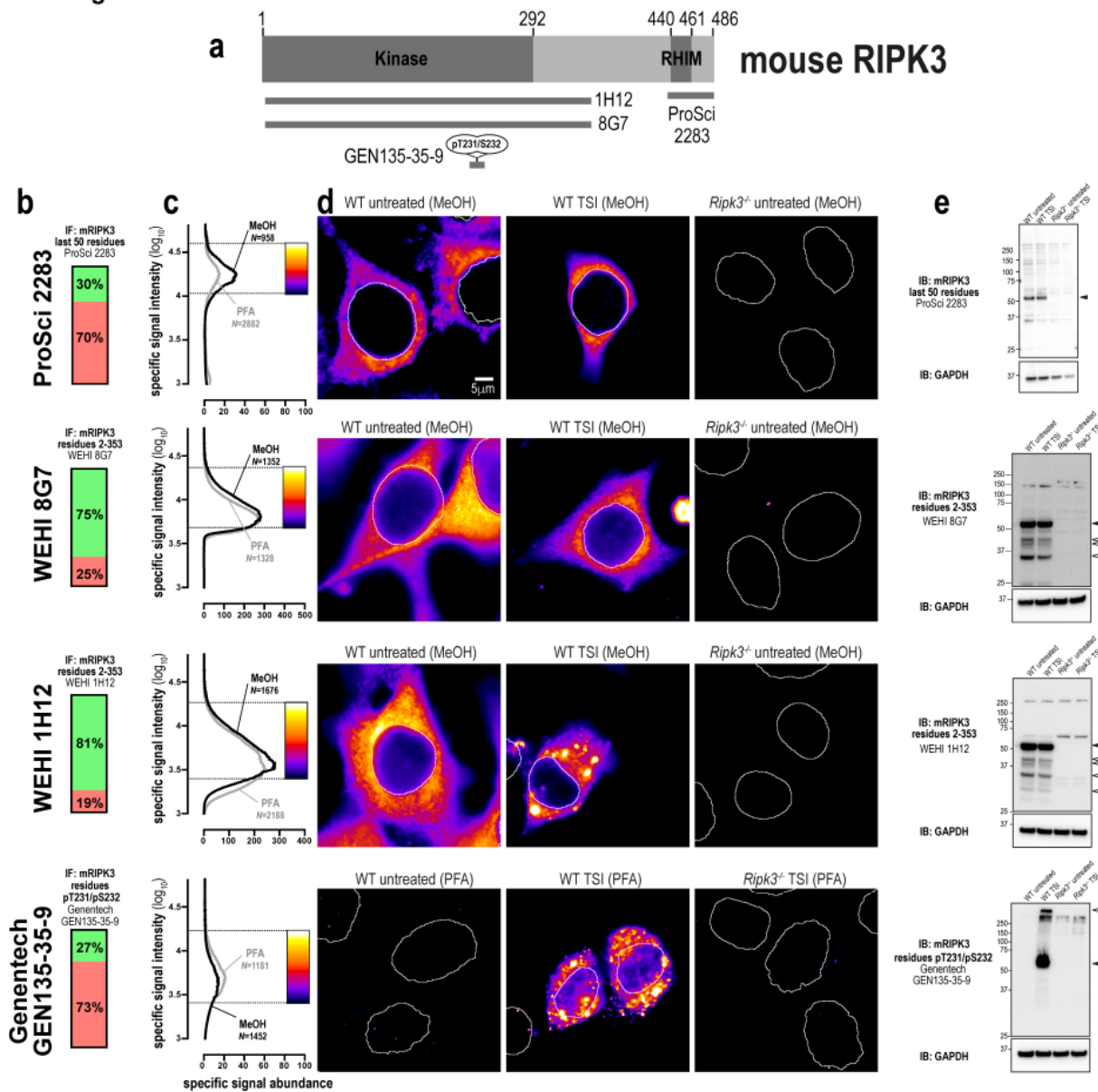**Figure 3: Three specific antibodies for imaging endogenous human RIPK1.**

**a** Human RIPK1 domain architecture showing the immunogens or epitopes for the tested anti-RIPK1 antibodies. **b** Quantitation of the percentage of gated signals for the tested RIPK1 antibodies. **c** Quantitation of specific signal abundance produced by the tested RIPK1 antibodies on methanol-fixed (MeOH) or paraformaldehyde-fixed (PFA) HT29 cells. The number of cells imaged (N) to generate each signal-to-noise curve is shown. **d** Micrographs of immunofluorescent signals for the tested RIPK1 antibodies on HT29 cells. As indicated by each pseudocolour look-up-table, only immunosignals within the respective gate in panel **c** were visualised. Data are representative of n=2 (Cell Signaling Technology clones D94C12 and D8I3A, BD Transduction laboratories clone 38/RIP) independent experiments. Nuclei were detected by Hoechst 33342 staining and are demarked by

36

853    white outlines in micrographs. **e** Immunoblot using the tested RIPK1 antibodies against wild-type

854    and *RIPK1$^{-/-}$* HT29 cell lysates. Closed arrowheads indicate the main specific band. Open arrowheads

855    indicate other specific bands of interest. Asterisks indicate non-specific bands that could otherwise

856    confound data interpretation. Immunoblots were re-probed for GAPDH as loading control.

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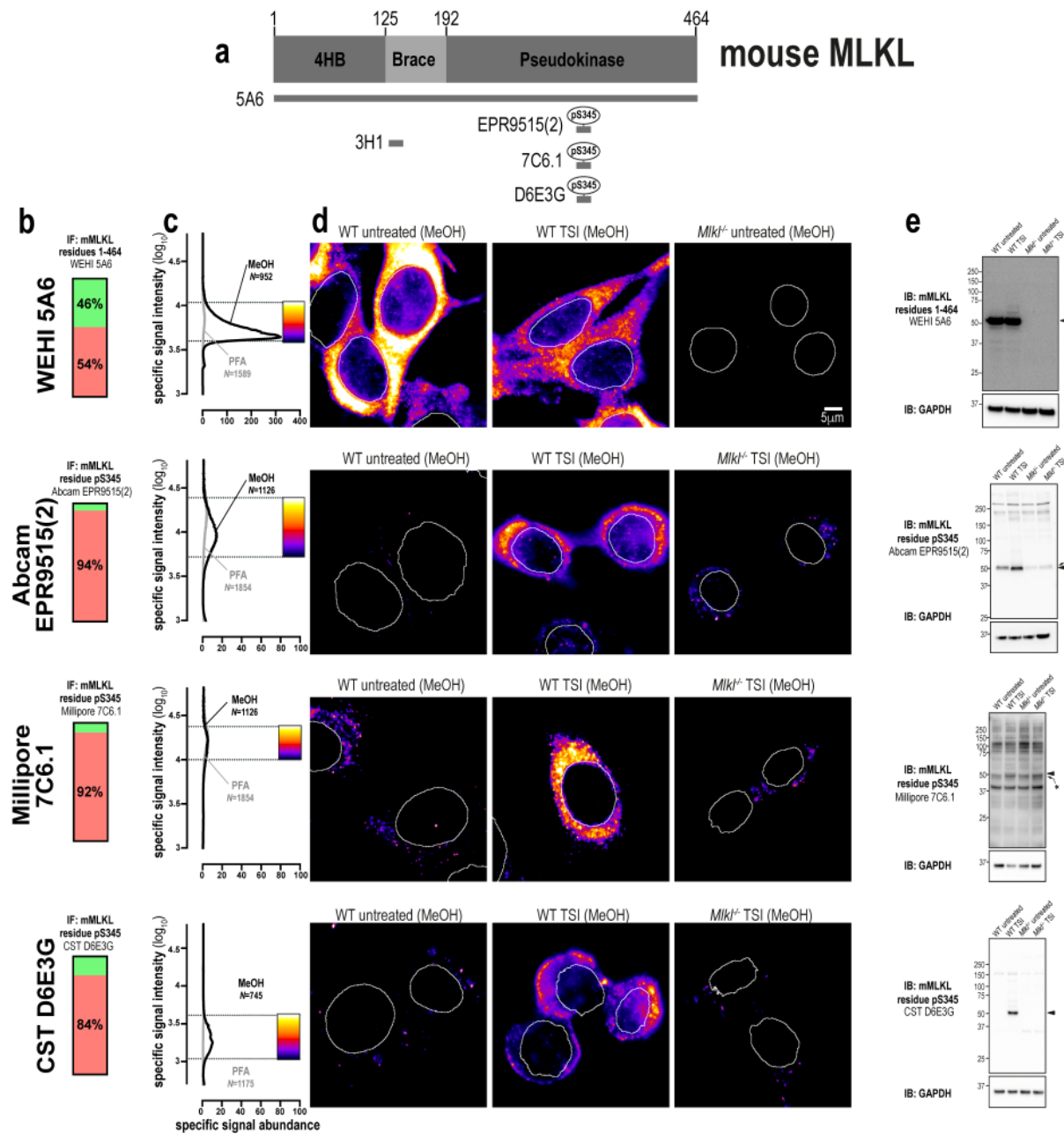
**Figure 4: A new monoclonal antibody to image endogenous mouse MLKL.**

**a** Mouse MLKL domain architecture showing the immunogens or epitopes for the tested anti-MLKL antibodies. **b** Quantitation of the percentage of gated signals for the tested MLKL antibodies. **c** Quantitation of specific signal abundance produced by the tested MLKL antibodies on methanol-fixed (MeOH) or paraformaldehyde-fixed (PFA) MDFs. The number of cells imaged (N) to generate each signal-to-noise curve is shown. **d** Micrographs of immunofluorescent signals for the tested

866      MLKL antibodies on MDFs. As indicated by each pseudocolour look-up-table, only immunosignals

867      within the respective gate in panel **c** were visualised. Data are representative of n=3 (in-house clone

868      5A6) and n=2 (Abcam clone EPR9515(2), Millipore MABC1158/clone 7C6.1 and Cell Signaling

869      Technology clone D6E3G) independent experiments. Nuclei were detected by Hoechst 33342

870      staining and are demarked by white outlines in micrographs. **e** Immunoblot using the tested MLKL

871      antibodies against lysates from wild-type and *Mlkl*$^{-/-}$ MDFs. Closed arrowheads indicate the main

872      specific band. Asterisks indicate non-specific bands that could otherwise confound data

873      interpretation. Immunoblots were re-probed for GAPDH as loading control.

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875

**Figure 5: Unlike human RIPK3, mouse RIPK3 is highly amenable to detection by immunofluorescence and immunoblotting.**

**a** Mouse RIPK3 domain architecture showing the immunogens or epitopes for the tested anti-RIPK3 antibodies. **b** Quantitation of the percentage of gated signals for the tested RIPK3 antibodies. **c** Quantitation of specific signal abundance produced by the tested RIPK3 antibodies on methanol-fixed (MeOH) or paraformaldehyde-fixed (PFA) MDFs. The number of cells imaged (N) to generate each signal-to-noise curve is shown. **d** Micrographs of immunofluorescent signals for the tested

884    RIPK3 antibodies on MDFs. As indicated by each pseudocolour look-up-table, only immunosignals
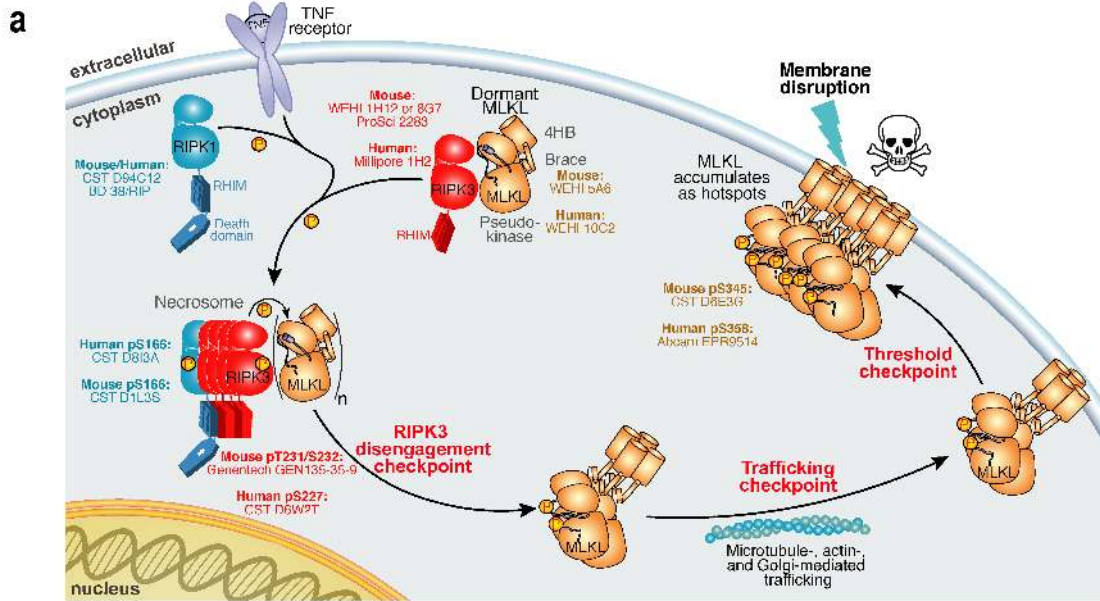
885    within the respective gate in panel **c** were visualised. Data are representative of n=3 (in-house clone

886    8G7 and Genentech clone GEN135-35-9) and n=2 (ProSci 2283, in-house clone 1H12) independent

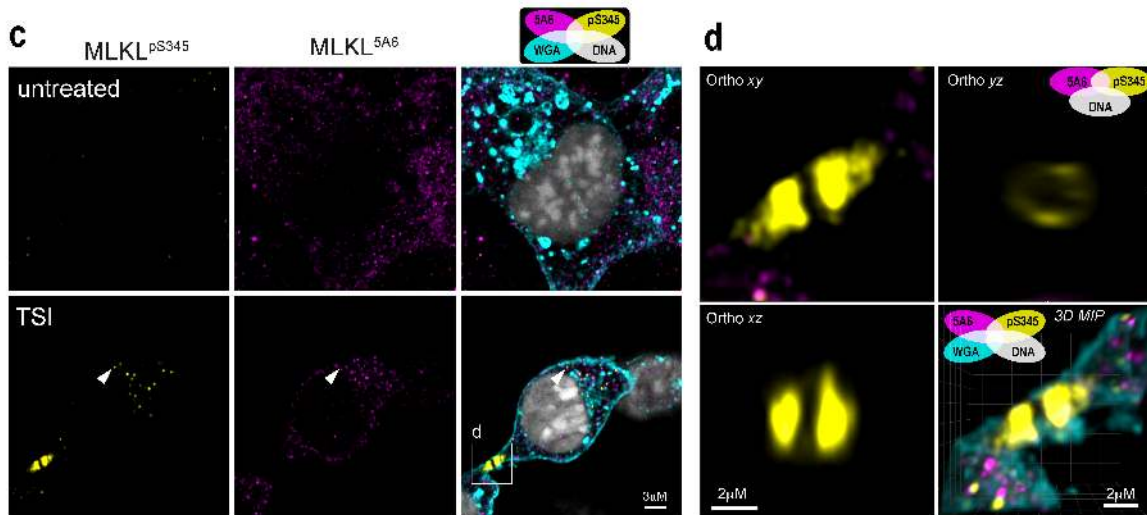887    experiments. Nuclei were detected by Hoechst 33342 staining and are demarked by white outlines in

888    micrographs. **e** Immunoblot using the tested RIPK3 antibodies against lysates from wild-type and

889    *Ripk3$^{-/-}$* MDFs. Closed arrowheads indicate the main specific band. Open arrowheads indicate other

890    specific bands of interest. Immunoblots were re-probed for GAPDH as loading control.

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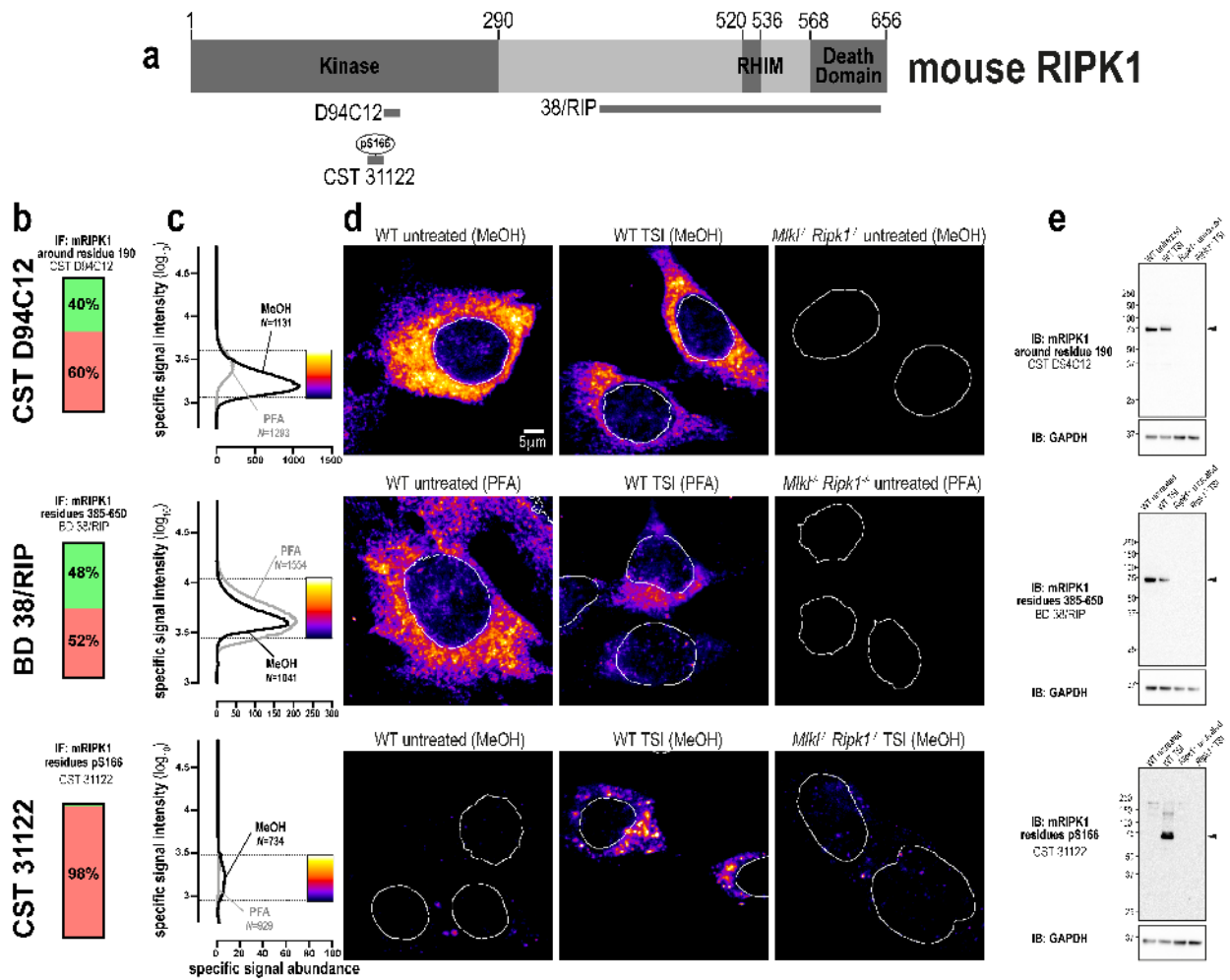
**Figure 6: Three specific antibodies for imaging endogenous mouse RIPK1.**

**a** Mouse RIPK1 domain architecture showing the immunogens or epitopes for the tested anti-RIPK1 antibodies. **b** Quantitation of the percentage of gated signals for the tested RIPK1 antibodies. **c** Quantitation of specific signal abundance produced by the tested RIPK1 antibodies on methanol-fixed (MeOH) or paraformaldehyde-fixed (PFA) MDFs. The number of cells imaged (N) to generate each signal-to-noise curve is shown. **d** Micrographs of immunofluorescent signals for the tested RIPK1 antibodies on MDFs. As indicated by each pseudocolour look-up-table, only immunosignals within the respective gate in panel **c** were visualised. Data are representative of n=2 (Cell Signaling Technology clones D94C12 and 31122, and BD Transduction Laboratories clone 38/RIP) independent experiments. Nuclei were detected by Hoechst 33342 staining and are demarked by

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904    white outlines in micrographs. **e** Immunoblot using the tested RIPK1 antibodies against lysates from

905    wild-type and *Ripk1⁻ᐟ⁻* MDFs. Closed arrowheads indicate the main specific band. Open arrowheads

906    indicate other specific bands of interest. Immunoblots were re-probed for GAPDH as loading control.

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**Figure 7: Optimised antibody cocktails for visualising endogenous necroptotic signaling in fixed human and mouse cells.**

912     **a** Cartoon summary of the currently-understood chronology of TNF-induced necroptosis.

913     Recommendations of validated antibodies for immunostaining various steps in the necroptotic

914     pathway are provided. **b** Summary of validated antibody cocktails and counterstains that can be

915     multiplexed to examine endogenous necroptotic signaling in fixed human and mouse cells. Successful

916     detection of specific signals relies on fixation in methanol, rather than crosslinking fixatives such as

917     paraformaldehyde. **c** Two-dimensional Airyscan micrographs of Wheat Germ Agglutinin (WGA)-

918     stained membranes, Hoechst-stained DNA and anti-MLKL immunosignals from clone 5A6 and clone

919     D6E3G on methanol-fixed wild-type MDFs that had been left untreated or TSI-treated for 60min.

920     Arrowhead exemplifies the small clusters of MLKL that form during necroptosis. The box indicates

921     the junctional accumulation of phospho-MLKL. **d** Three-dimensional orthogonal projections and

922     maximum intensity projection (MIP) of the boxed region from panel **c** showing a ring-like structure

923     adopted by phospho-MLKL at the WGA-stained plasma membrane. Accompanied by Supplementary

924     Video 1.